Kluyveromyces lactis Sir2p Regulates Cation Sensitivity and Maintains a Specialized Chromatin Structure at the Cryptic α-Locus

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ABSTRACT

In *Saccharomyces cerevisiae*, transcriptional silencing of the cryptic mating type loci requires the formation of a heterochromatin-like structure, which is dependent on silent information regulator (Sir) proteins and DNA sequences, called silencers. To learn more about silencing, we characterized the mating type loci from the yeast *Kluyveromyces lactis*. The *K. lactis MAT*, *HMR***a**, and *HMLα* loci shared flanking DNA sequences on both sides of the loci presumably acting as recombinational targets during mating type switching. *HMR***a** contained two genes, the **a***1* gene similar to the Saccharomyces **a***1* gene and the **a***2* gene similar to mating type genes from other yeasts. *K. lactis HMLα* contained three genes, the α*1* and α*2* genes, which were similar to their Saccharomyces counterparts, and a novel third gene, α*3*. A dam-methylase assay showed Sir-dependent, but transcription-independent changes of the chromatin structure of the *HMLα* locus. The *HMLα3* gene did not appear to be part of the silent domain because α3p was expressed from both *MATα3* and *HMLα3* and *sir* mutations failed to change the chromatin structure of the *HMLα3* gene. Furthermore, a 102-bp silencer element was isolated from the *HMLα* flanking DNA. *HMLα* was also flanked by an autonomously replicating sequence (*ARS*) activity, but the *ARS* activity did not appear to be required for silencer function. *K. lactis sir2* strains grown in the presence of ethidium bromide (EtBr) accumulated the drug, which interfered with the essential mitochondrial genome. Mutations that bypassed the requirement for the mitochondrial genome also bypassed the EtBr sensitivity of *sir2* strains. Sir2p localized to the nucleus, indicating that the role of Sir2p to hinder EtBr accumulation was an indirect regulatory effect. Sir2p was also required for growth in the presence of high concentrations of Ni^{2+} and Cu^{2+}.

MOST fungi have distinct cell types. In *Saccharomyces cerevisiae*, these cell types are the two haploid mating types, **a** and α, and haploid cells of the opposite mating types can fuse and form the third cell type, the **a**/α diploid. In haploid strains of *S. cerevisiae*, there are three loci that encode mating type information. The mating type is determined by the allele present in the expressed *MAT* locus. In addition, there are two transcriptionally silent loci, one containing the **a**- and the other containing the α-mating type information. These loci, called *HMR***a** and *HMLα*, are kept silent despite the presence of functional promoters and structural genes (Loo and Rine 1994; Lustig 1998). The proteins encoded by the *MAT* locus are transcription factors that either repress or activate the transcription of target genes. If the **a** and α information is expressed simultaneously, which occurs in a diploid cell, then mating pheromones and the receptors for the mating pheromones are not expressed (Herskowitz *et al.* 1992). This means that a diploid cell cannot mate. Haploid cells unable to silence the transcription of the *HM* loci due to a mutation in a gene required for silencing behave like a diploid in these aspects and are thus sterile.

The silencing of the cryptic mating type loci is an example of a position effect on gene expression, and these loci appear to be silenced by the formation of a repressive chromatin structure functionally analogous to heterochromatin. Other genes inserted into the cryptic mating type loci can be silenced, thus ruling out gene-specific mechanisms for silencing (Schnell and Rine 1986). Moreover, histones H3 and H4 are involved in silencing (Kayne *et al.* 1988) and the *HMR***a** locus is resistant to DNA-modifying enzymes both *in vivo* and *in vitro* (Singh and Klar 1992; Loo and Rine 1994). Position effects on gene expression are a widespread phenomenon, and in yeasts position effects have also been found close to telomeres (Gottschling *et al.* 1990) and centromeres (Allshire *et al.* 1995) and in the rDNA locus (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997). In other eukaryotes, intensively studied examples include X chromosome inactivation in mammalian females and the position-effect variegation observed in *Drosophila melanogaster* (Grigliatti 1991; Rastan 1994; Hennig 1999).

Several proteins have been identified that are required for silencing in *S. cerevisiae*. Some act by binding DNA

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sequences called silencers, which flank the cryptic mating type loci. The best-characterized silencer, *HMR-E*, contains binding sites for origin recognition complex (ORC), a protein complex involved in replication initiation, as well as binding sites for two widely used transcriptional activators, Rap1 and Abf1. Specific mutations in *ORC2*, *ORC5*, *RAP1*, and *ABF1* lead to the derepression of *HMR***a** (Sussel and Shore 1991; Foss *et al.* 1993; Micklem *et al.* 1993; Loo *et al.* 1995a,b). Other proteins seem to act on the silent loci by utilizing the silencer elements to establish a specialized chromatin structure, which is spread across the cryptic mating type loci. The most prominent members of these proteins are the silent information regulator (Sir) proteins, which are required for silencing at both the cryptic mating type loci and telomeres (Ivy *et al.* 1986; Rine and Herskowitz 1987; Aparicio *et al.* 1991). The Sir proteins are recruited to the cryptic loci by direct interaction to silencer binding proteins such as Rap1 (Moretti *et al.* 1994). Sir2, Sir3, and Sir4 also interact with each other and histones H3 and H4 to form a repressing chromatin structure that facilitates silencing (Hecht *et al.* 1995; Moazed *et al.* 1997). Sir proteins do not appear to interact directly with DNA. However, chromatin immunoprecipitation experiments show that both telomeric DNA and the cryptic mating loci contain Sir proteins as part of the chromatin at these loci (Strahl-Bolsinger *et al.* 1997).

This study contains the characterization of silencing of the cryptic α-locus from the budding yeast, *Kluyveromyces lactis*. Comparing the Kluyveromyces cryptic mating type loci with their Saccharomyces counterparts revealed both similarities and differences. Among the differences, *K. lactis HML*α contains an additional gene missing in *S. cerevisiae*. This α*3* gene showed α-specific expression, but was expressed from both *HML*α and *MAT*α in wild-type strains. A silencer element flanking *HML*α was found, and this element did not contain any apparent binding sites for Rap1, Abf1, or ORC. As in *S. cerevisiae*, Sir proteins controlled the chromatin structure at *HML*α. In the case of Sir2p, this protein appeared to control functions unrelated to mating type, telomeres, or rDNA.

MATERIALS AND METHODS

Cloning and sequencing of *HML*α and *HMR*a: *Escherichia coli* strain DH5α was transformed with a genomic *K. lactis* library in plasmid pAB24 (*2*μ*m*, *URA3*). Approximately 20,000 transformants were screened by colony hybridization using a PCR fragment corresponding to the *K. lactis* α*1* open reading frame (ORF) as probe. Several plasmids were recovered from colonies that hybridized to the probe. The DNA sequence of the insert of one of these isolates (M1) and also in part two overlapping isolates obtained from S. Fields (A1 and B1; Yuan *et al.* 1993) was determined on both strands by primer walking. Clones encoding *K. lactis HMR***a** were isolated in a similar fashion using a probe that was a PCR fragment that contained sequences from the α*1* gene 3′-flanking region including the R sequence (Figure 1). This probe corresponded to nucleotides 74–700 downstream of the α*1* stop codon. The sequence of two overlapping clones (B and K) was partially determined on both strands by primer walking. Sequencing was performed using a Prism sequencing kit (Applied Biosystems Inc., Foster City, CA) and a DNA sequencer (model 373; Molecular Dynamics, Sunnyvale, CA). These sequences have been submitted to the GenBank database under the accession nos. AF195066 (*HML*α) and AF195067 (*HMR***a**).

