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Mating Type Control in *Saccharomyces cerevisiae*: a Frameshift Mutation at the Common DNA Sequence, X, of the *HML*α Locus

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A mutation defective in the homothallic switching of mating type alleles, designated *hml*α*-2*, has previously been characterized. The mutation occurred in a cell having the *HO MAT***a** *HML*α *HMR***a** genotype, and the mutant culture consisted of ca. 10% **a** mating type cells, 90% nonmater cells of haploid cell size, and 0.1% sporogenous diploid cells. Genetic analyses revealed that nonmater haploid cells have a defect in the α*2* cistron at the *MAT* locus. This defect was probably caused by transposition of a cassette originating from the *hml*α*-2* allele by the process of the homothallic mating type switch. That the *MAT* locus of the nonmater cells is occupied by a DNA fragment indistinguishable from the Yα sequence in electrophoretic mobility was demonstrated by Southern hybridization of the *Eco*RI-*Hin*dIII fragment encoding the *MAT* locus with a cloned *HML*α gene as the probe. The *hml*α*-2* mutation was revealed to be a one-base-pair deletion at the ninth base pair in the X region from the X and Y boundary of the *HML* locus. This mutation gave rise to a shift in the open reading frame of the α*2* cistron. A molecular mechanism for the mating type switch associated with the occurrence of sporogenous diploid cells in the mutant culture is discussed.

Mating types, **a** and α, in *Saccharomyces cerevisiae* are specified by the *MAT***a** and *MAT*α alleles of the *MAT* locus on the right arm of chromosome III, 20 centimorgans from the centromere. Mating type information is also coded for by two additional loci, *HML* and *HMR*, on the left (*HML*) and right (*HMR*) arms of the same chromosome, each ca. 65 centimorgans distal to the centromere (12, 23). The *HML* and *HMR* loci each have two codominant alleles, *HML***a** and *HML*α and *HMR***a** and *HMR*α, like the *MAT***a** and *MAT*α alleles of the *MAT* locus. The mating type information in the *HML* and *HMR* loci is, however, silent, being under negative control of the gene products of four unlinked *SIR* genes (34; some of these genes have also been called *MAR* [22] or *CMT* [9, 18]). In homothallic cells, the mating types are interchangeable by switching from one mating type allele to the other. The switch is caused by transposition of genetic information (controlling element [11, 32] or cassette [17]) from either of the alleles of the *HML* and *HMR* loci into the *MAT* locus, with removal of preexisting information by the action of the *HO* gene product. The mating type switch occurs efficiently in mother cells that have undergone at least one cell division cycle and produces a pair of mother and daughter cells in which the *MAT* alleles are switched (16, 38). The resulting **a** and α cells in a single-spore culture mate to produce a zygote from which diploid nonmater cells of the heterozygous *MAT***a**/*MAT*α configuration are produced. Mating type interconversion is blocked as soon as the diploidization by zygote formation is complete, because expression of the *HO* gene is repressed when the heterozygosity of *MAT*α and *MAT***a** is established (20). In general, heterothallic cells have the *ho* allele (inactive allele of *HO*) and cells of like mating types mate only rarely (13; for a review, see reference 15).

Sequence analyses of the nucleotides in the cloned *MAT***a** and *HML*α genes revealed that these genes have an allele-specific sequence of either **a** or α information, Y**a** (642 base pairs [bp]) or Yα (747 bp), which is sandwiched between homologous regions, X (704 bp) and Z_1 (239 bp), in all of the *MAT*, *HML*, and *HMR* loci (1) (Fig. 1). Furthermore, W (723 bp) and Z_2 (88 bp) regions, respectively, lie outside the X and Z_1 regions in the *MAT* and *HML* loci, but not in *HMR*. It has been suggested that the switching occurs by replacement of a cassette from the donor locus (i.e., *HMR* or *HML*) into the recipient locus (i.e., *MAT*) by a mechanism which is initiated by a double-stranded DNA cut at the Y-Z boundary (40) and proceeds through direct pairing of the donor and recipient loci by chromosome folding (10) or by free diffusion of the cassette (31).

By mutagenesis of the ascospores of a homothallic strain having the *HO HML*α *HMR***a** genotype, Oshima and Takano (30) have isolated various mutants defective in the mating type switch. One of the mutations of the *HML*α gene, designated *hml*α*-2*, gives rise to a culture showing **a** mating type and has been characterized as a nonfunctional mutation at the *HML*α locus (31). Population analysis of the mutant culture by microscopic observation of cell size and shape and by sensitivity of individual cells to the α factor has revealed that the culture consists of ca. 10% **a** mating type cells, 90% nonmater cells of haploid cell size, and 0.1% sporogenous diploid cells. To explain this phenotype, Oshima and Takano (31) have suggested that although the *hml*α*-2* allele can produce a diffusible element carrying the wild-type α information, the element is defective in its insertion into the *MAT* locus. The sterile phenotype is caused by simultaneous expression of the *MAT***a** gene and the α mating type information of the free-diffusible element which is released from the repression of the *SIR* genes. The sporogenous diploid cells found in the *hml*α*-2* culture were attributed to zygote formation between **a** mating type cells and α mating type cells produced by occasional insertion of the diffusible *hml*α*-2* element into the *MAT* locus (hypothesis

