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A Bipartite DNA-Binding Domain in Yeast Reb1p

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The *REB1* gene encodes a DNA-binding protein (Reb1p) that is essential for growth of the yeast *Saccharomyces cerevisiae*. Reb1p binds to sites within transcriptional control regions of genes transcribed by either RNA polymerase I or RNA polymerase II. The sequence of *REB1* predicts a protein of 809 amino acids. To define the DNA-binding domain of Reb1p, a series of 5′ and 3′ deletions within the coding region was constructed in a bacterial expression vector. Analysis of the truncated Reb1p proteins revealed that nearly 400 amino acids of the C-terminal portion of the protein are required for maximal DNA-binding activity. To further define the important structural features of Reb1p, the *REB1* homolog from a related yeast, *Kluyveromyces lactis*, was cloned by genetic complementation. The *K. lactis REB1* gene supports active growth of an *S. cerevisiae* strain whose *REB1* gene has been deleted. The Reb1p proteins of the two organisms generate almost identical footprints on DNA, yet the *K. lactis REB1* gene encodes a polypeptide of only 595 amino acids. Comparison of the two Reb1p sequences revealed that within the region necessary for the binding of Reb1p to DNA were two long regions of nearly perfect identity, separated in the *S. cerevisiae* Reb1p by nearly 150 amino acids but in the *K. lactis* Reb1p by only 40 amino acids. The first includes a 105-amino-acid region related to the DNA-binding domain of the *myb* oncoprotein; the second bears a faint resemblance to *myb*. The hypothesis that the DNA-binding domain of Reb1p is formed from these two conserved regions was confirmed by deletion of as many as 90 amino acids between them, with little effect on the DNA-binding ability of the resultant protein. We suggest that the DNA-binding domain of Reb1p is made up of two *myb*-like regions that, unlike *myb* itself, are separated by as many as 150 amino acids. Since Reb1p protects only 15 to 20 nucleotides in a chemical or enzymatic footprint assay, the protein must fold such that the two components of the binding site are adjacent.

Reb1p is an abundant DNA-binding protein encoded by the *REB1* gene of the yeast *Saccharomyces cerevisiae* (29, 37, 38). The same protein has been studied in other laboratories under the names Y (14), GRF2 (7), and QBP (6). Reb1p binds to double-stranded DNA with the core consensus sequence CCGGGTA (7, 37, 38), present within transcriptional control regions of many unrelated genes (7, 38, 51). Accumulating evidence suggests that Reb1p may play a fundamental role in the architecture of the genome. Thus, at its binding site within the *GAL1-GAL10* intergenic region (52), Reb1p causes a localized exclusion of nucleosomes, with a concomitant phasing of the nucleosomes in the adjacent regions (14). This alteration in chromatin structure may facilitate the interaction of transcription factors with the transcriptional control regions surrounding the Reb1p-binding site.

A number of lines of evidence have implicated Reb1p in the regulation of transcription. It serves to potentiate the activity of a weak activator, such as Gcn4p, binding upstream of a TATA-less *HIS3* transcription unit (6). Similarly, it stimulates the activity of a T-rich element both in vivo and in vitro (7). In both cases, Reb1p is effective only when it binds within a short distance of the other activator. Thus, the physical presence of Reb1p may act as a potentiator of transcription, irrespective of the other factors involved. In several cases, Reb1p has been implicated in the transcription of yeast genes. Basal-level transcription of the *ILV1* gene depends on the presence of a Reb1p-binding site in the upstream region (40a). Interestingly, an Abf1p-binding site can substitute for the Reb1p site. Deletion of the Reb1p-binding site from the upstream activating site of the *TPI1* gene reduces transcription by 80% (45a).

There are two Reb1p-binding sites in the spacer region separating the rRNA genes. One lies within the enhancer that is present just downstream of each transcription unit. The second, 2 kb downstream, is 200 nucleotides upstream of the transcription initiation site, just beyond the promoter region, that has been mapped between positions −150 and +4 (8, 32, 39). Kulkens et al. (33) have recently shown that in the context of chromosomal DNA, although not in a plasmid (34), the binding of Reb1p plays an important role in the efficiency of transcription of the rRNA genes. Furthermore, in vitro evidence implicates Reb1p binding within the enhancer in termination of the rRNA transcript (34a).