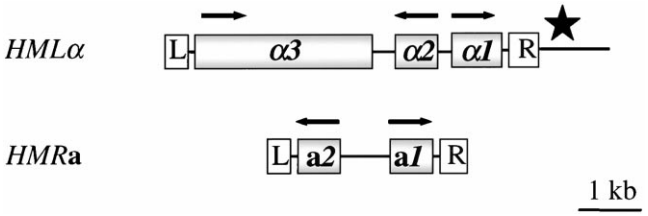

Figure 1.—Genomic organization of the *K. lactis HML*α and *HMR***a** loci. Arrows indicate the direction the genes are transcribed and the boxes labeled L and R correspond to the repeated sequences that were shared between the *HML*, *HMR*, and *MAT* loci. The star shows the position of the silencer.

Plasmid constructions: The pRS306 α*1::KanMX* plasmid (p350) was made in three steps. First a 3428-bp *Mun*I fragment from *HML*α containing clone M1 was cloned into the *Eco*RI site of pRS306 (Sikorski and Hieter 1989) forming p345. Plasmid 345 was subjected to *in vitro* mutagenesis (Kunkel 1985) using an oligonucleotide (5′-GGAGCATTCGATTG CATGCTTTGGCGTTAC-3′) that introduces a *Sph*I site at the α*1* gene start codon, which generated plasmid p348. A 900-bp PCR fragment containing the *KanMX* gene (Wach *et al.* 1994) with a *Sph*I site at the start codon and an *Eco*RI site downstream of the ORF was cloned into the corresponding sites of p348. The resulting vector, p350, thus exchanged amino acids 1–122 of the α*1* gene for a *KanMX* gene. The pRS306 α*3::LEU2* plasmid (p332) was made in three steps. A 4383-bp *Cla*I-*Eco*RI fragment from M1 containing the entire α*3* ORF was cloned into the corresponding sites of pRS306, resulting in vector p322. Then p322 was digested with *Mun*I, resulting in a deletion of internal α*3* sequences corresponding to amino acids 138–678, and the vector fragment was ligated to an oligonucleotide (5′-AATTAGATCT-3′), resulting in the conversion of the *Mun*I site into a *Bgl*II site. Into the resulting vector, a 2.9-kb *Bgl*II fragment containing the *LEU2* gene from vector pCXJ20 (Chen 1996) was cloned, thus resulting in vector p332 (pRS306 α*3::LEU2*). The pRS306 *hml*αΔp (p400) construct was generated in a single step by cloning two PCR fragments corresponding to the α*1* and α*2* genes into pRS306, exchanging the promoter region in between the two genes for a *Bam*HI-*Alw*I site. A 1747-bp *Xho*I-*Bam*HI PCR fragment containing the 3′ end of the α*3* gene plus the α*2* ORF and an 801-bp *Bam*HI-*Xba*I PCR fragment containing the α*1* ORF was combined with a *Xho*I-*Xba*I-digested pRS306 in a three-factor cloning, generating p400. To generate the pCXJ20-*GFP-SIR2* construct, in which the fusion gene is driven by a glycerol phosphate dehydrogenase gene promoter (*GPD*), a 2.0-kb *Mun*I-*Xba*I PCR fragment containing the entire *SIR2* gene (Chen and Clark-Walker 1994) with the *Mun*I site inserted at the start ATG codon was cloned into the *Eco*RI-*Xba*I sites of vector pJW192 (*2*μ*m TRP1 GPD-GFP*; Whistler 1996), thus generating an in-frame *GFP-SIR2* fusion gene. Then a 3.4-kb *Hin*dIII-*Xba*I fragment from the resulting vector was cloned into the corresponding sites of pCXJ20(*CEN ARS LEU2*), resulting in p280. Plasmid 280 complemented strains containing the *sir2::URA3* allele with respect to mating deficiency and

ethidium bromide (EtBr) sensitivity. Plasmid 291 was a 3.2-kb *Pst*I-*Nsi*I fragment from A1 cloned into the *Pst*I site of pCXJ18, and p300 was generated by cloning a 4.7-kb *Pst*I-*Bgl*II fragment from A1 into the *Pst*I-*Bam*HI sites of pCXJ18. PCR fragments with a *Bam*HI site in the 5′ end and a *Kpn*I site in the 3′ end were cloned into the corresponding sites of p291. The PCR fragments corresponded to different parts of the α*1* gene 3′-flanking DNA [the first base after the stop codon of the α*1* gene was defined as nucleotide (nt) 1], p324 (nt 521–1078), p339 (nt 521–902), p311 (nt 726–1571), p386 (nt 521–818), p357 (nt 563–1078), and p414 (nt 521–622). Some PCR fragments were also cloned into the *Bam*HI-*Kpn*I sites of pRS306 to assay for *ARS* activity, p302 (nt 356–1571), p316 (nt 521–1078), p333 (nt 521–902), and p305 (nt 726–1571). A 1.6-kb *Hin*dIII-*Xba*I fragment containing the *E. coli dam*$^+$ gene from vector pJR1830 (J. Rine, unpublished results) was cloned into the corresponding sites of pCXJ20 (*LEU2*) and pCXJ18 (*URA3*), generating vectors p365 and p401, respectively.

Yeast media and methods: Rich media, sporulation media, and conditions for matings were as described (Chen and Clark-Walker 1994; Adams *et al.* 1998). Specifically, matings were performed by mixing 2 × 5 μl of suspensions (A600 = 2) of the two mating partners on a 2 × 2-cm area on a YEPD plate. The mating plate was incubated for 18–24 hr at 30°, replica-plated onto selective medium, and incubated for ~72 hr before the results were scored. Preparation of yeast RNA/DNA and conditions for RNA and DNA blots were described before (Adams *et al.* 1998). Quantification of signals was performed using a Phosphorimager (Molecular Dynamics) and Imagequant software. In the dam-methylase assay, an [α-^{32}P]dCTP-labeled PCR fragment corresponding to the α*1* ORF was used as probe. To detect the *Alw*I site in the α*1* gene, the DNA (2 μg) was digested with *Eco*RV and *Alw*I for 4 hr at 37° and the 572-bp band (cut) was compared to the 957-bp (uncut) band. The *Alw*I site in between α*1* and α*2* genes was detected by digesting with *Cla*I, *Hin*dIII, and *Alw*I for 4 hr at 37° and the 520-bp (cut) and 760-bp (uncut) bands were compared. For the *Bcl*I site in the α*3* gene, the DNA was digested with *Cla*I (2 hr, 37°) and *Bcl*I (4 hr, 50°) and the 2.7-kb (cut) and 4.3-kb (uncut) bands were compared. This procedure was repeated with independent DNA preparations with very similar results. EtBr staining was performed by adding the drug at 0.5 μg/ml to logarithmically growing cultures (A600 = 0.5). The cells were harvested after 1–4 hr and prepared for fluorescence microscopy by staining with DAPI. Cation sensitivity assays were performed by adding a drug disc, to which 6 μl of a specific cation solution had been added previously, to a YEPD plate with a uniform lawn of the tester strain. The plate was incubated for 48 hr and the inhibition zone caused by the drug disc was measured. The following solutions were tested: saturated $CuSO_4$, 2 M $NiCl_2$, 2 M $MnCl_2$, 0.5 M $CoCl_2$, and saturated $PbSO_4$.