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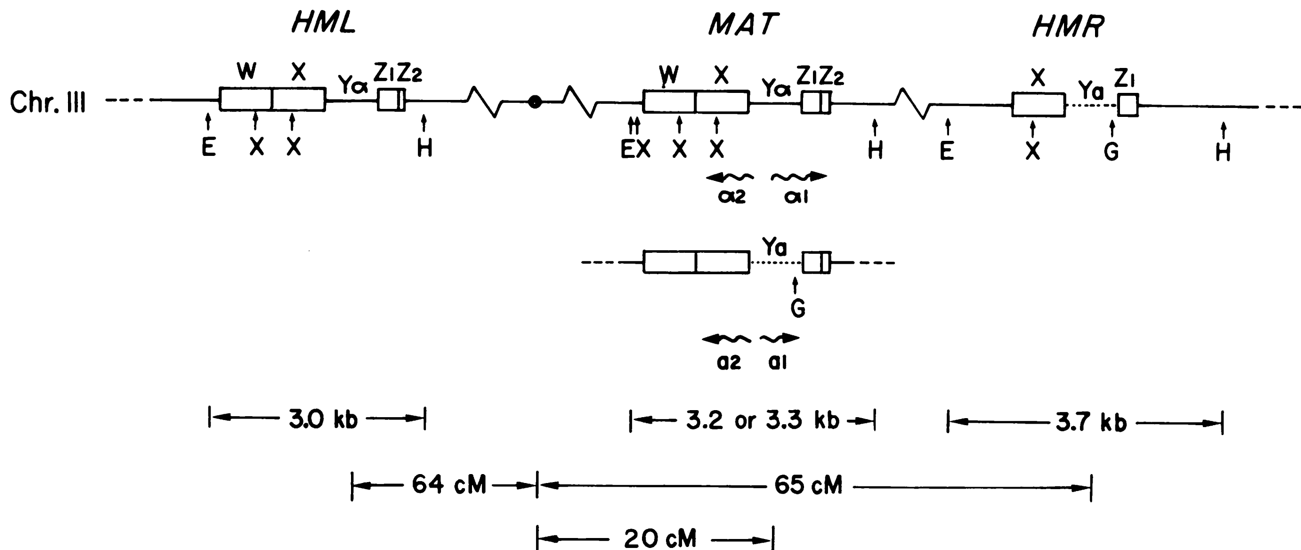

FIG. 1. Arrangement of the *HML*, *MAT*, and *HMR* genes on chromosome III. The regions, X, W, Z_1, Z_2, Ya, and Yα have, respectively, 704, 723, 239, 88, 642, and 747 bp (1). Restriction sites for *Eco*RI (E), *Hin*dIII (H), *Bgl*II (G), and *Xba*I (X) and that of transcripts corresponding to **a***1*, **a***2*, α*1*, and α*2* (wavy lines with arrow head) were compiled from the data of Strathern et al. (41) and Astell et al. (1). Map distances between *HML*, *MAT*, and *HMR* were those described by Harashima and Oshima (12). cM indicates centimorgans and the closed circle indicates centromere of the chromosome. Molecular sizes of the *Hin*dIII-*Eco*RI fragments carrying the *MAT*, *HML*, or *HMR* gene were estimated from the data shown in Fig. 2. The *MAT* fragments of 3.2 and 3.3 kb correspond, respectively, to those carrying *MAT***a** and *MAT*α.

I), whereas the appearance of **a** mating type cells was unexplained.

It is, however, possible to speculate as an alternative mechanism that the *hml*α*-2* mutation occurred in the X or Z_1 region flanking the α-specific region, Yα, rather than in Yα. The *hml*α*-2* DNA sequence could be transposed to the *MAT* locus through the switching process with substantial efficiency. The switched cells in which the *MAT* locus is installed with the *hml*α*-2* sequence are sterile due to defectiveness of the α function. However, the wild-type sequence of α information could be generated by, for example, occasional recombination between the site of the *hml*α*-2* mutation and the corresponding site of the wild-type *MAT***a** allele during the transposition. The α cell thus obtained would be able to mate with an **a** mating type cell, producing sporogenous diploid cells with the *MAT*α*/MAT***a** *hml*α*-2/hml*α*-2 HMR***a**/*HMR***a** genotype (hypothesis II).

This communication examines the above two hypotheses. The results indicated that hypothesis II is correct. This was further supported by sequence analysis which revealed that the *hml*α*-2* mutation is a single C · G bp deletion in the α*2* coding frame in the X region, nine bp distal to the X and Y boundary. To explain the rare occurrence of sporulating diploid cells, a model for the mating type switch is proposed.

MATERIALS AND METHODS

Strains and plasmids. The genotypes and sources or construction of *S. cerevisiae* strains are described in Table 1. *Escherichia coli* strain JA221 (F^- *leuB6* Δ*trpE5 lacY hsdR hsdM*$^+$ *recA*) (5) was used as the host for cloning yeast gene with pBR322 as the vector. Two DNA plasmids used were provided by J. B. Hicks. One of them, designated 43A, is a chimeric plasmid constructed by insertion of a 6.0-kilobase (kb) *Sau*3A fragment of *S. cerevisiae* DNA carrying the *HO* gene at the *Bam*HI site of the pBR322 region of YEp13 (10.7 kb) (4); the other, B2, is composed of a large *Eco*RI-*Hin*dIII fragment of pBR322 (4.3 kb) and an *Eco*RI-*Hin*dIII fragment (3.0 kb) of *S. cerevisiae* DNA bearing the *HML*α gene.

Media. Nutrient (YPAD) and minimal media for yeast and nutrient medium containing sodium ampicillin (Viccillin; Meiji Seika Kaisha Ltd., Tokyo, Japan; 50 μg/ml) for *E. coli* were as described previously (44). Sporulation of *S. cerevisiae* was carried out by smearing cells on an agar medium containing 0.5% anhydrous potassium acetate and 2% agar and incubating the cells for 2 days at 30°C.

Genetic methods. Frequency of *S. cerevisiae* sporulation was scored under the microscope by observing cells incubated on the sporulation medium at 30°C for 48 h after the inoculation from YPAD. Tetrad dissection was carried out with the aid of a micromanipulator after digestion of the ascus sac with snail gut juice (21). Mating types of *S. cerevisiae* strains were determined by cross streaking them on YPAD plates with standard **a** or α haploid strains KYC53 (**a**), KYC54 (α), S1586C (**a**), or 702 (α), with complementary auxotrophic markers to the strain to be tested and by replicating the cells with velveteen pads. Then, the cross-streaked cells were replicated onto minimal plates supplemented with appropriate nutrients after overnight incubation at 30°C, to test complementation of auxotrophic markers by the mating of these strains. Hybrids were constructed by mass mating or by protoplast fusion as described below. The hybrid construction was confirmed by the complementation of auxotrophic markers of parent strains which were crossed or fused and (if possible) by examining the marker segregation in the tetrad segregants from the hybrids. For the protoplast fusion, cells were grown in 4 ml of YPAD medium overnight. All of the cells of the culture (ca. 4×10^8) were washed with 3 ml of 0.6 M KCl solution and suspended in 3 ml of enzyme solution containing 0.1 mg of Zymolyase 60,000 (Kirin Brewery Co., Tokyo, Japan) per ml in 0.6 M KCl containing 10 μl of 2-mercaptoethanol and incubated at 30°C for 1 to 3 h depending on the strain. The protoplasts

