Functional Diversity of Silencers in Budding Yeasts

Jimmy O. O. Sjöstrand, Andreas Kegel, and Stefan U. Åström*

Developmental Biology, Wenner-Gren Institute, Arrhenius Laboratories E3, Stockholm University, SE-106 91 Stockholm, Sweden

Received 20 May 2002/Accepted 21 May 2002

We studied the silencing of the cryptic mating-type loci HMLα and HMRα in the budding yeast Kluyveromyces lactis. A 102-bp minimal silencer fragment was defined that was both necessary and sufficient for silencing of HMLα. Mutagenesis of the silencer revealed three distinct regions (A, B, and C) that were important for silencing. Recombinant K. lactis ribosomal DNA enhancer binding protein 1 (Reb1p) could bind the silencer in vitro, and point mutations in the B box abolished both Reb1p binding and silencer function. Furthermore, strains carrying temperature-sensitive alleles of the REB1 gene derepressed the transcription of the HMLα1 gene at the nonpermissive temperature. A functional silencer element from the K. lactis cryptic HMRα locus was also identified, which contained both Reb1p binding sites and A boxes, strongly suggesting a general role for these sequences in K. lactis silencing. Our data indicate that different proteins bind to Kluyveromyces silencers than to Saccharomyces silencers. We suggest that the evolution of silencers is rapid in budding yeasts and discuss the similarities and differences between silencers in Saccharomyces and Kluyveromyces.

Two copies of the same gene can display different expression levels, even though the two genes are present in the same nucleus. As a consequence of the nuclear environment surrounding a gene, specialized chromatin structures can influence how accessible the promoter of the gene is to the transcriptional machinery. This phenomenon, called “position effect,” has been observed in many organisms, and intensively studied examples include X-chromosome inactivation in mammals (49), position effect variegation in Drosophila (23), and silencing of the cryptic mating-type loci in yeasts (40). In yeasts, loci that exhibit position effects are characterized by chromatin structures that are inaccessible to DNA-modifying enzymes (39, 58), and the chromatin of these loci contains hypoacetylated histones (11).

In the budding yeast Saccharomyces cerevisiae, position effects have been observed at the cryptic mating-type loci HMLα and HMRα (29, 45, 51), at telomeres (22), and at the ribosomal DNA (rDNA) locus (12, 19, 59). At the cryptic mating-type loci, this phenomenon is called “silencing” and requires DNA elements close to the inactivated loci and dedicated proteins. Because these DNA elements mediate transcriptional repression, the opposite effect of enhancers, they are called “silencers” (9). The polycomb response element in Drosophila (14, 57) and sequences near the cryptic mating-type cassettes in Schizosaccharomyces pombe (64) are other examples of silencers, demonstrating that silencers are not unique to budding yeast. Silencers can be required for establishing and/or maintaining inactive chromatin, but exactly how silencers accomplish this is not known.

The best-studied silencer is the Saccharomyces HMR-E silencer. HMR-E consists of a combination of binding sites for Rap1p, the origin recognition complex (ORC), and Abf1p (10, 55). Rap1p and Abf1p are transcription factors that bind to many promoters in yeast (13, 55). ORC is required for the initiation of replication (8), and HMR-E is in fact a chromosomal replication origin (52). It is believed that the combination of these binding sites presents a surface that nucleates the formation of a higher-order chromatin structure that spreads, thus repressing nearby promoters. Sir proteins (Sir2 to -4) are essential for silencing (26, 50) and appear to be part of the protein-DNA complex that defines silent chromatin (62). The Sir3 and Sir4 proteins are recruited to the silent domains by direct interaction with Rap1p (43). Euchromatic genes juxtaposed to telomeric sequences are also silenced in a Sir-dependent manner (3). Telomeres contain multiple Rap1p binding sites, and the Rap1p molecules bound to these sites are probably sufficient to recruit Sir proteins to telomeres. The single Rap1p binding site at HMR-E, however, is probably not sufficient for Sir recruitment, but also other interactions occur, such as the interaction between Sir proteins and ORC (18, 65). Sir3p and Sir4p also interact with each other (42) and with histone tails (24), providing additional evidence that multiple protein-protein interactions are required for silencing. HMR-E shows signs of redundancy, since deletion of the Rap1p, Abf1p, or ORC binding sites individually does not abolish silencing, but deletion of two of these sites simultaneously does (10). The Sir2 protein is a histone deacetylase (25, 34, 60) and probably contributes to the deacetylation of histone tails in silent chromatin (11).

Much less is known about silencing in Kluyveromyces. Kluyveromyces lactis also contains cryptic mating-type loci, and the cryptic α-locus (HMLα) requires Sir2p and Sir4p to remain silent (5). Both Sir2p and Sir4p are also required for maintaining a specialized chromatin structure at HMLα, as would be expected (4). Moreover, both Sir2p and Sir4p are functionally interchangeable with their Saccharomyces counterparts (5, 16), but K. lactis and S. cerevisiae Sir4p have remarkably divergent amino acid sequences. We previously defined silencers close to both HMLα and HMRα (4), but the features and possible protein binding sites of these silencers remained unknown.