Strain constructions: The strains used in this study are listed in Table 1. Strain SAY119 was generated by crossing strain CK213 with SAY102 followed by tetrad analysis. Strains with *matα1::KanMX* or *hmlα1::KanMX* alleles were generated by a two-step gene disruption procedure (Scherer and Davis 1979) by transforming SAY119 (*MAT*α) and CK213 (*MAT***a**) with *Pac*I-linearized p350. This procedure generated strains carrying either the *matα1::KanMX* or the *hmlα1::KanMX* disruptions and the allele present in a particular strain was identified by DNA blot hybridizations, generating strains SAY128 (*matα1::KanMX*), SAY129 (*MAT*α *hmlα1::KanMX*), and SAY130 (*MAT***a** *hmlα1::KanMX*). An identical procedure was used to generate the *matα3::LEU2* and *hmlα3::LEU2* alleles except that strain SAY119 was transformed with *Pac*I-linearized p332, generating strains SAY120 (*hmlα3::LEU2*) and SAY121 (*matα3::LEU*). The double mutant *matα3::LEU2 hmlα3::LEU2* strain was generated in two steps. Crossing strain SAY120 with CK213 generated a *MAT***a** *hmlα3::LEU2* strain (SAY122). SAY122 was crossed to SAY121 and from this cross the double mutant *matα3::LEU2 hmlα3::LEU2* strain (SAY124) was recovered. Since *HML*α and *MAT*α were tightly linked, no nonparental ditypes with respect to the *LEU2* gene were found in this cross so the double mutant strain was identified by a DNA blot hybridization using genomic DNA from haploids arising from a meiosis with a tetratype segregation. Plasmid 400 was linearized with *Pac*I and transformed into CK213 to generate the *MAT***a** *hmlα*Δp strain SAY186 by a two-step gene disruption procedure. Crossing SAY186 to CK57-7A (*MAT*α *sir2::URA3*) and SAY 96 (*MAT*α *sir4::LEU2*), respectively, generated the *hmlα*Δp *sir2::URA3* (SAY189) and *hmlα*Δp *sir4::LEU2* (SAY191) double mutant strains. The *hmlα1::KanMX sir2* double mutant strains, SAY155 and SAY156, were spontaneous G418-resistant isolates from strains SAY129 and SAY130, respectively. These isolates behave as *sir2* null alleles with respect to mating defects and EtBr sensitivity and are complemented by a single copy plasmid carrying *SIR2*.

RESULTS

The *K. lactis* cryptic α-locus (*HMLα*) contains three genes: To learn more about mating type in *K. lactis*, we cloned and characterized a locus encoding α-mating type information. We used a functional homologue of *S. cerevisiae* α*1* from Kluyveromyces (Yuan *et al.* 1993) to screen a *K. lactis* genomic plasmid library to identify a complete α-locus. This locus contained three putative genes (Figure 1). The α*1* gene, encoding a protein of 261 amino acids sharing 30% identity with *S. cerevisiae* α1p, can functionally complement a *S. cerevisiae* α*1*$^-$ mutant (Yuan *et al.* 1993). A second gene was found that encoded a protein that shared 31% identity with Saccharomyces α2p. As in *S. cerevisiae*, the α*1* and α*2* mating type genes in *K. lactis* were divergently transcribed from a common promoter region. On the 3′ end of the coding strand for α*2*, a third large open reading frame was found, which we called α*3*. The putative peptide encoded by this gene was 897 amino acids and lacked all significant homology to other proteins in GenBank. Since we previously determined that *K. lactis* contains both an expressed and a silent α-locus (Åström and Rine 1998), we had to determine which of these loci we had cloned. The restriction map of our sequence combined with DNA blot analysis of *MAT***a** and *MAT*α strains showed that the locus sequenced by us was the cryptic α-locus (data not shown), which we thus call *K. lactis HML*α.

To compare the sequences shared between *MAT*α and *HML*α, as a prelude to identifying a silencer, DNA blots were performed with various DNA probes from the α*1*, α*2*, and α*3* genes. The blots showed that all three genes were duplicated in the *K. lactis* genome and thus present at both the *MAT*α and *HML*α loci. Furthermore, these data revealed that *MAT*α and *HML*α shared at least 304 bp, but <885 bp of flanking DNA upstream of the α*3* start codon (as drawn) and ≥370

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
WM52V4	*MAT*α *ade1 adeX his7 uraA1*	Chen and Clark-Walker (1994)
CK213-4C	*MAT***a** *lysA1 trp1 leu2 metA1 uraA1*	Chen and Clark-Walker (1994)
CK56-7A	*MAT*α *uraA1 ade1 mgi1-1*	Chen and Clark-Walker (1994)
CK164	CK56-7A *sir2::URA3*	Chen and Clark-Walker (1994)
CK57-7A	*MAT*α *uraA1 ade1 sir2::URA3*	Chen and Clark-Walker (1994)
SAY90	CK213 *sir4::LEU2*	Åström and Rine (1998)
SAY96	*MAT*α *uraA1 leu2* or *LEU2 ade1 lysA1 trp1* or *TRP1 sir4::LEU2*	Åström and Rine (1998)
SAY99	*MAT***a** *uraA1 metA1 sir2::URA3*	Åström and Rine (1998)
SAY102	*MAT***a** *uraA1 leu2 lysA1 metA1 trp1 sir2::URA3*	Åström and Rine (1998)
SAY105	*MAT***a** *uraA1 leu2* or *LEU2 lysA1 sir4::LEU2*	Åström and Rine (1998)
SAY119	*MAT*α *uraA1 ade1 leu2 trp1 metA1*	This study
SAY120	SAY119 *hml*α*3::LEU2*	This study
SAY121	SAY119 *mat*α*3::LEU2*	This study
SAY122	*MAT***a** *uraA1 leu2 lysA1 trp1 metA1 hml*α*3::LEU2*	This study
SAY124	*mat*α*3::LEU2 uraA1 ade1 leu2 trp1 metA1 hml*α*3::LEU2*	This study
SAY128	SAY119 *mat*α*1::KanMX*	This study
SAY129	SAY119 *hml*α*1::KanMX*	This study
SAY130	CK213 *hml*α*1::KanMX*	This study
SAY155	SAY129 *sir2*	This study
SAY156	SAY130 *sir2*	This study
SAY186	CK213 *hml*αΔp	This study
SAY189	*MAT***a** *uraAI leu2 trp1* or *TRP1 sir2::URA3 hml*αΔp	This study
SAY191	*MAT***a** *uraAI leu2 trp1 lysAI metAI sir4::LEU2 hml*αΔp	This study

bp, but <750 bp downstream of the α*1* stop codon (as drawn).

The *K. lactis* cryptic a-locus contains two genes: When a probe corresponding to the α*1* 3′-flanking DNA was used on DNA blots from genomic *K. lactis* DNA, three bands were present. Two bands were of invariant size, but one band's size was cell type specific, and thus varied between *MAT***a** and *MAT*α strains (data not shown). We speculated that this flanking sequence was shared among the *MAT*, *HML*, and *HMR* loci and that the variable sized band corresponded to a *MAT* locus containing restriction fragment. This speculation was later confirmed when the silent **a**-locus (*HMR***a**) was cloned from a genomic library, using a probe corresponding to the α*1* 3′-flanking DNA. The sequence of *HMR***a** revealed that *HML*α and *HMR***a** shared common flanking sequences on both sides (Figure 1), and we call these sequences L (left) and R (right; Figure 1). The L sequence on the left side of both *HML*α and *HMR***a** (as drawn) was 250 bp long and the R sequence on the right side of both *HML*α and *HMR***a** (as drawn) was 360 bp long. Presumably the L and R sequences act as homologous blocks for resolving recombination intermediates during mating type interconversion. Further sequence analysis of *K. lactis HMR***a** revealed the presence of two ORFs. One of these ORFs (228 amino acids) shared 46% identity in the last 50 carboxyl-terminal amino acids with the Saccharomyces **a**1 protein. This domain corresponded to the homeodomain of Saccharomyces **a**1p. The sequence of the *K. lactis* **a***1* gene indicated the presence of an intron in the gene. The intron splice donor/acceptor sites were in frame with the potential unspliced message, so further experiments are required to determine the length of the **a**1 protein. The other ORF (256 amino acids) was not similar to the Saccharomyces **a**2 gene. Rather this gene shared similarities to genes from the mating type loci of other yeasts, such as the sporulation minus regulator 2 (*SMR2*) gene from *Podospora anserina* (Debuchy *et al.* 1993) and the *MAT 1-3* gene from *Gibberella fujikuroi*. The proteins encoded by these genes all contain a high mobility group (*HMG*) DNA binding motif, characteristic of proteins that bind DNA in the minor groove.