TABLE 1. List of *S. cerevisiae* strains[a]

Strain	Mating type	Genotype[b]	Source or construction method
J-3-5A	**a**	*MAT***a** *ho HML*α *HMR***a** *lys2 thr4*	Oshima and Takano (30)
J-3-8D	α	*MAT*α *ho HML*α *HMR***a** *lys2 thr4*	Oshima and Takano (30)
J-137-6B	**a**	*MAT***a** *HO hml*α*-2 HMR***a** *lys2 thr4 trp1*	Oshima and Takano (31)
T-1171-5D	**a**/α	*MAT***a**/*MAT*α *HO/HO HML*α/*HML*α *HMR***a**/*HMR***a** *lys2/lys2 thr4/thr4*	Oshima and Takano (30)
T-1851-2D	**a**/α	*MAT***a**/*MAT*α *HO/HO HML*α/*HML*α *HMR***a**/*HMR***a** *ade1/ade1 arg4/arg4 his4/his4 leu2/leu2 lys2/lys2 trp1/trp1*	Oshima and Takano (30)
DS12-10B	**a**	*MAT***a** *ho HML*α *HMR***a** *ade2 his4 leu2 trp1*	Our stock
HT19-8B	**a**	*MAT***a** *ho HML***a** *HMR***a** *sir3-4 arg4 his4 leu1 leu2 ura3*	Our stock
K167	α	*mat***a**(Am) *ho HML*α *hmr***a**(Am) *mar2-3 ade8-10 cry1 lys1-1 met13 tyr7*	Obtained from A. J. S. Klar
KYC53	**a**	*MAT***a** *ho trp3 ura1 ura2*	Our stock
KYC54	α	*MAT*α *ho lys1 trp3 ura1 ura2*	Our stock
KYC56	α	*MAT*α *ho HML*α *HMR***a** *his6 leu1 pho8 trp4*	Our stock
S-14-9C-1A	α	*MAT*α *HO HML*α *HMR*α *his4 leu2 lys2*	Oshima and Takano (31)
S1586C	**a**	*MAT***a** *ho arg1 ura1*	Our stock
702	α	*MAT*α *ho arg1 his1 trp2*	Our stock
KA1-7A	α	*MAT*α *ho hml*α*-2 HMR***a** *his6 lys2 thr4 trp4*	Segregant from J-137-6B × KYC56 cross. Ascus types from tetrad analysis of KA1-7A × J-137-6B cross were 2 **a**:2 nonmater, 2 **a**:1 nonmater:1 α or 2 **a**:2 α. Thus, KA1-7A has the indicated genotype.
KA13-1D	α	*MAT*α *HO hml*α*-2 HMR*α *lys2 thr4 trp1*	α mating type segregant of an ascus showing 2 non mater (i.e., *HO HML*α *HMR***a**):2 α segregation from J-137-6B × S-14-9C-1A cross
KA15-1A	**a**	*MAT***a** *ho hml*α*-2 HMR***a** *lys2 thr4 trp1 trp4*	Segregant from KA1-7A × J-137-6B cross
KA45-20A	**a**	*MAT***a** *ho HML***a** *HMR***a** *sir3-4 arg4 his4 leu1* (or *leu2*) *lys2 ura3*	Segregant from J-3-8D × HT19-8B cross. The *sir3-4* allele in KA45-20A was confirmed by sporulation of diploid constructed by cross with K167.
KA47-12D	Ste[c]	*MAT***a** *ho hml*α*-2 HMR***a** *sir3-4 his4 his6 leu2 lys2*	Segregant from KA45-20A × KA1-7A cross from an ascus showing 1 **a**:2 α:1 nonmater segregation. Since the two α segregants should have the *SIR3*^{+} allele, the genotype of the remaining two clones including KA47-12D could be assigned as indicated.
KA47-20D	Ste	*MAT***a** *ho hml*α*-2 HMR***a** *sir3-4 his4 leu2 lys2 trp4 ura3*	Segregant from KA45-20A × KA1-7A cross from an ascus showing 1 **a**:2 α:1 nonmater segregation. Since the two α segregants should have the *SIR3*^{+} allele, the genotype of the remaining two clones including KA47-20D could be assigned as indicated.
KA63-5A	**a**	*MAT***a** *HO hml*α*-2 HMR***a** *ade1 arg4? leu2 lys2 thr4 trp1*	Segregant showing **a** mating type from T-1851-2D × KA13-1D cross
KA70-23B	**a**/α	*MAT***a**/*MAT*α *HO/HO HML*α/*HML*α *HMR***a**/*HMR***a** *leu2/leu2 lys2/lys2*	Constructed by repeated crosses and tetrad analyses of the crosses among DS12-10B, T-1171-5D, and J-3-5A
KA74-4C	**a**/α	*MAT***a**/*MAT*α *HO/HO hml*α*-2/hml*α*-2 HMR***a**/*HMR***a** *leu2/leu2 lys2/lys2*	Homothallic diploid clone derived from a spore of KA70-23B × J-137-6B cross
KA79-8B	**a**	*MAT***a** *ho hml*α*-2 HMR***a** *leu2 lys2 thr4 trp1* (or *trp4* or both)	Segregant from KA15-1A × KA74-4C cross
KA79-8B-α2	Ste	*mat*α*-2 ho hml*α*-2 HMR***a** *leu2 lys2 thr4 trp1* (or *trp4* or both)	Constructed from KA79-8B by mating type switch with the *HO* function through transformation of plasmid 43A and curing of it

[a] Table includes the strains used for the construction of tester strains.
[b] The genetic symbols are those described by Broach (3).
[c] Ste indicates the nonmater phenotype due to a certain mutation.

were separated from the enzyme solution by gentle centrifugation (500 × *g* for 5 min) and suspended in 1 ml of 0.6 M KCl. Then 1 ml each of two protoplast suspensions was mixed, the mixture was centrifuged, and the protoplasts were suspended in 1 ml of solution containing 30% polyethylene glycol 4,000 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 50 mM $CaCl_2$. The suspension was incubated at 30°C for 20 min and centrifuged again. The supernatant was discarded, 1 ml of 0.6 M KCl solution was added to the precipitated protoplasts, and a 0.1-ml portion of the protoplast suspension was poured onto the surface of a minimal plate appropriately supplemented with nutrients and covered with the same medium containing 3% agar. Transformation of *E. coli* with plasmid DNAs was carried out by the method of Morrison (27), and *S. cerevisiae* transformation by the procedure of Beggs (2), except that Zymolyase 60,000 at 0.1 mg/ml was used for protoplast preparation instead of Helicase.