Reb1p has been purified, and its gene has been cloned and sequenced (7, 29, 38). It encodes a protein of 809 amino acids. The *REB1* gene is essential for the growth of *S. cerevisiae* (29). Within Reb1p is a region that resembles the DNA-binding domain of the oncoprotein Myb (29). The Myb DNA-binding motif is present in a family of proteins that are conserved throughout eukaryotes (4, 20, 36, 40, 44). It consists of three tandem repeats of 51 to 52 amino acids near the N terminus of the protein (1, 43, 44). Each repeat contains three tryptophans separated by 18 to 19 amino acids, with approximately 12 amino acids between each repeat. Only the second and the third repeats are required for DNA binding (25).

We now show, using a series of deletions, that the DNA-binding domain of Reb1p comprises nearly the entire C-terminal half of the protein, including the Myb-like DNA-binding motif. To further define the salient structural features of Reb1p, its homolog from the related yeast *Kluyvero-*

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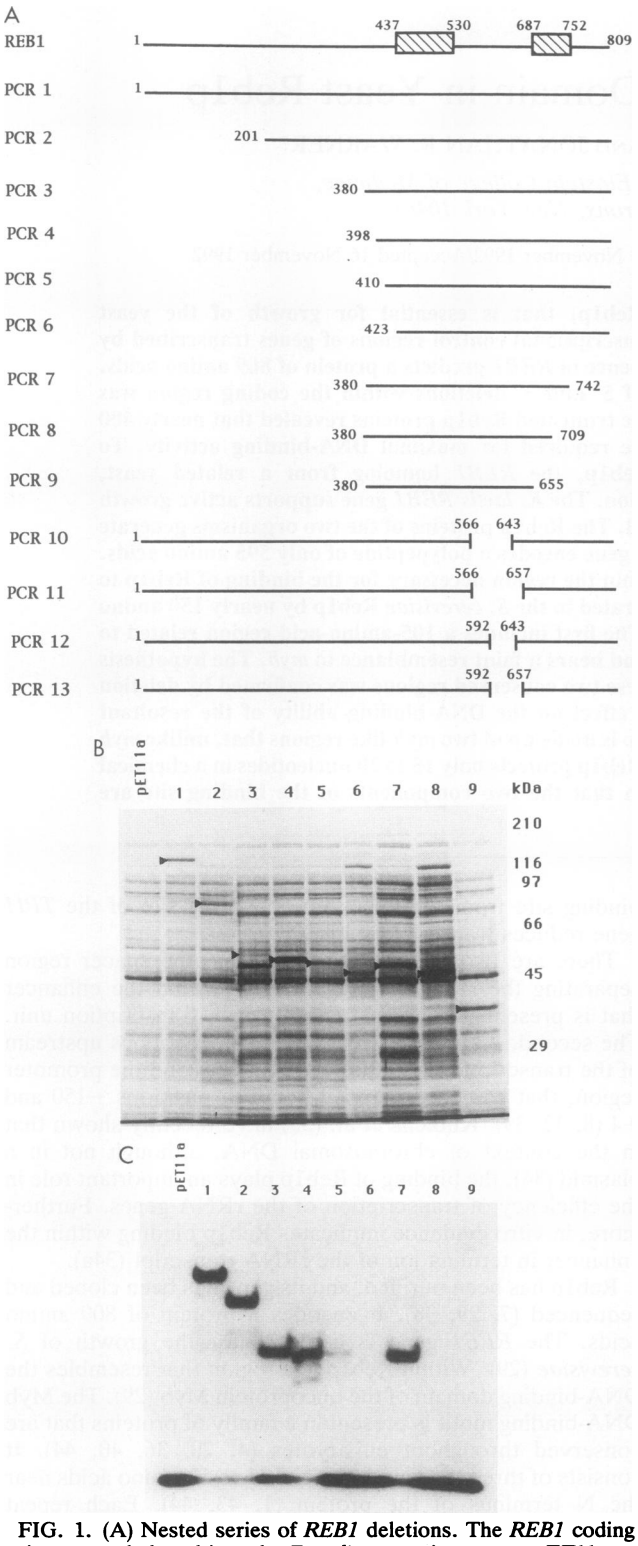