Matα1p is required for α-mating proficiency: To learn more about the function of the genes encoded by the α-locus, we generated strains in which the α*1* gene at *MAT* and *HML* was replaced by a *KanMX* gene, which encodes a protein that mediates resistance against the aminoglycoside Geneticin. The replacement was constructed such that the promoter of the α*1* gene transcribed the *KanMX* gene (see materials and methods). Strains with *mat*α*1::KanMX* or *hml*α*1::KanMX* alleles were then tested for mating ability with a *MAT***a** tester strain. The *mat*α*1::KanMX* mutant exhibited a large mating defect, whereas the *hml*α*1::KanMX* mutant was indistinguishable in mating efficiency from the wild-type control strain (Figure 2A). Thus, Matα1p was required for α-mating proficiency and the *HML*α*1* gene did not contribute to the α1 protein function. It was interesting to note that in quantitative mating determi-

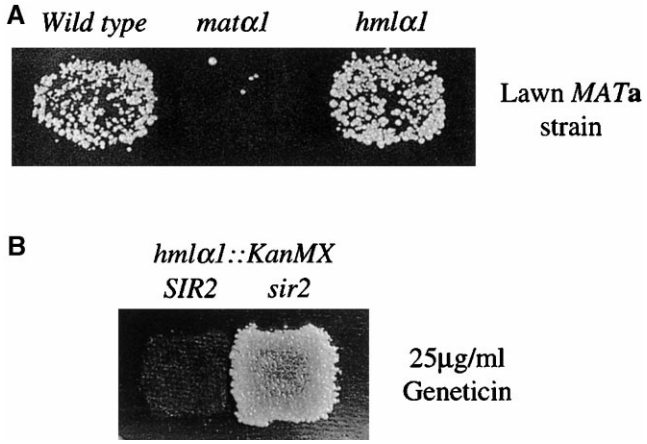

FIGURE 2.—(A) The *MATα1* but not the *HMLα1* gene was required for α-mating proficiency. Patch matings of strains SAY119 (*MAT*α), SAY128 (*matα1::KanMX*), and SAY129 (*hmlα1::KanMX*) are shown. The *MAT***a** mating type tester strain was CK213 and diploids were selected on synthetic dextrose plates supplemented with uracil, leucine, tryptophane, and methionine (SD + Ura, Leu, Trp, and Met). (B) The silencing of the *hmlα1::KanMX* gene required Sir2p. Patches of strains SAY129 (*hmlα1::KanMX*) and SAY155 (*hmlα1::KanMX sir2*) were replica-plated onto rich media (YEPD) containing 25 μg/ml Geneticin and incubated for 72 hr at 30°.

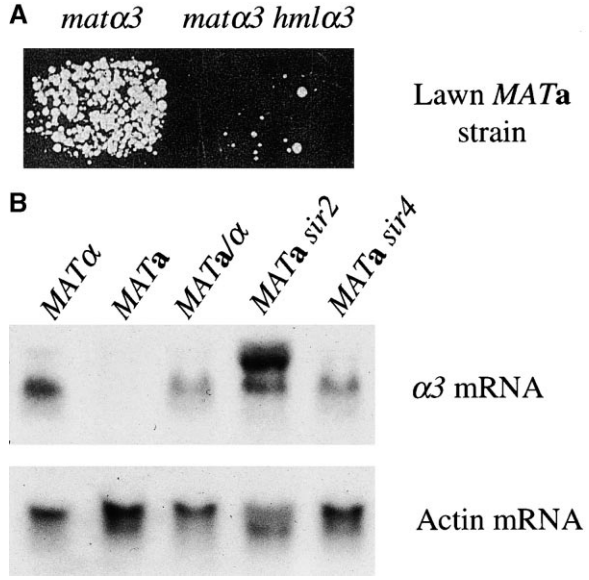

FIGURE 3.—(A) The *MATα3* and *HMLα3* genes were redundant for a function that ensured optimal α-mating proficiency. Patch matings of strains SAY121 (*matα3::LEU2*) and SAY124 (*matα3::LEU2 hmlα3::LEU2*) are shown. The *MAT***a** mating type tester strain used was CK213 and diploids were selected on SD + Ura, Lys, Trp, and Met. (B) The *α3* gene was an α-specific gene that required both Sir2p and Sir4p to remain transcriptionally repressed. RNA blot hybridization of *K. lactis* total RNA from strains WM52 (*MAT*α), CK213 (*MAT***a**), CK213XWM52 (*MAT***a**/α), SAY99 (*MAT***a** *sir2::URA3*), and SAY90 (*MAT***a** *sir4::LEU2*). The blot was probed with a radiolabeled PCR fragment corresponding to the middle portion of the *α3* gene (top), and then the blot was stripped and reprobed with an actin probe (bottom). The migration of the *α3* transcript indicated a size of 2.5–3.0 kb, consistent with the size of the *α3* ORF.

nations with a large surplus of the *MAT***a** strain, the *matα1::KanMX* strain did not show a severe mating defect (data not shown). This behavior is fundamentally different from the corresponding Saccharomyces mutant strains, in which *α1*$^-$ null mutants are unable to mate even in the presence of a large excess of *MAT***a** cells.

Silencing at *HML* is not specific to α-genes: The phenotypic difference between *α1::KanMX* insertions at *MATα1* and *HMLα1* was most likely due to the transcriptional silencing of the *HML*α locus. Consistent with this model, the *hmlα1::KanMX* strain was sensitive to Geneticin (Figure 2B), thus implying that the *KanMX* gene was subject to repression when integrated at the *HML*α locus. An alternative hypothesis was that the *hmlα1::KanMX* allele was nonfunctional, due perhaps to the PCR procedure employed to generate the disruption construct. To distinguish between these two possibilities we combined the *hmlα1::KanMX* allele with a mutation in the *sir2* gene and compared the growth of the *hmlα1::KanMX* strain with the double mutant *hmlα1::KanMX sir2* strain on media containing Geneticin. The double mutant strain grew on plates containing 25 μg/ml of Geneticin, but the *hmlα1::KanMX* strain did not (Figure 2B). This result thus confirmed that the *KanMX* gene present at *HML*α was functional, yet not expressed. Thus, the silencing at *HML*α was not gene specific but was Sir2p dependent.

Either Matα3p or Hmlα3p is required for α-mating proficiency: The *α3* gene at *K. lactis HMLα* is missing from both the *MAT* and *HML* loci of *S. cerevisiae*, which prompted us to investigate the function and regulation of the *α3* gene. A *α3* null allele was made, in which approximately two-thirds of the *α3* ORF was exchanged for a functional *LEU2* gene. This construct was exchanged for the wild-type *α3* gene at both the *MAT*α and *HML*α loci, thus generating *matα3::LEU2* and *hmlα3::LEU2* strains. By analogy to the results presented above, we expected that the *hmlα3::LEU2* strain would require the addition of leucine to the medium for optimal growth due to silencing at *HML*α, whereas the *matα3::LEU2* strain would not. Both the *matα3::LEU2* and *hmlα3::LEU2* strains were complete leucine prototrophs (data not shown), indicating either that the *HMLα3* gene was not silenced or that the *LEU2* promoter was resistant to silencing.