Biochemical methods. *S. cerevisiae* genomic DNA was prepared from cells grown on YPAD medium by the method of Hereford et al. (14). Bacterial plasmid DNA was purified from cleared lysate (6) by CsCl-ethidium bromide banding.

Electrophoresis of DNA was carried out on slab gels (15 by 18 by 0.5 cm) prepared by pouring melted agarose (0.7 or 1%) at 2 V/cm overnight or 5 V/cm for 4 h in 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA or on acrylamide slab gels (7.5 or 4%) in 0.09 M Tris-borate buffer (pH 8.3) containing 2.5 mM EDTA at 10 V/cm for 4 h (7.5%) or at 4 V/cm for 40 h (4%). DNA bands were stained with ethidium bromide and photographed under long-wave (365 nm) UV light irradiation. Restriction endonucleases were purchased from Takara Shuzo Co. Ltd., Kyoto, Japan and New England Biolabs, Inc., Beverly, Mass., and DNAs were digested in the buffers recommended by the suppliers. Partially purified T4 ligase was prepared in our laboratory by the method of Weiss (45) and used for ligation of DNA by the method of Tanaka and Weisblum (42), with or without pretreatment with *E. coli* alkaline phosphatase which was generously provided by A. Nakata. ^{32}P-labeled DNA was prepared by nick translation by the method of Rigby et al. (33). Transfer of DNA from an agarose gel to a nitrocellulose sheet (PKG SMWOP 00 10; Millipore Corp., Bedford, Mass.) was performed by the method of Southern (35), and DNA hybridizations were performed as described by Jeffreys and Flavell (19) at 65°C for 18 h in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), containing 0.02% polyvinylpyrrolidone (Wako), 0.02% Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N.J.), 0.02% bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.), 50 μg of calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.), and 0.1% sodium dodecyl sulfate. Colony hybridization of *E. coli* was by the method of Grunstein and Hogness (8). Sequence determinations of DNA were carried out by the method of Maxam and Gilbert (26) by ^{32}P labeling the 5′ ends of DNAs with T4 polynucleotide kinase (Takara Shuzo Co. Ltd.).

RESULTS

Genetic characterization of *hmlα-2* mutation. The *hmlα-2* culture is reported to consist of 90% sterile haploid, 10% **a** mater haploid, and 0.1% sporulating diploid cells (31). We confirmed by single-cell isolation that the single-cell cultures always consisted of either pure diploid cells showing high frequencies of sporulation or mixed populations of the same three different cell types as the original culture, J-137-6B. To determine which of the hypotheses for the *hmlα-2* mutation holds, we tested the phenotype of a strain having the *ho mat***a***l hmlα-2 hmr***a***l sir* genotype. According to the α1-α2 hypothesis (39) for the control of cell type, if the *hmlα-2* mutation gives rise to a defect in the transposition or integration process but not in the regulatory function of α as predicted by hypothesis I, the strain should exhibit the α mating ability by the in situ expression of the information of the *hmlα-2* allele due to the *sir* mutation. On the other hand, if the *hmlα-2* mutation occurred in a common region, i.e., Z_1 or X, of the *HMLα* locus and the region is involved in either the *α1* or *α2* cistron, the strain should show the nonmater phenotype, because none of the α- and **a**-specific genes would be expressed in the case of a defect in the *α1* cistron, whereas all of the α- and **a**-specific genes would be expressed in the case of a defect in the *α2* cistron (hypothesis II). To breed such strains, KA47-12D and KA47-20D, which have the *ho MAT***a** *hmlα-2 HMR***a** *sir3* genotype (phenotype is weak **a** mating type) were crossed by mass mating with strain K167, which bears the *ho mat***a***l HMLα hmr***a***l mar2-3* (*mar2-3* is an allele of the *sir3* mutation [A. J. S. Klar, personal communication]) genotype (phenotype is α), and diploids (KA50 and KA51) were selected by complementation of auxotrophic markers. Among the meiotic segregants from the diploids, we expected to find segregants with the *ho mat***a***l hmlα-2 hmr***a***l sir3* (or *mar2-3*) genotype. Their expected phenotypes would be α according to hypothesis I or nonmater according to hypothesis II. Thus, the predicted segregations in asci of KA50 and KA51 on tetrad analysis differ for the above alternative hypotheses. The result was consistent with the patterns expected for hypothesis II (Table 2).

To see whether the *hmlα-2* mutation occurred in the *α1* or *α2* cistron of the *HMLα* locus, protoplast fusions were performed between strain KA47-12D or KA47-20D, the same strains as in the previous experiment, and strain HT19-8B, which has the *ho MAT***a** *HML***a** *HMR***a** *sir3* genotype (phenotype is **a**). From the above two combinations, i.e., KA52 and KA53, several independent fusants were isolated and tested for sporulation. According to the α1-α2 hypothesis, the *α2* and **a***1* functions are necessary for sporulation. Therefore, if the *hmlα-2* mutation occurred at the *α2* cistron, the fusants should not sporulate, whereas if it occurs at the *α1* cistron, they should. Sixty-four independent fusants, 32 each from the KA52 and KA53 combinations, were tested, and none showed sporulation. This observation strongly suggested that the *hmlα-2* mutation occurred at the *α2* cistron of the *HMLα* gene.