To better understand the architecture of a silencer, we have
FIG. 1. Three discrete sites were required for silencer function. (A) DNA sequence of the HMLα silencer and mutations generated. The top line represents the wild-type sequence, and lines 1 to 10 represent mutations generated in the silencer. Changed bases are underlined. Reb1 shows the 2-bp substitution in the Reb1p recognition sequence. Lines c1 to c4 represent the DNA fragments used for competition in the gel mobility shift experiment. A, B, and C (shaded boxes) indicate the sequences defined to be essential for silencer function. Silencer function in the mating assay is indicated on the right. (B) Mating assay for silencer function. A tester mater strain (MATα WM52V4) was mated to a MATa strain (CK213-4C) containing different plasmids. These plasmids contained the HMLα locus either lacking a silencer (p291; Reb1) or containing a wild-type silencer (p413; Reb1). Mutant silencers were as indicated in panel A. Diploids were selected on synthetic dextrose plates. (C) Introduction of ORC binding sites did not improve the silencer function of A and B box mutations. A mating assay was performed as described in panel B. Squares 3, 6, and 9 indicate that the adjacent ORC binding site was included on the plasmid. A plasmid without an insert is denoted as the vector. (D) Schematic drawing of the HMLα and HMRα silencers. Sequence analysis with MatInspector software revealed the presence of both A boxes (solid arrowhead) and B boxes (Reb1 binding-sites) (open arrowhead) at both the HMLα and HMRα silencers.
analyzed the *K. lactis* *HMLα* silencer. We found three discrete sequences within the silencer that were essential for silencing. One of these sequences was a Reb1p DNA binding site, and we could show that Reb1p itself was important for silencing. Similarly to Rap1p, Reb1p is a Myb domain transcription factor (27), suggesting that evolution has used Myb domain-containing proteins repeatedly to generate silencers.

**MATERIALS AND METHODS**

**Plasmid construction.** Plasmids p291 (pCXJ18-HMLα without silencer) p300 (pCXJ18-HMLα plus 1.6-kb silencer), p326 (pCXJ18-HMLα plus 719-bp silencer), and p413 (pCXJ18-HMLα plus 102-bp minimal silencer) were described previously (4). Plasmid p243 was p291 with a 301-bp BamHI-KpnI PCR fragment originating from the *HMLα* flanking region, corresponding to nucleotides 425 to 726 (the first base after the stop codon of the α1 gene was defined as nucleotide 1). A BamHI-KpnI silencer fragment from p413 and p326 was cloned into the corresponding sites of pRS806 (56), generating plasmids plp1.6 and p318, respectively. Phagemids were produced from plp1.6 and p318, and then the mutations indicated in Fig. 1 were introduced with the appropriate oligonucleotides by in vitro mutagenesis (24). The mutant silencers were cloned back into the BamHI vector (BamHI-KpnI) after the mutations had been confirmed by DNA sequencing. Plasmid pAB24-REB1 (from J. Warner, Albert Einstein College of Medicine, New York, N.Y.) was used as a template to generate a 2.2-kb REB1 PCR fragment containing SacI-XbaI sites at the ends, and this fragment was cloned into the SacI-XbaI sites of pCX20 (LEU2 CEN) (15). A PCR fragment including the Myb domain of *REB1* was cloned into a T-vector (pGEM-T; Promega). Phagemids were produced, and the mutations indicated in Fig. 4 were generated with the corresponding oligonucleotides by in vitro mutagenesis (32). The mutations were confirmed by DNA sequencing, and from the resulting plasmids, a 415-bp XhoI-BsuBI fragment was exchanged for the corresponding fragment of pCX20-REB1. The resulting plasmids were p500 (reb1 RRK to AAA, C), p502 (reh1 EED to AAA, reb1-1), and p504 (reh1 EE to AAA, reb1-2). A pCX20-SREB1 plasmid was generated by cloning a 2.8-kb SacI-BamHI PCR fragment corresponding to the entire Saccharomyces *REB1* gene, including the promoter region, into the corresponding sites of pCX20. A maltose binding protein (MBP)-Reb1 fusion protein was generated by cloning an EcoRI-SalI PCR fragment corresponding to amino acids 1 to 595 of Reb1p into the corresponding sites of pMALC2 (New England Biolabs).

**Strain construction.** The genotypes of all strains used in this study are indicated in Table 1. All disruptions were confirmed on DNA blots. A diploid strain generated by crossing strains CK213 and SAY119 was transformed with HindIII-linearized pRS806-reb1::LEU2 to generate a REB1::reb1::LEU2 heterozygous diploid by a two-step gene disruption procedure (53). Tetrad analysis of this diploid revealed that viability segregated 2:2, and all survivors were leucine auxotrophs, indicating that the REB1 gene was essential. The reb1::LEU2 allele was converted into a reb1::hisG allele by using plasmid pNYK55 (2). Plasmid pAB24-REB1 (URA3) (44) was introduced into the resulting REB1::reb1::hisG diploid strain, and tetrad analysis was performed. 5-Fluoro-orotic acid (5-FOA)-sensitive progeny were identified, resulting in strains SAY205 and SAY206. Strain SAY208 was generated by crossing SAY205 to SAY130 followed by tetrad analysis. Disruptions depended on either *S. cerevisiae REB1* or mutant derivatives of *K. lactis REB1* were generated by introducing LEU2 plasmids with the tested gene into SAY206, and the transformants were plated on synthetic complete medium lacking leucine but containing 5-FOA (5-FOA-LEU). The result of this plasmid shuffling procedure was that the pAB24-REB1 plasmid was exchanged for a LEU2 plasmid containing a mutant REB1 gene.