To distinguish between these two possibilities, we first tested the mating proficiency of both *matα3::LEU2* and *hmlα3::LEU2* strains (Figure 3A; data not shown). Surprisingly, neither strain showed a mating defect. Given that the *HMLα3* and *MATα3* genes are identical in sequence we investigated the possibility that both genes were expressed. We thus generated a double mutant *matα3::LEU2 hmlα3::LEU2* strain by mating two single mutant strains. The resulting diploid, homozygous for *leu2*, was sporulated and tetrad analysis revealed linkage

between *MAT*α and *HML*α of 10 cM based upon 31 parental ditypes, 8 tetratypes, and no nonparental ditypes from a total of 39 tetrads. We then tested the double mutant *mat*α*3::LEU2 hml*α*3::LEU2* strain in a mating assay and found that this strain mated with a low efficiency (Figure 3A). Thus, the α*3* gene appeared to be required for efficient mating as an α-cell and both the *MAT*α*3* and *HML*α*3* genes seemed to be expressed.

To investigate the regulation of the α*3* gene we performed RNA blots, probing for the α*3* transcript, on RNA from different cell types (Figure 3B). The experiment showed that the α*3* transcript was present in both the α- and **a**/α-cell types, but not in the **a**-cell type. Furthermore, *MAT***a** strains that contained mutations in either *sir2* or *sir4* also expressed the α*3* transcript. We noted that the α*3* transcript was a doublet band and in the *sir2* strain the slower migrating band was more abundant than in the wild-type *MAT*α strain, but at the moment we do not understand the significance of this observation. Thus, α*3* was an α-specific gene expressed from both *MAT*α and *HML*α.

Expression of α2p inhibits mating of *MAT*a strains: In Saccharomyces, simultaneous expression of **a** and α information leads to the formation of the **a**1/α2 heterodimer, which represses the transcription of haploid-specific genes (Herskowitz *et al.* 1992). Among the haploid-specific genes are those encoding the trimeric G-protein required for mating pheromone receptor signaling, whose repression leads to the sterility of cells expressing the **a**1/α2 repressor. As in Saccharomyces, introduction of a plasmid encoding the α1 and α2 genes into *K. lactis MAT***a** strains inhibited mating, indicating that *K. lactis* mating was also subject to **a**/α repression (see below). Plasmids carrying only the α1 gene did not inhibit mating of *MAT***a** strains, indicating that it was α2p and not α1p that inhibited mating. To confirm this notion, we tested the mating proficiency of two double mutant strains. One strain contained the *hml*α*1::KanMX* allele and the other strain contained a promoter deletion of the entire region between the α*1* and α*2* genes, *hml*αΔp (see materials and methods), and thus expressed neither α1p nor α2p. These two *hml*α mutant alleles were combined with *sir2* mutations in *MAT***a** strains and the mating proficiencies of the resulting strains were determined (Figure 4). Only the *hml*αΔp allele suppressed the mating defect caused by the *sir2* mutation, thus confirming that it was the expression of α2p and not α1p that inhibited mating of the *MAT***a** strain. Since the *HMR***a** locus contained an **a***1*-like gene (Figure 1) we propose that diploid *K. lactis* strains also contain an **a**1/α2 repressor.

A silencer and an ARS flanked *K. lactis HML*α: In Saccharomyces, the transcriptional silencing of *HML*α and *HMR***a** requires flanking DNA elements called silencers. To investigate whether DNA elements flanking the *K. lactis HML*α locus were required for silencing, we developed a plasmid-based assay for silencing. This

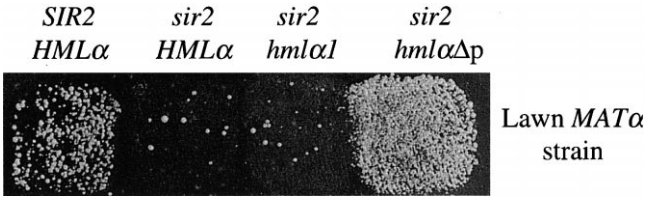

Figure 4.—Expression of α2p inhibited mating of *MAT***a** strains. Patch matings of strains CK213 (*MAT***a**), SAY124 (*MAT***a** *sir2::URA3*), SAY156 (*MAT***a** *sir2 hml*α*1::KanMX*), and SAY189 (*MAT***a** *sir2::URA3 hml*αΔp) are shown. The *MAT*α mating type tester strain used was WM52. Diploids were selected on SD + Ura plates.

assay measured the mating proficiency of a *MAT***a** strain containing plasmids with *HML*α sequences (Figure 5A). Since the simultaneous expression of **a** and α information inhibits mating in *K. lactis*, mutant plasmids that inhibited mating of the *MAT***a** strain contained expressed α*1* and α*2* genes. The *HML*α DNA fragments tested contained the entire α*1* and α*2* genes, but included only half of the α*3* gene (Figure 5B). A plasmid (p291) that contained only 78 bp of DNA downstream of the α*1* gene could inhibit mating of the *MAT***a** strain, but a plasmid (p300) that included 1.6 kb of flanking DNA did not affect the mating ability of the tester strain. Thus, within these 1.6 kb there was a DNA sequence that inhibited transcription of *HML*α in a *cis*-dominant manner. Furthermore, this putative silencer was not likely to be located within the first 370 bp downstream of the α*1* gene, since this flanking sequence was shared between the *MAT*α and *HML*α loci. To pinpoint further the potential silencer element, PCR fragments of the DNA flanking *HML*α were cloned into p291, and the resulting plasmids were tested for their ability to inhibit mating of *MAT***a** strains. This analysis showed that a 102-bp fragment (p414), corresponding to nucleotides 521–622 downstream of α*1* gene, completely retained silencing activity in this assay. An additional deletion of 50 bp of this minimal silencer (p357) completely abolished silencing (Figure 5B).

The *S. cerevisiae HMR-E* and *HMR-I* silencers are also origins of replication. To investigate if this feature of a silencer was conserved between yeasts, we tested if the DNA flanking the *K. lactis HML*α locus contained autonomously replicating sequence (*ARS*) activity. Different DNA fragments from the *HML*α flanking DNA were cloned into a yeast vector lacking origins of replication (pRS306) and transformed into *K. lactis*. The transformation frequency of vector without insert was very low due to the inability of the transformants to replicate the plasmid DNA. Several plasmids containing *K. lactis* DNA increased the transformation frequency by several orders of magnitude (Figure 5B), which indicated that an *ARS* was present close to *HML*α. These transformants grew on selective media, but lost the selectable marker following nonselective growth. Interestingly, the *ARS*