To confirm hypothesis II, we transformed cells of strain KA79-8B, (*ho MAT***a** *hmlα-2 HMR***a**) with plasmid 43A, which carries the *LEU2*$^+$ and *HO* genes. Transformants were selected by the prototrophic phenotype for leucine (Leu$^+$), cultured in YPAD broth, and plated onto a YPAD plate after appropriate dilution. Colonies appearing after incubation at 30°C for 2 days were replica plated onto a Leu test plate of minimum medium supplemented with appropriate nutrients but not with leucine. Several colonies showing leucine auxotrophy (Leu$^-$) were isolated and tested for their mating ability. If hypothesis I is correct, most of the Leu$^-$ colonies (cured of the 43A plasmid bearing the *HO* gene) should show **a** mating type, because the *hmlα-2* element is only rarely inserted into the *MAT* locus. In contrast, if hypothesis II is correct, an individual cell would exhibit

TABLE 2. Segregation of ascus types in asci of diploids KA50 and KA51 constructed by the *MAT***a** *ho hmlα-2 HMR***a** *sir3 mat***a***l ho HMLα hmr***a***l sir3* cross

Category	No. of segregations in the following patterns:		
	2 Ste[a]:2α	3 Ste:1α	4 Ste:0α
Observed[b]	3	31	38
Hypothesis I[c]	20.8	44.0	7.2
Hypothesis II[c]	7.2	38.3	28.4

[a] Sterile segregants showed haploid size and were unable to sporulate.
[b] Pooled data from the two diploids, KA50 and KA51.
[c] In hypothesis I, the *hmlα-2* allele was assumed to have normal functioning of α information. The chi-square value was 135.6 with 2 df ($P < 0.01$). In hypothesis II, the *hmlα-2* allele was assumed to be defective in the *α1* or *α2* function. The chi-square value was 4.6 with 2 df ($0.10 > P > 0.05$). The expected segregations were calculated by assuming that the mating type locus is located at 20 centimorgans on the right arm of chromosome III and those of the *HML* and *HMR* loci are at 64 or 65 centimorgans left and right, respectively, distal to the centromere on the same chromosome (12).

either the sterile or the **a** mating type depending upon whether the defective α (α2) cassette from *hmlα-2* or the **a** cassette from *HMR***a** was inserted into the *MAT* locus when the *HO* gene on plasmid 43A was present in the cell. Of 92 Leu^- colonies tested for their mating ability, 79 were sterile, and the remaining 13 exhibited **a** mating type; cells of both types showed haploid cell size. These observations also supported hypothesis II but not hypothesis I. The observed ratio (79:13) of sterile and **a** clones roughly accorded with the 90:10 ratio of α-factor-insensitive versus α-factor-sensitive cells of the mutant culture described by Oshima and Takano (31).

Analysis by Southern hybridization. To confirm the transposition of the cassette from *hmlα-2*, we physically examined the *MAT* gene of the *hmlα-2* mutant. If the sterile phenotype was caused by integration of the *hmlα-2* mutant cassette into the *MAT* locus, as predicted by hypothesis II, nonmater cells (90% of the population) should have the *HO matα-2 hmlα-2 HMR***a** genotype, the remaining cells (10% of the population) of **a** mating type should have the *HO MAT***a** *hmlα-2 HMR***a** genotype, and the low frequency (0.1%) of sporogenous diploid cells should have the *HO/HO MAT***a***/MATα hmlα-2/hmlα-2 HMR***a***/HMR***a** genotype. If, on the other hand, the *hmlα-2* mutation is responsible for the defect in the switching process and the nonmater phenotype is caused by simultaneous expression of the α information of the diffusible *hmlα-2* fragments and the **a** information at the *MAT* locus (hypothesis I), sterile cells (90% of the population) should have the *HO MAT***a** *hmlα-2 HMR***a** genotype along with the free, diffusible *hmlα-2* element, which is probably a DNA fragment of the *hmlα-2* sequence. The remaining **a** mating type cells (10%) and sporogenous cells (0.1%) should carry the *HO MAT***a** *hmlα-2 HMR***a** genotype and the *HO/HO MAT***a***/MATα hmlα-2/hmlα-2 HMR***a***/HMR***a** genotype, respectively. Thus, 90% of the cells in the mutant population should have physically the same sequence as the *MATα* allele at the *MAT* locus by hypothesis II, whereas almost all the cells should have the *MAT***a** allele by hypothesis I.

To test the above expectations, DNAs were extracted from cells of the mutant strain J-137-6B and the wild-type diploid strain T-1851-2D. DNAs were double digested with *Eco*RI and *Hin*dIII, and the fragments were electrophoresed on agarose gel. Since, like the *HML* and *HMR* loci, the *MAT* locus does not have *Hin*dIII and *Eco*RI restriction sites inside the locus (41) (Fig. 1), these loci can be distinguished from each other by agarose gel electrophoresis. The DNA fragments were transferred to a nitrocellulose sheet by Southern blotting and hybridized with ^{32}P-labeled B2 plasmid DNA, which bears the whole sequence of the *HMLα* gene, as the probe. Since the *HMLα* locus contained the X and Z_1 sequences homologous for all the *HML*, *MAT*, and *HMR* loci and the W and Z_2 regions common to the *HML* and *MAT* loci (Fig. 1), the *HMLα* fragment was able to hybridize with DNA fragments containing the *HML*, *MAT*, and *HMR* loci. The Yα region, however, extends for 747 bp, whereas the Ya region is 642 bp. This difference makes the migration of restriction fragments containing the Yα sequence slower than those containing Ya for all of the *MAT*, *HMR*, and *HML* loci. In the case of the wild-type strain, two hybridization bands at sites corresponding to the *MATα* and *MAT***a** fragments were visible (Fig. 2). But, in the case of the *hmlα-2* mutant, we observed an intense band corresponding to the *MATα* fragment along with a faint band of the *MAT***a** sequence (hardly visible on Fig. 2). This observation indicates that the nonmater cells in the *hmlα-2* culture have physically the same sequence as the *MATα* locus. This fact

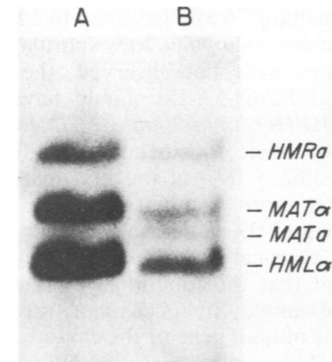

FIG. 2. Migration of DNA fragments encoding the mating-type genes on agarose gel electrophoresis. DNAs of the *hmlα-2* mutant (J-137-6B; lane A) and the wild-type diploid strain (T-1851-2D; lane B) were digested with *Eco*RI and *Hin*dIII. The fragments electrophoresed on agarose gel were transferred onto a nitrocellulose filter by Southern blotting, hybridized with the ^{32}P-labeled plasmid B2 DNA under moderately stringent conditions, and autoradiographed with Kodak X-Omat RP film. Lane A was loaded with approximately twice the amount of DNA as for lane B. Correspondence of bands to *HMLα*, *HMR***a**, *MATα*, and *MAT***a** is indicated on the right-hand margin.

further supports hypothesis II, indicating that the *hmlα-2* sequence was substantially inserted into the *MAT* locus.