**Yeast media and methods.** DNA manipulations, RNA-DNA blots, DNA sequencing, and media for growth of yeast and bacteria followed standard protocols (1, 6). Matting assays were also performed as described previously (5). For the gel mobility shift assay, the binding reaction was performed in a mixture of 20 mM HEPES-KOH (pH 7.6), 10% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.05% NP-40, 70 ng of poly(dI-dC) per μl, 300 μg of bovine serum albumin per ml, 20,000 cpm of 32P-labeled DNA probe (0.1 to 0.5 ng of 102-bp minimal silencer), and approximately 15 μg of crude extract or 10 ng of purified MBP-Reb1 protein. For competition assays, unlabeled competitor DNA was added at approximately 30-fold molar excess over the probe. The reaction mixture (15 μl) was incubated at 30°C for 15 min. The samples were then analyzed by non-denaturing polyacrylamide gel electrophoresis (40:1 acrylamide/bisacrylamide ratio) with Tris-acetate (TAE) (6) as gel and electrophoresis buffer. The gel was dried and autoradiographed. Total RNA was prepared from late-logarithmic-phase-growing cells (SAY206 plus plasmids) grown at 30 or at 36°C for approximately 14 h. Strains dependent on the temperature-sensitive reb1 alleles [reb1(Ts)] were viable after 14 h at 36°C, but had ceased to divide.

**RESULTS**

Three discrete sequence elements were important for silencer function. Previously, we identified a minimal silencer element, 102 bp long, that was both necessary and sufficient for silencing of *HMLα* in a silencing assay. This assay measured the mating proficiency of a MATα strain containing plasmids with *HMLα* sequences (4). Since the simultaneous expression of α and α information inhibits mating in *K. lactis*, mutant plasmids that inhibited mating of the MATα strain contained expressed α1 and α2 genes. The *HMLα* DNA fragments tested contained the entire α1 and α2 genes, but only included half of the α3 gene. If a silencer fragment was included on this plasmid, the α genes were not expressed, and the strain mated efficiently (4). To pinpoint short sequences within the minimal silencer that were important for silencing and thus might define protein-binding sites, we performed an extensive mutational analysis of the minimal silencer. Ten mutant derivatives of the silencer were generated by oligonucleotide-directed mutagenesis. For convenience, the altered base pairs were changed into a *HindIII* site (Fig. 1A). By using the plasmid-based assay for silencing, we determined that three of these mutant silencers could no longer support silencing in the mating assay. The remaining seven mutant silencers had mating efficiencies similar to that of the wild-type silencer (Fig. 1B). We call the sequences that were defined as important for silencing by this assay the “A, B, and C boxes.”

In *Saccharomyces*, the HMR-E silencer is partly redundant for the Abf1p, Rap1p, and ORC binding sites. Thus, a silencer lacking only one of these elements is still functional, but if two of the sites are mutated simultaneously, then silencing is abolished (10). Since we previously found an *ARS* sequence (ORC binding site) close to the minimal silencer at *HMLα*, we wanted to determine whether the A, B, and C boxes were equally important for silencing when the *ARS* sequence was included on the silencer fragment. We thus generated the A, B, and C box mutations in a silencer fragment that was 712 bp long and thus included the *ARS* sequence (4). In the silencing assay, none of these mutant silencers were completely functional, thus indicating that the *ARS* sequence was not redundant with the A, B, or C boxes for silencer function (Fig. 1C). In the case of the C box mutation, a slight improvement of silencing was observed when the longer silencer fragment was used, but we assume that this improvement may be due to cryptic C boxes in the longer fragment (see Discussion). Thus, we defined three short sequence elements that were important for silencing of *HMLα* and found no evidence for involvement of ORC.

Previously, we defined a 301-bp fragment close to the *K. lactis* *HMRα* locus that could act as a silencer in our mating assay. In *Saccharomyces*, the *HMRα* and *HMLα* silencers are similar and consist of similar protein binding sites. We wanted to investigate if sequences similar to the A, B, and C boxes could be found in the *K. lactis* HMRα silencer. The HMRα was thus analyzed with the MatInspector software, and we found two good matches to both the A box sequence and
three good matches to the B box sequence (Fig. 1D). The HMRα silencer did not contain sequences similar to those of the C box. Since A and B box sequences were found in both silencers, they were excellent candidates for protein binding sites that mediated silencing in *Kluyveromyces*.

**K. lactis extracts contained a protein that bound to the B box.** Since we suspected that the silencer contained protein binding sites, we performed a DNA mobility shift assay, using the minimal silencer and protein extracts from *K. lactis*. One major, slower-migrating mobility shift and one minor, faster-migrating mobility shift were observed (Fig. 2). To determine whether the binding was sequence specific and what part of the silencer these activities recognized, we added competing DNA to the band shift reaction. A 30-fold excess of unlabeled wild-type silencer abolished the slower-migrating mobility shift, but had less impact on the faster-migrating mobility shift. The type silencer abolished the slower-migrating mobility shift, but to the band shift reaction. A 30-fold excess of unlabeled wild-type silencer these activities recognized, we added competing DNA into an