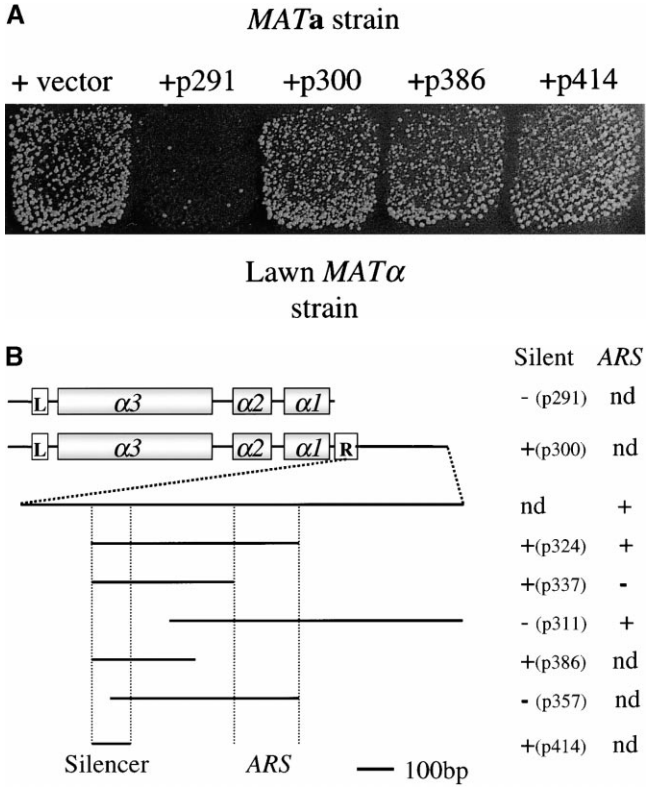

FIGURE 5.—(A) A plasmid-based assay for silencer function. Patch matings of strain CK213 transformed with the indicated plasmids. Vector indicates the pCXJ18 (*CEN ARS URA3*) plasmid, p291 is pCXJ18 with the *HMLα1* and *2* genes without flanking DNA, p300 was p291 + 1.6 kb of flanking DNA, p386 was p291 + 297 bp of flanking DNA, and p414 was p291 + the 102-bp minimal silencer. The *MAT*α mating type tester strain used was WM52 and diploids were selected on SD plates. (B) A silencer and an *ARS* activity flanked *HMLα*. Schematic drawing of the *HMLα* locus indicating the PCR fragments used to pinpoint the localization of the silencer and *ARS* activity. − means that the plasmid abolished mating of *MAT***a** strains CK213 (Silent) or that the DNA fragment was unable to confer a high transformation efficiency to pRS306 (*ARS*). + means that the plasmid did not affect mating of *MAT***a** strain CK213 (Silent) or that the DNA fragment conferred a high transformation efficiency to pRS306 (*ARS*). nd, not determined. The names of the plasmids are indicated in parentheses.

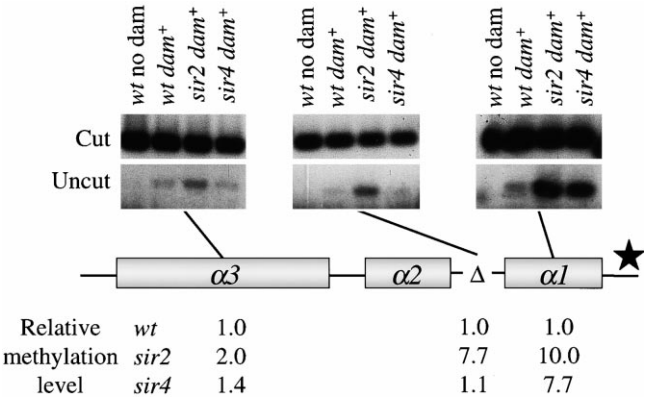

FIGURE 6.—Sir2p and Sir4p maintained a specialized chromatin structure at *HMLα*. Dam-methylase accessibility assay of chromosomal DNA from strains SAY186 (*hmlα*Δp), SAY186 + p365 (pCXJ20-*dam*$^+$), SAY189 (*hmlα*Δp *sir2::URA3*) + p365, and SAY191 (*hmlα*Δp *sir4::LEU2*) + p401 (pCXJ18-*dam*$^+$). A *Bcl*I site in the α*3* gene (left), an *Alw*I site between the α*1* and α*2* genes (middle), and an *Alw*I site in the α*1* gene (right) were assayed for the efficiency of enzyme digestion. For comparisons, the bands generated from when the enzyme did cut are shown (cut, 12-hr exposure) above the panels in which the site was protected by dam-methylation (uncut, 24-hr exposure). The *HMLα* locus is shown schematically, Δ indicating the deletion of the promoter region between the α*1* and α*2* genes, and the star indicating the silencer. The methylation levels relative to strain SAY186 + p365 are shown below.

was found close to the minimal silencer element, but was not necessary for silencing activity (Figure 5B). Thus, *K. lactis HMLα* was flanked by a silencer element and by a functionally separable *ARS* activity.

Sir2p and Sir4p were required for maintaining a specialized chromatin structure at *HMLα* in the absence of transcription: Unlike other fungi, budding and fission yeasts lack endogenous DNA methylation. This absence allows one to investigate the chromatin structure at different loci, by expressing foreign DNA methylases and assaying the accessibility of specific sequences to these methylases (SINGH and KLAR 1992). We used such an assay to determine the effect of *sir2* and *sir4* mutations on the chromatin structure of *HMLα*. Others have shown (SINGH and KLAR 1992) that actively expressed genes are more accessible to the *E. coli* dam-methylase than are silenced genes. Since both *sir2* and *sir4* mutations result in a partial derepression of *HMLα1* expression (ÅSTRÖM and RINE 1998) we generated a transcriptionally compromised *HMLα* allele to distinguish between effects on transcription from effects on chromatin structure that were independent from transcription. We thus generated an *HMLα* allele, in which the entire promoter region of the divergently transcribed α*1* and α*2* genes was replaced with an *Alw*I site. Then we combined this *hmlα*Δp allele with *sir2* and *sir4* mutations and introduced the *dam*$^+$ gene on a plasmid. Dam-methylase accessibility was determined by digesting chromosomal DNA from these strains with restriction enzymes that were sensitive to a methyl group at its target site. We investigated such sites at three different positions (Figure 6), an *Alw*I site in the middle of the α*1* open reading frame, an *Alw*I site between the α*1* and α*2* genes, and a *Bcl*I site present in the α*3* gene. Bands generated from DNA molecules in which *Alw*I or *Bcl*I were unable to cut the chromosomal DNA were normalized to the total DNA in each lane. We could thus determine the methylation levels in *sir2* and *sir4* mutant strains relative to the level in a wild-type strain. Both the *sir2* and *sir4* strains showed increased accessibility of the *Alw*I site present in the α*1* gene compared to the wild-type strain. This was expected since both of these genes are required for complete silencing of *HMLα1*

transcription. More surprising was the effect observed at the *Alw*I site in between the α*1* and α*2* genes. In this case only the *sir2* strain showed increased accessibility, whereas the *sir4* mutant strain showed similar accessibility compared to the wild-type strain. The *Bcl*I site found in the α*3* open reading frame showed less than twofold differences in accessibility between all strains tested. These data thus indicated that the silencing was weak in the α*3* region of *HML*α, which was expected since the genetic analysis indicated that α3p was expressed from the *HML*α locus in silencing-proficient strains. Moreover, these data indicated that *sir2* and *sir4* mutations affected the chromatin structure to different extents at *HML*α.

Sir2p regulated cation sensitivity in *K. lactis*: *K. lactis sir2* strains are hypersensitive to the DNA intercalating drug EtBr (Chen and Clark-Walker 1994). To investigate what this sensitivity might be due to, we grew *sir2* and wild-type strains in the presence of EtBr. Microscopic examination of *sir2* cells grown in the presence of EtBr revealed that the drug accumulated inside of the cells in a punctate pattern, similar to mitochondrial staining (Figure 7A). This accumulation was rapid, being visible by 1 hr after the addition of the drug to logarithmically growing cultures. At identical EtBr concentrations the wild-type strain showed only diffuse low-level staining (Figure 7A), showing that Sir2p function blocked accumulation of EtBr inside the yeast cells. EtBr preferentially intercalates into mitochondrial DNA in living cells. Thus, the role of Sir2p in preventing EtBr accumulation is consistent with Sir2p's role in maintaining the mitochondrial genome in the presence of EtBr (Chen and Clark-Walker 1994). *K. lactis* is a so-called "petite-negative yeast," meaning that, unlike *S. cerevisiae*, respiratory-deficient *K. lactis* strains cannot be isolated under normal circumstances because the mitochondrial genome is essential in *K. lactis* (Clark-Walker and Chen 1996) even for growth on fermentable carbon sources. There are, however, a group of genes called mitochondrial genome integrity (*mgi*) genes, that, when mutated, allow growth of *K. lactis* in the absence of the mitochondrial genome (Chen and Clark-Walker 1993). Thus, if the sole reason *sir2* mutants in *K. lactis* were EtBr sensitive was because EtBr caused the loss of the mitochondrial genome, then *mgi1 sir2* double mutant strains should grow in the presence of EtBr. Indeed, the *mgi1-1* mutation completely suppressed the EtBr sensitivity of *sir2* strains (Figure 7B). The *mgi1-1 sir2* double mutant strain was, in fact, more resistant to EtBr than a wild-type strain. The *mgi1-1 sir2* strain still accumulated EtBr and consequently all *mgi1-1 sir2* cells grown in the presence of EtBr were converted into respiratory-deficient petites (data not shown). Taken together these data showed that the toxic effect of EtBr in *sir2* strains was solely due to the interference of the mitochondrial genome. The molecular explanation for how Sir2p stops EtBr accumulation remains unclear, but since a functional Sir2-Gfp fusion protein was localized exclusively to the nucleus of *K. lactis* (Figure 7C), it would appear that the effect of Sir2p in this respect is mediated by a role of that protein outside of the mitochondria, such as regulating a transport function. We tested whether this potential transport defect could affect the growth of *sir2* strains in the presence of other compounds. It was found previously that the DNA intercalating drug Berenil, which like EtBr is a cation, inhibited growth of *sir2* strains (Chen and Clark-Walker 1994). Therefore we tested the relative sensitivities of a *sir2* strain either transformed with plasmid alone or transformed with a plasmid encoding a functional *SIR2-GFP* fusion gene to several different cations. The *sir2* strain showed increased sensitivity to Cu^{2+} and Ni^{2+} ions, but not to Mn^{2+}, Zn^{2+}, or Pb^{2+} ions (Figure 7D; data not shown). Thus, Sir2p appeared to regulate the transport of several cations in *K. lactis*.