Frequency of sporogenous nonmater cells in the *hmlα-2* culture. The population of sporogenous diploid cells in a culture of the *hmlα-2* mutant is ca. 0.1% (31). However, this frequency may not represent the frequency of $MAT\alpha^+$ occurrence at the first mating type switch immediately after spore germination, because the cassette transposition might occur continuously in the subsequent growth of the *MAT***a** *HO hmlα-2 HMR***a** cells. To estimate the frequency of $MAT\alpha^+$ occurrence during the cassette transfer from the *hmlα-2* locus to the *MAT***a** locus, we investigated tetrad segregants from a sporogenous diploid strain isolated from a *HO MAT***a** *hmlα-2 HMR***a** mutant. If the $MAT\alpha^+$ allele did not appear at the first switching after spore germination, all the tetrad clones of an ascus showed a segregation of 2 high-sporulating nonmater:2 **a** clones with low sporulation frequency, but if the $MAT\alpha^+$ allele is produced immediately after the spore germination in one of the **a** spores in an ascus, the segregation will be 3 high-sporulating nonmater:1 **a** clone with low sporulation; and if it is produced in both the **a** spores, a 4 high-sporulating nonmater:0 **a** segregation would be expected. We picked up such a diploid cell in a culture of J-137-6B with the aid of a micromanipulator. This strain bears the genotype *MAT***a***/MATα HO/HO hmlα-2/hmlα-2 HMR***a***/HMR***a**. To avoid the possibility of having picked up the *MAT***a***-α-2/MATα HO/HO hmlα-2/hmlα-2 HMR***a***/HMR***a** genotype, we then selected a high-sporulating diploid clone, J-137-6B-D1, from an ascus of the primary diploid after self-sporulation. (The *MAT***a***-α-2* allele is a *MAT***a** allele with an undetectable *α-2* lesion donated by the previous insertion of the *hmlα-2* element and subsequent replacement of an *HMR***a** element.) Of 62 asci examined by self-sporulation of J-137-6B-D1, all showed a segregation pattern of 2 high-sporulating nonmater:2 **a** clones of low sporulation. We also picked up a similar diploid, KA63-5A-D4, from the cell population of KA63-5A, which has the same genotype for the mating type and homothallic genes as J-137-6B. Of 77 asci dissected after selfsporulation of the diploid, only one ascus showed 3 high-sporulating nonmater:1 **a** clone showing low sporulation,

whereas the remaining 76 asci gave rise to 2 high-sporulating nonmater:2 **a** clones showing low sporulation. Since the other ascus types were not observed, the diploid strains J-137-6B-D1 and KA63-5A-D4 should have the genotype *MAT***a***/MATα HO/HO hmlα-2/hmlα-2 HMR***a***/HMR***a**, and a supposed *MATα*$^+$ allele does occur with a frequency of ca. 0.3% (1 × 100/2[62 + 77]) at the first mating type switch immediately after spore germination.

Base sequencing of the *hmlα-2* allele. The above findings, along with the physical structure of the *MAT* and *HML* genes, suggested that the functional *MATα* allele may be produced, for example, by occasional recombination between the *hmlα-2* mutant gene or the cassette from it and the corresponding DNA sequence of the wild-type *MAT***a** gene. Since a *matα2* cell cannot mate with an **a** cell, and a *matα2/MAT***a** cell cannot sporulate according to the α1-α2 hypothesis, the sporogenous diploid cells found in the *hmlα-2* culture might be produced by zygote formation between an original **a** mating type cell and such a newly generated α mating type cell. Since the frequency of occurrence of the supposed *MATα* cell was high enough (0.1 to 0.3%) to neglect the mutational mechanism, we inferred a recombinational mechanism. This argument further suggested that the *hmlα-2* mutation was situated in a region common to the *HMLα* and *MAT***a** loci. Since the *hmlα-2* mutation is an *α2* mutation, and the *α2* cistron extends over portions of the specific Yα region and the X region (1, 25, 29, 41), the *hmlα-2* mutation might be situated in the X region. To confirm these arguments, we cloned a 3.0-kb fragment containing the mutant *hmlα-2* allele, and the base sequence of the DNA fragment was determined.

The genomic DNA extracted from strain J-137-6B (*HO MAT***a** *hmlα-2 HMR***a**) was double digested with *Eco*RI and *Hin*dIII, and the digest was electrophoresed on a polyacrylamide slab gel. It is known that the entire DNA sequence coding for the *HMLα* gene resides in a 3.0-kb *Eco*RI-*Hin*dIII DNA fragment (Fig. 1). The DNA sample of the *hmlα-2* mutant also showed a band at molecular size of 3.0 kb, as seen in Fig. 2. The 3.0-kb fragment was eluted from the polyacrylamide gel and mixed with the large *Eco*RI-*Hin*dIII fragment of pBR322 pretreated with *E. coli* alkaline phosphatase. Then cells of *E. coli* JA221 were transformed with the DNA mixture after treatment with T4 ligase. About 2,000 transformants showing the ampicillin resistance phenotype were isolated and analyzed by in situ hybridization on a nitrocellulose filter with the ^{32}P-labeled *Eco*RI-*Hin*dIII fragment prepared from plasmid B2 as probe. After several trials, six transformants were found to harbor DNA molecules which hybridized with the probe. To determine whether the *Eco*RI-*Hin*dIII fragments connected with pBR322 contained either the Ya- (642 bp) or Yα- (747 bp) specific regions in the *HML*, *HMR*, or *MAT* locus, their migration on an agarose gel and their specific restriction with *Bgl*II and *Xba*I (Fig. 1) were examined. Results indicated that one of the six chimeric plasmids carried the *HML* gene, whereas the remaining five carried *MAT* (data not shown).