| Strain      | Genotype                                      | Source or reference |
|-------------|-----------------------------------------------|---------------------|
| K. lactis   |                                               |                     |
| WM52V4      | MATα ade1 adeX his7 uraA1                     | 16                  |
| CK213-4C    | MATα lysA1 rpl1 leu2 metA1 uraA1             | 16                  |
| SAY119      | MATα uraA1 ade1 leu2 trp1 metA1              | 4                   |
| SAY130      | CK213 hmlA::KanMX                            | 4                   |
| SAY156      | SAY130 sr2                                   | 4                   |
| SAY205      | MATα uraA1 ade1 leu2 trp1 metA1 reb1::hisG + pAB24-REB1 | This study          |
| SAY206      | MATα uraA1 leu2 trp1 metA1 reb1::hisG + pAB24-REB1 | This study          |
| SAY208      | MATα uraA1 leu2 lysA1 trp1 metA1 reb1::hisG hmlA::KanMX + pAB24-REB1 | This study          |
| S. cerevisiae J343 | MATα ade2-1 his3-11.15 leu2-3,112 trp1-1 ura3-1 can1-100 reb1::LEU2 + pAB24-KIREB1 | J. Warner, Albert Einstein College of Medicine, New York, N.Y. |

---

**TABLE 1. Yeast strains used in this study**

---

bound to the silencer element, and the position of the shifted complex was consistent with that of the shift of the silencer fragment with *K. lactis* protein extracts, considering that the recombinant protein contained an MBP moiety (Fig. 3A). Also the binding of the recombinant MBP-Reb1p protein to the silencer was specific, since a 30-fold excess of unlabeled silencer DNA almost abolished binding. We thus conclude that the HMLα silencer contained a Reb1p binding site.

Next we wanted to determine if the silencer DNA binding activity from *K. lactis* extracts really corresponded to Reb1p. An alternative hypothesis was that *K. lactis* contained another DNA binding protein, which binds to a sequence similar to the Reb1p DNA binding site. To determine this, we generated a strain containing a deletion of the genomic *K. lactis* REB1 gene (see Materials and Methods). Tetrad analysis of a heterozygous REB1/reb1::hisG diploid indicated that REB1 was an essential gene, since the viability of the meiosis segregated 2:2. This lethality could be complemented, however, if the heterozygous REB1/reb1::hisG diploid was transformed with a plasmid containing the *REB1* gene. We thus generated a haploid strain containing a reb1::hisG-null allele that was rescued by a *URA3* plasmid containing the *REB1* gene. The possibility that Reb1p was only required for spore germination and not vegetative growth was tested by plating this haploid strain on medium containing 5-FOA. Since 5-FOA selects against the vegetative growth was tested by plating this haploid strain on medium containing 5-FOA. The *REB1* gene was required for vegetative growth of *K. lactis*, since the haploid reb1::hisG strain was unable to cut loose the *URA3-REB1* plasmid and thus grow on 5-FOA medium. Others previously showed that *K. lactis* *REB1* complemented *S. cerevisiae* reb1 strains (44). We wanted to test if the reverse was true and thus made the *K. lactis* reb1::hisG strain dependent on *Saccharomyces* *REB1* with plasmid shuffling (see Materials and Methods). The resulting strain was viable and displayed no growth rate defect. *K. lactis* and *S. cerevisiae* *REB1* thus cross-complemented each other in both organisms. Next we took advantage of the fact that the *K. lactis* and *S. cerevisiae* *REB1* genes encode proteins of different sizes. *Kluyveromyces* Reb1p is only 595 amino acids long, whereas *Saccharomyces* Reb1p is 810 amino acids long. If the silencer DNA binding activity that was present in *K. lactis* protein extracts corresponded to Reb1p, then we would expect to se a slower-migrating gel mobility shift with protein extract from...
Reb1p (36), which has the same recognition sequence as K. lactis and S. cerevisiae Reb1p proteins share only 40\% sequence identity, and we expected that the K. lactis REB1 gene had to be mutationally compromised in positions conserved between Saccharomyces and Kluyveromyces in order to generate conditional alleles. We thus performed a limited alanine-scanning mutagenesis of charge clusters (67) (at least three charged amino acids in a row) that were conserved between the two yeasts. All of the conserved charge clusters were found in the two Myb domains of Reb1p, which are responsible for DNA binding. To avoid generating mutations that would abolish DNA binding and thus generate a completely inactive Reb1p molecule, we took advantage of a previous study that solved the structure of the Myb domain of a mouse protein called “c-myb” (46, 47). Utilization of this structure to predict the structure of the REB1 Myb domains revealed that several of the conserved charge clusters probably were present in the potential DNA binding helices. We chose to generate mutations predicted to be on the surface of the first Myb domain (Fig. 4A). Three mutants bearing targeted mutations in this domain were generated, two of which had a phenotype (Fig. 4A). A K. lactis reb1::hisG strain was made dependent on these alleles by plasmid shuffling, and the resulting strains were tested for growth at high temperature. Compared to the strain containing wild-type REB1, strains expressing the reb1 EED$_{346}$-to-AAA$_{346}$ allele (reb1-1) and the reb1 EEE$_{398}$-to-AAA$_{398}$ allele (reb1-2) were unable to grow at 35°C, but grew normally at 30°C (Fig. 4B). We had thus generated two temperature-sensitive alleles in the REB1 gene. We also attempted to isolate reb1(Ts) alleles by a random mutagenesis approach. A plasmid containing the REB1 open reading frame was mutagenized with hydroxylamine and tested for a conditional phenotype in the reb1::hisG strain. By this procedure, we isolated one additional reb1(Ts) allele, which we call “reb1-3.” DNA sequencing of this allele revealed that the REB1 open reading frame contained five missense mutations. By subcloning different fragments of the reb1-3 allele into a wild-type REB1 gene, we determined that the mutations responsible for the conditional growth phenotype mapped to the first Myb domain (H$_{327}$ to T$_{327}$ and K$_{392}$ to E$_{392}$; Fig. 4). We did not investigate which one of these two mutations (or both) generated the temperature sensitivity.