DISCUSSION

This study presented the first characterization of the *HMR***a** and *HML*α loci in the yeast *K. lactis*. The *HML*α locus contained three genes that were transcriptionally silent in *MAT***a** cells and silencing of all three genes required Sir2p and Sir4p (Figure 3; Åström and Rine 1998). Given the sequence conservation between the Saccharomyces and Kluyveromyces α*1* and α*2* genes and the fact that the *K. lactis* α*1* gene also is functionally interchangeable between yeasts (Yuan *et al.* 1993), these two genes probably have similar or identical roles in the two yeasts. Consistent with this notion, the α*1* gene was required for mating proficiency of α-strains in *K. lactis*, just as α1p is required for mating proficiency of α-strains in Saccharomyces. *K. lactis* is likely to form an **a**1/α2 heterodimer that inhibits haploid-specific gene expression in **a**/α diploids, since *MAT***a** and *HMR***a** contained a gene homologous to the Saccharomyces **a***1* gene and expression of α2p inhibited mating in *MAT***a** strains. The third gene at *K. lactis HML*α did not show significant similarities to other known genes. Single mutant *hml*α*3::LEU2* or *mat*α*3::LEU2* strains did not show a mating defect, but a *hml*α*3::LEU2 mat*α*3::LEU2* double mutant strain did. This indicated that the α*3* gene promoted the α-mating phenotype and was expressed from both *HML*α and *MAT*α. Moreover, a DNA methylase assay, which measured transcriptionally independent effects on the chromatin structure of *HML*α, revealed that a *Bcl*I site present in the α*3* gene was much less affected by the absence of Sir2p or Sir4p than an *Alw*I site in the α*1* gene. Thus, the α*3* gene did not appear to be part of the silent domain at *HML*α. The reason for the absence of the α*3* transcript in *MAT***a** cells is most likely due to the lack of α1p, since preliminary observations indicated that the α*3* transcript was absent in a *mat*α*1::KanMX* strain.

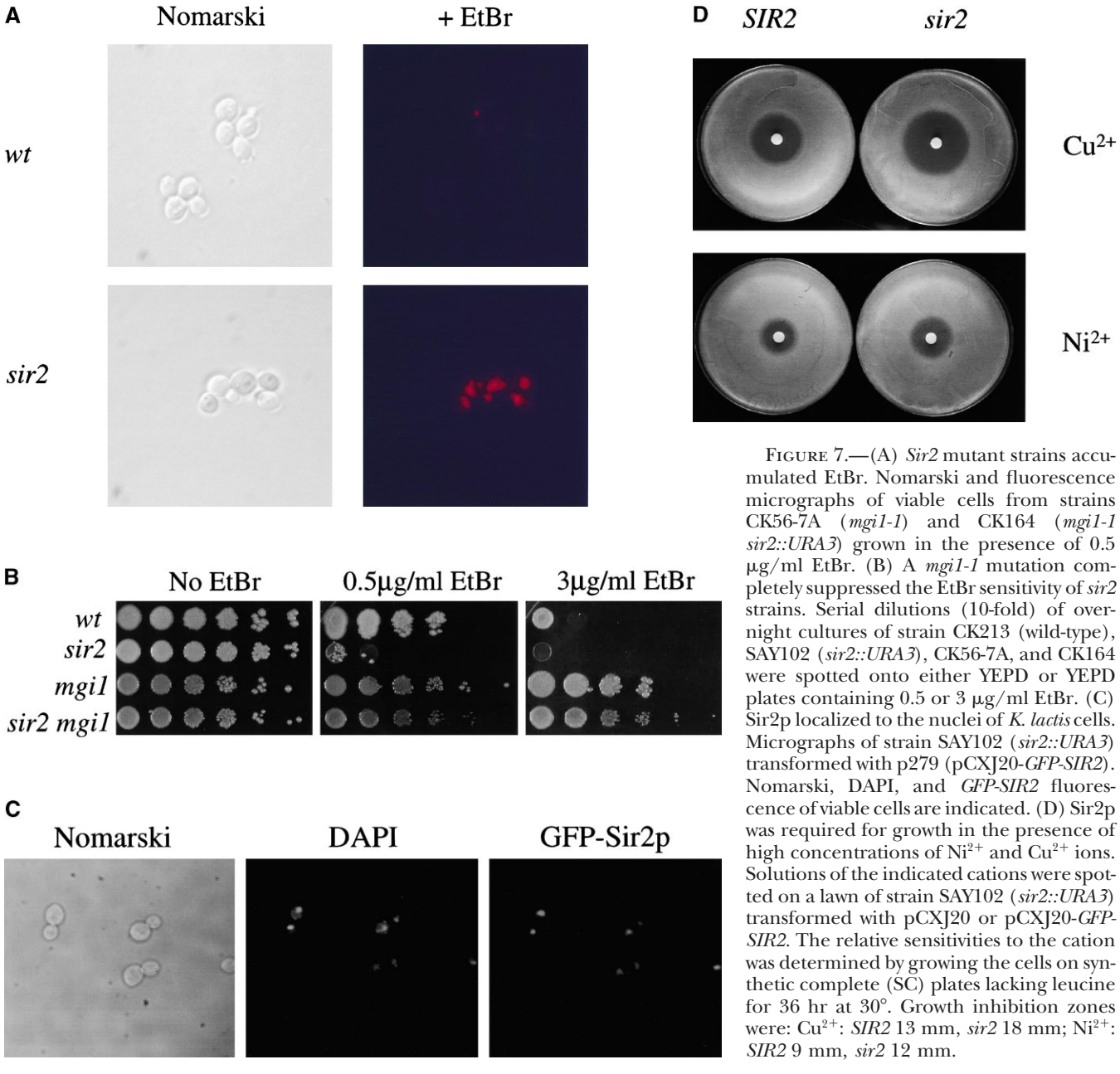

FIGURE 7.—(A) *Sir2* mutant strains accumulated EtBr. Nomarski and fluorescence micrographs of viable cells from strains CK56-7A (*mgi1-1*) and CK164 (*mgi1-1 sir2::URA3*) grown in the presence of 0.5 μg/ml EtBr. (B) A *mgi1-1* mutation completely suppressed the EtBr sensitivity of *sir2* strains. Serial dilutions (10-fold) of overnight cultures of strain CK213 (wild-type), SAY102 (*sir2::URA3*), CK56-7A, and CK164 were spotted onto either YEPD or YEPD plates containing 0.5 or 3 μg/ml EtBr. (C) Sir2p localized to the nuclei of *K. lactis* cells. Micrographs of strain SAY102 (*sir2::URA3*) transformed with p279 (pCXJ20-*GFP-SIR2*). Nomarski, DAPI, and *GFP-SIR2* fluorescence of viable cells are indicated. (D) Sir2p was required for growth in the presence of high concentrations of Ni^{2+} and Cu^{2+} ions. Solutions of the indicated cations were spotted on a lawn of strain SAY102 (*sir2::URA3*) transformed with pCXJ20 or pCXJ20-*GFP-SIR2*. The relative sensitivities to the cation was determined by growing the cells on synthetic complete (SC) plates lacking leucine for 36 hr at 30°. Growth inhibition zones were: Cu^{2+}: *SIR2* 13 mm, *sir2* 18 mm; Ni^{2+}: *SIR2* 9 mm, *sir2* 12 mm.