The plasmid having the *HML* gene, designated pMS20, might contain the *hmlα-2* gene, because the cloned DNA was prepared from the *hmlα-2* mutant. To confirm this, a *Hin*dIII fragment of pJDB219 (2), which contains the replication origin of 2-μm DNA and the *LEU2* gene of *S. cerevisiae*, was inserted into the *Hin*dIII sites of pMS20 and of plasmid B2. Then, the yeast strain KA79-8B-α2, which has the *ho mata-2 hmlα-2 HMR***a** genotype, was transformed with these chimeric plasmids. Of seven Leu$^+$ transformants, no cells of α mating type could be found with the plasmid constructed

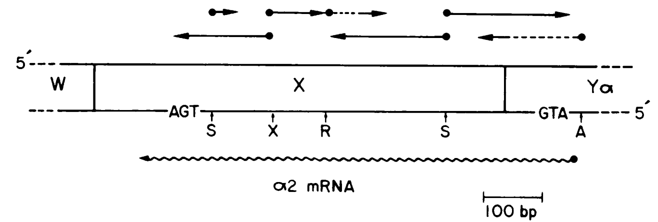

FIG. 3. Restriction map and strategy for sequencing the *hmlα-2* gene. The extent and direction of sequencing are indicated by the arrows. All the DNA fragments sequenced were 5' end labeled (closed circles). A, R, S, and X indicate the restriction sites for *Alu*I, *Rsa*I, *Sau*96I, and *Xba*I, respectively.

with pMS20, whereas the plasmid constructed with B2, which contains the wild-type *HMLα* gene, could confer α mating type to the yeast host. This indicates that the cloned gene on pMS20 is indeed *hmlα-2*.

The entire nucleotide sequence of the putative *α2* reading frame (1) on the *hmlα-2* gene was determined by the strategy shown in Fig. 3, and the result was compared with that of the wild-type *HMLα* allele described by Astell et al. (1). We found two apparent differences between the mutant and wild-type *HMLα* sequences (Fig. 4). One is the deletion of a single C · G pair in the *hmlα-2* DNA at position 1,419 of the wild-type sequence (i.e., the 9th base pair from the X-Y boundary); the other is a nucleotide variation from the A · T pair of the wild-type gene to C · G in the *hmlα-2* mutant at position 1,336 of the wild-type sequence (the 92nd base pair from the X-Y boundary). Since position 1,419 is involved in the open reading frame of the *α2* protein, deletion of this base causes a frame shift of the triplet code and consequently generates a nonsense codon, TAA, at positions 1,373 to 1,375 of the wild-type sequence of the *HML* locus. (The coding order is in the reverse direction to the base numbering of this region.) Thus, the *hmlα-2* gene is expected to produce a polypeptide which is about one fourth of the molecular size of that of the wild-type gene. On the other hand, it appears unlikely that the A · T to C · G change at position 1,336 contributes to the *hmlα-2* phenotype, because the same C · G pair variation was found on the *MAT* locus in a certain strain (1). Thus, we concluded that the deletion of one bp at position 1,419 is responsible for the *hmlα-2* mutant phenotype.

DISCUSSION

The results clearly negated the hypothesis I, because the major population of the *hmlα-2* cultures was nonmater cells having physically the same molecular size as *MATα* at the *MAT* region. Thus, it is possible to conclude that the nonmater cell has the *matα2* genotype which is probably transposed from the *hmlα-2* mutant gene and, therefore, that the *hmlα-2* cassette was substantially transposed to the *MAT* locus. However, sporogenous diploid cells were always found in the *hmlα-2* culture at a low frequency (ca. 0.1%) as described by Oshima and Takano (31). Because the *matα2/MAT***a** diploid cells cannot sporulate, these sporogenous cells must have the functional allele of *MATα*.

The *hmlα-2* mutation was revealed to be a 1-bp deletion at the ninth bp from the X-Y boundary in the X region, and the mutation gives rise to a shift of the open reading frame in the *α2* cistron. Although we also found a 1-bp divergence from A · T to C · G pair in the *hmlα-2* mutant at position 92 from the X-Y boundary in the X region, the same sequence