At temperatures below the restrictive temperature, strains carrying the reb1-1, reb1-2, and reb1-3 alleles were not silencing deficient, since these strains did not derepress an hmlA::KanMX marker gene (assayed on plates containing Geneticin). Then, we compared the mating efficiency of a strain containing the wild-type REB1 gene to strains containing reb1(Ts) alleles (Fig. 4C). The reb1-1 and reb1-2 strains remained viable for at least 24 h at the restrictive temperature (data not shown), making it possible to test the mating efficiency at both the permissive and restrictive temperatures. At the permissive temperature, the reb1(Ts) strains did not have a mating defect, but at the restrictive temperature, neither the reb1-1 strain nor the reb1-2 strain could mate (Fig. 4C). This result was consistent with the finding that Reb1p was essential for silencing, but it was also possible that this was a pleiotropic phenotype caused by another function of Reb1p, distinct from silencing. Therefore, we wanted to test whether silencing

FIG. 2. A fragment containing a consensus Reb1p binding site efficiently competed for binding to the silencer by a K. lactis protein extract. An electrophoretic mobility shift assay showing the ability of unlabeled parts of the silencer to compete with a labeled 102-bp minimal silencer for binding to a K. lactis protein extract was performed. The competing DNA fragments (30-fold molar excess; Fig. 1A) are schematically depicted below. A Reb1p binding site was present in competing fragment c3, but not present or only partially present in the overlapping fragments c2 and c4. The free silencer fragment (solid arrowhead) and shifted silencer fragment (open arrowhead) are indicated.

![Diagram of competition with fragment K. lactis extract](image-url)

the reb1::hisG strain that exclusively expressed Saccharomyces Reb1p. This notion was confirmed (Fig. 3B), and we thus concluded that the HMLα silencer contained a Reb1p binding site, which was recognized both by endogenous Reb1p and Saccharomyces Reb1p.

Reb1p was required for silencing. We noted that the Reb1p binding site overlapped with the previously defined B box sequence and wanted to learn if Reb1p binding was important for silencing. Since the REB1 gene is essential in K. lactis, we could not assay silencing in a reb1-deficient strain. Instead, we generated a silencer that contained two point mutations (in boldface) in the Reb1p binding site (Fig. 1) by changing CCGGGTA into CCGG. The result was consistent with the finding that Reb1p was essential for silencing in K. lactis.

To confirm that Reb1p indeed was required for silencing, we wanted to generate conditional alleles of the REB1 gene, so that we could inactivate Reb1p in living cells and investigate if this would lead to the derepression of the cryptic HMLα locus. An interesting observation was that K. lactis strains exclusively expressing Saccharomyces Reb1p displayed normal mating and did not derepress a KanMX marker gene at HMLα (data not shown), demonstrating that the Saccharomyces protein was proficient for silencing. The K. lactis and S. cerevisiae Reb1p proteins share only 40\% sequence identity, and we expected that the K. lactis REB1 gene had to be mutationally compromised in positions conserved between Saccharomyces and Kluyveromyces in order to generate conditional alleles. We thus performed a limited alanine-scanning mutagenesis of charge clusters (67) (at least three charged amino acids in a row) that were conserved between the two yeasts. All of the conserved charge clusters were found in the two Myb domains of Reb1p, which are responsible for DNA binding. To avoid generating mutations that would abolish DNA binding and thus generate a completely inactive Reb1p molecule, we took advantage of a previous study that solved the structure of the Myb domain of a mouse protein called “c-myb” (46, 47). Utilization of this structure to predict the structure of the REB1 Myb domains revealed that several of the conserved charge clusters probably were present in the potential DNA binding helices. We chose to generate mutations predicted to be on the surface of the first Myb domain (Fig. 4A). Three mutants bearing targeted mutations in this domain were generated, two of which had a phenotype (Fig. 4A). A K. lactis reb1::hisG strain was made dependent on these alleles by plasmid shuffling, and the resulting strains were tested for growth at high temperature. Compared to the strain containing wild-type REB1, strains expressing the reb1 EED$_{346}$-to-AAA$_{346}$ allele (reb1-1) and the reb1 EEE$_{398}$-to-AAA$_{398}$ allele (reb1-2) were unable to grow at 35°C, but grew normally at 30°C (Fig. 4B). We had thus generated two temperature-sensitive alleles in the REB1 gene. We also attempted to isolate reb1(Ts) alleles by a random mutagenesis approach. A plasmid containing the REB1 open reading frame was mutagenized with hydroxylamine and tested for a conditional phenotype in the reb1::hisG strain. By this procedure, we isolated one additional reb1(Ts) allele, which we call “reb1-3.” DNA sequencing of this allele revealed that the REB1 open reading frame contained five missense mutations. By subcloning different fragments of the reb1-3 allele into a wild-type REB1 gene, we determined that the mutations responsible for the conditional growth phenotype mapped to the first Myb domain (H$_{327}$ to T$_{327}$ and K$_{392}$ to E$_{392}$; Fig. 4). We did not investigate which one of these two mutations (or both) generated the temperature sensitivity.