Our analysis suggested that the α3 protein was required for optimal α-mating, but the exact function of α3p was not revealed. Based on the knowledge of α-specific genes in Saccharomyces, it seems reasonable to assume that α3p was either involved in α-factor maturation or **a**-factor receptor activity. Since the identity of neither α-factor nor the **a**-factor receptor is known in *K. lactis* we have been unable to test these ideas. *MAT* loci from several other yeasts have been identified and most of the genes encoded by these loci correspond to transcriptional regulators. Recently however, the sequence of the mating type-like (*MTL*) loci from the asexual yeast *Candida albicans* revealed the presence of genes encoding oxysterol binding proteins, phosphatidylinositol kinases, and poly(A) polymerases (HULL and JOHNSON 1999). In Candida, it is not known if the genes in the *MTL* loci are involved in a mating type-like process, but this observation illustrates that the genes encoded by *MAT* loci in yeasts cannot always be immediately connected to mating.

The silencer we identified downstream of the α*1* gene was apparently sufficient to silence the α*1* and α*2* genes in a plasmid assay. At face value these data indicate that in *K. lactis HML*α is flanked by only one silencer and not two, like *HML*α in *S. cerevisiae*. It should be noted that at *HML*α in *S. cerevisiae* either silencer is sufficient to silence *HML*α. In Saccharomyces, boundary elements have been described close to *HMR***a** that protect euchro-

matic genes close to *HMR***a** from heterochromatin (Donze *et al.* 1999). However, more experiments are required to determine whether or not *K. lactis* has discrete boundary elements and if so, where they are located at *HMLα*. The first long ORF downstream of the *K. lactis HMLα1* gene was found ~3.0 kb from the *α1* stop codon. An RNA blot analysis using a probe corresponding to our partial sequence of this gene revealed mRNA expression in both SIR^+ and sir^- cells, indicating that the silent domain did not extend into the promoter of this gene (data not shown). Since this gene was transcribed on the opposite strand from the *HMLα1* gene we did not determine the exact distance to its promoter.

The dam-methylase assay that measured transcriptionally independent effects on the chromatin structure of *HMLα* revealed a difference between strains lacking Sir2p and Sir4p. Both strains showed increased dam accessibility in the *HMLα1* gene, but only the *sir2* strain showed increased accessibility at a site in between the *HMLα1* and *HMLα2* genes. This result was consistent with *sir2* mutations derepressing *HMLα1* transcription to a higher degree than *sir4* strains do (Åström and Rine 1998). One explanation for this effect is that Sir4p does not spread along the chromatin, starting from the silencer, to the same extent as Sir2p. Others showed previously that in Saccharomyces Sir3p could spread from telomeres further than either sir2p or Sir4p, indicating that not all Sir proteins may be part of the same complex (Hecht *et al.* 1996; Strahl-Bolsinger *et al.* 1997).

Silencing in both Saccharomyces and Kluyveromyces requires silencer elements. Other similarities are that Sir proteins are required for silencing, and marker genes integrated at *HMLα* can be silenced in a Sir-dependent manner, in both yeasts. Downstream of the *HMLα1* gene we identified a 102-bp fragment that was sufficient to mediate silencing. In the same assay a 300-bp DNA fragment from the *HMR***a** locus, corresponding to a region located downstream of the **a***1* gene, could also mediate silencing of the *HMLα* locus in the plasmid assay (data not shown). Thus, both silent loci appear to be flanked by silencers. Comparisons of the DNA sequences of these two regions should facilitate the identification of DNA sequence motifs that are important for silencing. An *ARS* activity close to the cryptic mating type loci appeared to be evolutionarily conserved between yeasts, suggesting that ORC binding was important for silencing also in Kluyveromyces. However, we could delete the sequences that mediated the *ARS* activity and still retain full silencer activity. This observation did not exclude the possibility that ORC binding plays a role in silencing. For example, at the *S. cerevisiae HMR-E* silencer, the deletion of the Rap1p, Abf1p, or ORC binding sites individually does not abolish silencing, but the deletion of two of the sites simultaneously does (Brand *et al.* 1987). Perhaps the *K. lactis HMLα* silencer shows similar redundancy. The consensus DNA binding sites for *K. lactis* Rap1p and Abf1p are known. However, the 102-bp silencer did not contain any close matches to the consensus binding sites for Rap1p or Abf1p. Thus, silencers in *K. lactis* must be quite different compared to the Saccharomyces silencers, and studying these silencers should thus lead to a deeper understanding of both the architecture of a silencer and the mechanism of silencing.

K. lactis sir2 strains grow slowly compared to wild-type strains (data not shown), a phenotype that is not observed in Saccharomyces *sir2* strains. Thus, the *SIR2* gene has different and perhaps more important functions in *K. lactis* compared to *S. cerevisiae*. One of these differences was that *K. lactis* Sir2p was required for hindering accumulation of EtBr inside of the cell. A mutation that bypassed the need for the mitochondrial genome also relieved the EtBr sensitivity of *sir2* strains. The effect that Sir2p had on EtBr accumulation was likely to be indirect, since Sir2p appeared to be exclusively localized to the nucleus. *Sir2* strains were also more sensitive to both Cu^{2+} and Ni^{2+} ions compared to a wild-type strain, suggesting that Sir2p regulated either the intake or efflux of a subset of cations in *K. lactis*. Perhaps a gene required for the transport of cations is close to a telomere in *K. lactis* and the absence of Sir2p may derepress such a gene, similar to the effect *sir2* mutations have on telomeric position effect (TPE) in *S. cerevisiae*. However, *sir4* mutations in *K. lactis* are slightly more resistant to EtBr compared to the wild type. Mutations in *sir4* affect the telomere length in *K. lactis* and are thus likely to affect TPE. If Sir2p has a role in cation transport in *K. lactis*, it would appear to perform this function independently of Sir4p. The EtBr sensitivity of *sir2* strains cannot be an indirect effect of the simultaneous expression of **a** and α information, since *MAT***a** *sir2* *hmlα*Δp strains still were sensitive to EtBr. A more likely model is thus that Sir2p has a different regulatory role in *K. lactis*, perhaps as a part of a complex with other molecules. Such distinct Sir2p-containing complexes have been observed already in Saccharomyces, in which Sir2p has a Sir4p-independent role in regulating the chromatin structure at the rDNA locus (Smith *et al.* 1998; Straight *et al.* 1999).

Since *SIR2*-like genes are present in organisms ranging from archea to mammals, it is likely that Sir2p-like molecules are involved in many different processes. In this study we have demonstrated one such role in what appears to be regulation of cation transport. Further studies of Sir proteins and the cryptic mating type loci in *K. lactis* are likely to reveal more interesting features of heterochromatin and new dimensions to the role of silencing proteins.

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