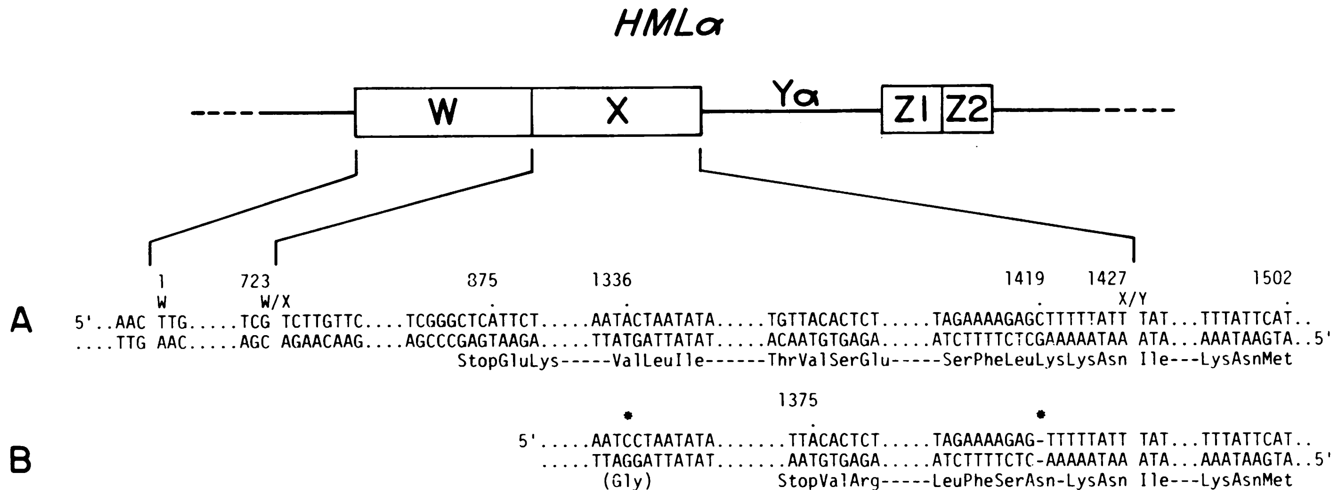

FIG. 4. Nucleotide sequence of the region coding for the α*2* cistron of the *hml*α*-2* gene. The corresponding wild-type region (A) is adopted from Astell et al. (1). The bp indicated by asterisks in the mutant DNA (B) were deleted or altered from the wild-type sequence. The amino acid sequences of the wild-type and mutant α*2* cistrons are shown on a line below the coding sequences. The nucleotide and amino acid sequences at the regions shown by the dotted or broken lines were omitted.

divergences have been found at the same position of the *MAT* locus by Astell et al. (1). Because the C · G pair at position 1,336 (position 92 from the X-Y boundary) is conserved in *MAT*α and *MAT***a**, whereas *HML*α has the A · T pair, Astell et al. (1) assumed that the left-hand boundary of the sequence transposed from *HML*α is between 1,336 and 1,427 (the X-Y boundary). However, present observation that the bp at position 1,336 of *hml*α*-2* mutant is C · G, and that the DNA segments of Astell et al. bearing *MAT*α and *MAT***a** were cloned from an independent strain from that bearing *HML*α (1, 28), we cannot at present decide whether or not position 1,336 is included in the transposable segment. The bp divergence at position 1,336 may depend on a strain difference.

That a part of the α*2* cistron extends over a homologous region for the *MAT*α and *MAT***a** genes was suggested by genetic study by Sprague et al. (36); *MAT*α$^+$ recombinants were produced at high frequency (ca. 2×10^{-3}) by meiotic recombination in *mat*α*2*/*MAT***a** diploid cells, but at low frequency (ca. 5×10^{-5}) in the *mat*α*1*/*MAT***a** diploid cells. These arguments accord well with the observations by physical analyses of the mating type genes and their transcriptional products (25, 29, 43), that the α*2* cistron extends over the X and Yα regions, whereas most of the coding region of α*1* cistron is involved in the Yα sequence. Since the same *mat*α*2* mutation, *mat*α*2-1*, used by Sprague et al. (36) was effectively switched to *MAT*α by the homothallic conversion (37), the mutational site of this allele may also be part of the transposable segment. These observations indicate that a part of the homologous region, X, is also involved in the cassette.

In the homothallic switching of the *mat***a** missense allele with an *hmr***a** nonsense donor, Klar et al. (24) have observed a high frequency of *MAT***a**$^+$ recombinants (12 of 32 cases tested). They also have observed a similarly high frequency (7 in 43 cases tested) of *MAT***a**$^+$ in the reverse combination of the same mutant alleles. To explain the high frequency of *MAT***a**$^+$, these authors have proposed an intermediate step in mating type interconversion, which allows recombination between donor and recipient DNAs. In contrast, we observed the supposed *MAT*α$^+$ allele through the transposition process in a frequency of ca. 0.3% of the event. It should be noted, however, that the experiments performed by Klar et al. (24) involved transposition of a homologous cassette from *HMR* to *MAT*, whereas the cassette transposition in the present study is heterologous, from *hml*α to *MAT***a**.

In explaining the low frequency of *MAT*α$^+$ appearance in the present study, the recent communication of Strathern et al. (40) is of particular interest. These authors have described the mating type conversion to be initiated by a double-stranded cut at the Y-Z boundary of *MAT*, and then the mating-type-specific region on the *MAT* DNA is digested immediately or after a brief life to allow the recombination between the donor and preexistent recipient cassette. DNA synthesis on the recipient (i.e., *MAT*) locus starts from the Y-Z boundary, the left end of the cassette in the X region, or both, in favor of the donor DNA as a template with the aid of the sequence homologies of the bases at the Z and X regions. In the experiments by Klar et al. (24), the donor *hmr***a** DNA and the recipient *mat***a** DNA, however, were homologous even at the Y regions, and, therefore, both strands may have been able to form a double-stranded DNA molecule, which might be resistant to digestion. The heteroduplex formation might be followed by mismatch-repair at the mutation sites. If mismatch-repairs occur independently for both the nonsense and missense sites on the heteroduplex formed with, for example, the *hmr***a** nonsense and *mat***a** missense DNA strands, and because the mismatch-repair is thought to occur effectively during meiotic DNA duplication and gene conversion in *S. cerevisiae* (7), it is possible to expect that 25% of heteroduplexes will give rise to the *MAT***a**$^+$ allele. The expected frequency of *MAT*α$^+$ coincides fairly well with the observation by Klar et al. (24).

Although the *hml*α*-2* mutation is located in the X region, the low frequency of the possible *MAT*α$^+$ appearance in comparison with that of Klar et al. (24) may be due to the stability of the DNA heteroduplexes formed at the Y region. In the regular mating type switch, the combination of the donor and recipient DNAs is heterologous, because the Y**a** and Yα regions have to be exchanged, and no stable heteroduplex DNA is formed. Thus, the recipient DNA will be quickly degraded soon after the double-stranded cut. This

fact further suggests that the mating type information of the donor locus will be transferred to the *MAT* locus without modification by the influence of the preexistent recipient DNA in the heterologous combination, as observed in the regular mating type switch.

In the case of the *hml*α*-2* mutation, the mutation site is on the cassette and simultaneously in the X region, which is homologous with that of the recipient DNA. Therefore, it is possible that the region of the *hml*α*-2* mutation will form, even though in a low frequency, a heteroduplex DNA between the donor and recipient DNAs. This will be followed by mismatch-repair and give rise to the *MAT*α^{+} allele in the convertant.

However, it is possible to speculate as another alternative mechanism that the left end of the cassette is variable and the *hml*α*-2* mutation site is situated close to the left end. Thus, the switching event in a certain cell might cease before transfer of the *hml*α*-2* site. These arguments remain to be investigated.

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