At temperatures below the restrictive temperature, strains carrying the reb1-1, reb1-2, and reb1-3 alleles were not silencing deficient, since these strains did not derepress an hmlA::KanMX marker gene (assayed on plates containing Geneticin). Then, we compared the mating efficiency of a strain containing the wild-type REB1 gene to strains containing reb1(Ts) alleles (Fig. 4C). The reb1-1 and reb1-2 strains remained viable for at least 24 h at the restrictive temperature (data not shown), making it possible to test the mating efficiency at both the permissive and restrictive temperatures. At the permissive temperature, the reb1(Ts) strains did not have a mating defect, but at the restrictive temperature, neither the reb1-1 strain nor the reb1-2 strain could mate (Fig. 4C). This result was consistent with the finding that Reb1p was essential for silencing, but it was also possible that this was a pleiotropic phenotype caused by another function of Reb1p, distinct from silencing. Therefore, we wanted to test whether silencing...
of \(HML\alpha\) was lost when strains carrying the \(reb1\) (Ts) alleles were shifted to the nonpermissive temperature. RNA was prepared from wild-type, \(reb1-1\), \(reb1-2\), and \(reb1-3\) strains grown at 30°C and then shifted to the nonpermissive temperature (36°C, in liquid) thus inactivating Reb1p in the temperature-sensitive strains. An RNA-blot analysis was then performed with the \(HML\alpha\) gene as a probe (Fig. 4D). This analysis revealed that the \(reb1\) (Ts) strains grown at 36°C derepressed the cryptic \(HML\alpha\) gene. The wild-type strain did not derepress \(HML\alpha\) transcription at 36°C. These data thus confirmed the notion that Reb1p was essential for silencing in \(K. lactis\).

**DISCUSSION**

In this study, we characterized the \(K. lactis HML\alpha\) silencer. We found three discrete sequences that were essential for silencing and identified one of them as a Reb1p binding site. We found no evidence for the involvement of Rap1p or ORC binding sites. Additionally, Reb1p binding sites are not found...
Reb1p was essential for silencing in *K. lactis*. (A) Schematic drawing of three *reb1(Ts)* alleles. *K. lactis* Reb1p (595 amino acids long) contains two Myb domains (shaded boxes, top row), and the amino acid substitutions generated in the *reb1(Ts)* mutants are indicated below. The *reb1*-1 and *reb1*-2 alleles were generated by site-directed mutagenesis, and the *reb1*-3 allele was generated by hydroxylamine mutagenesis. Amino acid numbers in which the mutations were generated are also indicated. (B) The *reb1* alleles generated were temperature sensitive. Serial dilutions (10-fold) of a *K. lactis reb1::hisG* strain (SAY206) dependent on plasmid-encoded Reb1p were spotted onto rich medium (yeast extract-peptone-dextrose) at 30 and 35°C. The plasmid contained either the wild-type *REB1* gene or the *reb1*-1, *reb1*-2, and *reb1*-3 temperature-sensitive alleles.
in silencer sequences in Saccharomyces. The S. cerevisiae and K. lactis species are both classified as hemiascomycetes and belong to the same order, Saccharomycetales (61). They are more closely related to each other than is related to budding yeasts, such as Candida albicans and Yarrowia lipolytica (7). Given this close evolutionary relationship, it was surprising that the silencers appear so different in the two yeasts. A straightforward interpretation would be that the evolution of silencers is rapid. Others showed that the conservation of transcription factors among 13 different hemiascomycetous yeasts were lower than would be expected (20). They speculated that rapid evolution of transcription factors was somehow selected during speciation. The difference between Saccharomyces and Kluyveromyces silencers could thus be partly explained by rapid evolution of regulatory factors. Several similarities exist, however, and below we will discuss similarities and differences between the Saccharomyces and Kluyveromyces silencers.

Even though a functional ARS element was found close to the HMLα silencer in K. lactis, this ORC binding site did not contribute to silencing. The A and B box mutations completely abolished silencing, even in the presence of the ORC-binding site. The C box mutation, however, was partially suppressed by including the DNA fragment that contained the ARS activity. Not only was the ORC-binding site included on this fragment, but flanking DNA was included as well, and we prefer an explanation in which cryptic C boxes were present in these flanking sequences. The C box, defined by the mutagenesis of the silencer, bears a weak resemblance to an Abf1p binding site. In the DNA sequence between the minimal silencer and the ORC-binding site, there are in fact other sites that show a closer resemblance to an Abf1p binding site. It does not seem to be the case that the C box to an Abf1p binding site is weak, and thus it would be premature to conclude that Abf1p binds to this site. Moreover, in the HMRα silencer, we could not find sequences that resembled Abf1p binding sites or the C box, indicating that the protein binding to the C box was only important for silencing of HMLα and not HMRα.

The A box, found both in the HMLα and HMRα silencers, was similar to a binding site for the Saccharomyces Ume6p protein (63). One of the two A boxes in the HMRα silencer was in fact a perfect match to the Ume6p consensus sequence. In some gel mobility shift experiments (see, for example, Fig. 3C) using K. lactis protein extracts, we observed protein-DNA complexes that were migrating slower than the Reb1p-silencer complex. This observation is consistent with the idea that other proteins bind to the silencer, but such proteins are of low abundance or require different conditions for optimal DNA binding. In Saccharomyces, Ume6p is required for repressing transcription from meiosis-specific promoters during vegetative growth (48, 63), but has not been implicated in silencing. Ume6p recruits the Sin3p-Rpd3p complex to meiotic promoters (28), and therefore we tested whether Rpd3p was important for silencing in Kluyveromyces. Strains containing an rpd3- null allele showed improved silencing, indicating that Rpd3p counteracted silencing in Kluyveromyces (data not shown). Thus, if the A box was a Ume6p binding site, then recruitment of Rpd3p to this binding site cannot be important for maintaining silencing. It is still possible that the A box binds Ume6p, but Ume6p in this context recruits a complex distinct from the Rpd3p-containing complex. In Saccharomyces, Ume6p can in fact recruit a chromatin-remodeling complex containing the ISWI2 gene product (21). We are currently investigating the role of the K. lactis UME6 and ISWI2 genes in silencing.

In this study, we showed that the B box was a binding site for the Reb1 protein, and by using point mutations in the B box and conditional alleles of the REB1 gene, we concluded that Reb1p binding to the silencer was essential for silencing. The Reb1 protein contains two so-called “Myb domains.” The Myb domain is characterized by regularly spaced tryptophan residues, separated by 18 or 19 amino acids (46). This domain forms a structure related to, but distinct from, the canonical helix-loop-helix motif. The tryptophan repeat structure appears to be a general motif, utilized in the many members of the Myb gene family. It has already been noted that proteins that bind to eukaryotic telomeres often contain Myb-like domains (54). In humans (Trf1) (66), the budding yeasts S. cerevisiae and K. lactis (Rap1) (30, 38, 41), and the fission yeast (Taz1) (17), Myb domain proteins bind to telomeres. Moreover, in the case of S. pombe Taz1p and S. cerevisiae Rap1p, the interaction with telomeres contributes to telomeric position effect (17, 33, 43). In this study, we show that yet another Myb domain protein contributes to silencing in K. lactis. Thus, the Myb domain may have features that make it suited for silencers, since Myb domain proteins are required for silencing in such a diverse set of organisms.

It is tempting to speculate about the role, if any, of Reb1p in the phenomenon rDNA silencing. Reb1p binding to the rDNA locus to at least two different binding sites ensures correct transcriptional termination by RNA polymerase I (35, 36) and probably also affects the expression of rRNA (31). The rDNA locus can also impart position effects on RNA polymerase II-transcribed genes artificially placed in this locus (12, 19, 59). This phenomenon, rDNA silencing, requires Sir2p, but not the other Sir proteins (19, 59). To our knowledge, no silencer has been identified in the rDNA locus. Given the role of Reb1p in Kluyveromyces silencing, it would appear to be worth the effort to test the role of Reb1p in rDNA silencing in Saccharomyces.

Further study of the K. lactis HMLα and HMRα silencers
promises to be a fruitful avenue of research to learn more about characteristics of and variations among these DNA elements. It is likely that the A and C boxes correspond to protein binding sites, and the identification of the proteins binding to these sites is a high priority. This study shows that two evolutionarily related yeasts have quite different silencer elements to impart silencing of their cryptic mating-type loci. Furthermore, the K. lactis silencers cannot functionally complement S. cerevisiae HMR-E (A. Ehrenhofer-Murray, personal communication). Learning more about Klyveromyces silencers should further our understanding about how silencer elements work in general.

ACKNOWLEDGMENTS

J. Warner is acknowledged for plasmids and strains. We thank U. Saier for helping with the prediction of the structure of the Reb1p Myb domain. S.U. is supported by grants from the Swedish Natural Science Research Council (B-AA/BU 11279-306).

REFERENCES

1. Adams, A. D., E. Gottschling, C. A. Kaiser, and T. Stearns. 1998. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

2. Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics 116:541–545.

3. Aparicio, O. M., B. L. Billington, and D. E. Gottschling. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell 66:1279–1287.

4. Åström, S. U., A. Kegel, J. O. Sjöstrand, and J. Rine. 2000. Klyveromyces lactis Sir2 regulates cation sensitivity and maintains a specialized chromatin structure at the alpha-alpha locus. Genetics 156:81–91.

5. Åström, S. U., and J. Rine. 1998. Theme and variation among silencing proteins in Saccharomyces cerevisiae and Klyveromyces lactis. Genetics 148:1021–1029.

6. Ausselb, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 2002. Current protocols in molecular biology. John Wiley and Sons, New York, NY.

7. Barns, S. M., D. J. Lane, M. L. Sogin, C. Bibeau, and W. G. Weisburg. 1991. Evolutionary relationships among pathogenic Candida species and a cladistic analysis. J. Bacteriol. 173:2250–2255.

8. Bell, S. P., and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature 357:128–134.

9. Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth. 1987. A method for gene disruption that is supported by grants from the Swedish Natural Science Research Council (B-AA/BU 11279-306).

10. Buchman, A. R., and R. D. Kornberg. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82:488–492.

11. Buchman, A. R., and R. D. Kornberg. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82:488–492.

12. BYU, M. A. Rose, S. G. Holmes, C. D. Allis, and J. R. Broach. 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. 7:592–604.

13. Bryk, M., M. Banerjee, M. Murphy, K. E. Knudsen, D. J. Garfinkel, and M. J. Curiel. 1997. Transcriptional silencing of Tel1 elements in the RDN1 locus of yeast. Genes Dev. 11:255–269.

14. Buchman, A. R., and R. D. Kornberg. 1985. A yeast ARS-binding protein activates transcription synergistically in combination with other weak activating factors. Mol. Cell. Biol. 18:887–897.

15. Chan, C. S., L. Rastelli, and V. Pirrotta. 1997. Origin recognition complex, SIR1, and the S phase requirement for silencing. Science 276:1547–1551.

16. Cooper, J. P., E. R. Nimmo, R. C. Allshire, and T. R. Cech. 1997. Regulation of telomerase length and function by a Myb-domain protein in fusion yeast. Nature 385:744–747.

17. Cooper, J. P., E. R. Nimmo, R. C. Allshire, and T. R. Cech. 1997. Regulation of telomerase length and function by a Myb-domain protein in fusion yeast. Nature 385:744–747.

18. Fox, C. A., A. E. Ehrenhofer-Murray, S. Loo, and J. Rine. 1997. The origin recognition complex, SIR1, and the S phase requirement for silencing. Science 276:1547–1551.

19. Fritz, C. E., K. Verschueren, R. Strich, and R. Easton Espósito. 1997. Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J. 16:6495–6509.
a helix-turn-helix-related motif with conserved tryptophans forming a hydrophobic core. Proc. Natl. Acad. Sci. USA 89:6428–6432.
47. Ogata, K., H. Kanai, T. Inoue, A. Sekikawa, M. Sasaki, A. Nagadoi, A. Sarai, S. Ishii, and Y. Nishimura. 1993. Solution structures of Myb DNA-binding domain and its complex with DNA. Nucleic Acids Symp. Ser. 29:201–202.
48. Park, H. D., R. M. Luche, and T. G. Cooper. 1992. The yeast UME6 gene product is required for transcriptional repression mediated by the CAR1 URS1 repressor binding site. Nucleic Acids Res. 20:1909–1915.
49. Rastan, S. 1994. X chromosome inactivation and the Xist gene. Curr. Opin. Genet. Dev. 4:292–297.
50. Rine, J., and I. Herskowitz. 1987. Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. Genetics 116:9–22.
51. Rine, J., J. N. Strathern, J. B. Hicks, and I. Herskowitz. 1979. A suppressor of mating-type locus mutations in Saccharomyces cerevisiae: evidence for and identification of cryptic mating-type loci. Genetics 93:877–901.
52. Rivier, D. H., and J. Rine. 1992. An origin of DNA replication and a transcription silencer require a common element. Science 256:743–748.
53. Scherer, S., and R. W. Davis. 1979. Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Natl. Acad. Sci. USA 76:4951–4955.
54. Shore, D. 1997. Telomeres. Different means to common ends. Nature 385:676–677.
55. Shore, D., and K. Nasmyth. 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell 51:721–732.
56. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122:19–27.
57. Simon, J., A. Chiang, W. Bender, M. J. Shimell, and M. O’Connor. 1993. Elements of the Drosophila bithorax complex that mediate repression by Polycomb group products. Dev. Biol. 158:131–144.
58. Singh, J., and A. J. Klar. 1992. Active genes in budding yeast display enhanced in vivo accessibility to foreign DNA methylases: a novel in vivo probe for chromatin structure of yeast. Genes Dev. 6:186–196.
59. Smith, J. S., and J. D. Boeke. 1997. An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev. 11:241–254.
60. Smith, J. S., C. B. Brachmann, I. Celic, M. A. Kenna, S. Muhammad, V. J. Starai, J. L. Avalos, J. C. Escalante-Semerena, C. Gruenmeyer, C. Wolberger, and J. D. Boeke. 2000. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA 97:6658–6663.
61. Souciet, J., M. Aigle, F. Artigueneave, G. Blandin, M. Bolotin-Fukuhara, E. Bon, P. Brottier, S. Casaregola, J. de Monignore, B. Dujon, P. Durrens, C. Gaillardin, A. Lepingle, B. Llorente, A. Malpertuy, C. Neuveglise, O. Oziou-Kalogeropoulos, S. Potier, W. Saurin, F. Tekai, C. Toffano-Nioche, M. Wesselowski-Louvel, P. Wincker, and J. Weissman. 2000. Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies. FEBS Lett. 487:3–12.
62. Strahl-Bolsinger, S., A. Hecht, K. Luo, and M. Grunstein. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. 11:83–93.
63. Strich, R., R. T. Surosky, C. Steber, E. Dubois, F. Messenguy, and R. E. Esposito. 1994. UME6 is a key regulator of nitrogen repression and meiotic development. Genes Dev. 8:796–810.
64. Thon, G., K. P. Bjerring, and I. S. Nielsen. 1999. Localization and properties of a silencing element near the mat3-M mating-type cassette of Schizosaccharomyces pombe. Genetics 151:945–963.
65. Triolo, T., and R. Stemm-Blanc. 1996. Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature 381:251–253.
66. van Steensel, B., and T. de Lange. 1997. Control of telomere length by the human telomeric protein TRF1. Nature 385:740–743.
67. Wertman, K. F., D. G. Drubin, and D. Botstein. 1992. Syst. mutational analysis of the yeast ACT1 gene. Genetics 132:337–350.