FIG. 1. (A) Nested series of *REB1* deletions. The *REB1* coding region was subcloned into the *E. coli* expression vector pET11a as a series of deletions synthesized by PCR. The full-length *REB1* gene encodes a protein of 809 amino acids with one region that resembles the DNA-binding domain of Myb rather closely (hatched box on the left) and another that resembles it less closely (hatched box on the right). PCR1 represents the full-length Reb1p. PCR2 to PCR6 contain deletions of increasing size, starting at the N terminus of the protein. PCR7 to PCR9 contain both N- and C-terminal deletions. PCR10 to PCR13 are based on PCR2, with additional internal deletions. The N- or C-terminal amino acid residues of each construct are indicated. (B) Expression of Reb1p and its derivatives in *E. coli*. SDS-PAGE analysis of induced expression of truncated Reb1p proteins in bacterial extracts was performed. The coding sequence of *REB1* and of truncated derivatives was cloned behind the T7 promoter in the *E. coli* vector pET11A (see Materials and Methods). The crude protein extracts were analyzed by SDS-PAGE (10% polyacrylamide gel). The Reb1p derivatives are indicated by arrowheads in lanes representing PCR1 to PCR9 in panel A. Note that in sample PCR3, we consistently observe that most of the product is shorter than expected, presumably as a result of proteolytic activity. Comparison of the migration of the complex with that of PCR4 (C) suggests that only the full-length, fainter band indicated by the arrowhead binds the DNA. Positions of molecular weight markers myosin, β-galactosidase, phosphorylase *b*, bovine albumin, egg albumin, and carbonic anhydrase are indicated on the right. (C) Gel retardation analysis of truncated Reb1p proteins. Gel retardation assays were performed with the samples analyzed in panel B, using 12.5% as much extract. The radiolabeled, double-stranded oligonucleotides JW107 and JW106 were used as a probe for binding activity.

myces lactis was cloned by genetic complementation. The *K. lactis REB1* gene (termed *Kl-REB1*) encodes a protein that has only 40% sequence identity and is substantially smaller than the *S. cerevisiae* protein. Yet Kl-Reb1p yields almost an identical DNA footprint and supports cell growth at nearly the same rate. Comparison of the sequences of the two proteins suggested that the DNA-binding domain of Reb1p is bipartite. Analysis of the effects of internal deletions within Reb1p confirmed this idea.

MATERIALS AND METHODS

Strains. The basic yeast strain used was W303 (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) obtained from R. Rothstein (Columbia University). Strain J342 is a derivative of W303 in which the *REB1* gene has been disrupted by insertion of *LEU2*; growth is supported from a plasmid carrying *REB1* under control of the *GAL1* promoter (29). Strain J343 is like J342 except that growth is supported by a plasmid carrying the *Kl-REB1*. *Escherichia coli* BL21(DE3) is described in reference 47.

Expression of *REB1* and its derivatives in *E. coli*. Polymerase chain reaction (PCR) amplification of *REB1* was performed by using a pair of oligonucleotides forming the 5′ and the 3′ borders of the coding region for each mutant. The oligonucleotide forming the 5′ border contained an *Nde*I site that includes the codon for the initiator methionine and was used to insert the *REB1* coding region in frame (47). The oligonucleotide forming the 3′ border contained a *Bam*HI linker. Following digestion with *Nde*I and *Bam*HI, each PCR product was inserted into the vector pET11a (47), cut with *Nde*I and *Bam*HI.

To construct *REB1* mutants with internal deletions (PCR10 to PCR13), a series of PCR amplifications utilizing four different oligonucleotides was used. Each mutant consisted of two PCR half-reactions linked in frame by a *Sal*I restriction site. The antisense oligonucleotides used to form the 5′ half of *REB1* started at either nucleotide 1683 or nucleotide 1716 (29), each containing a *Sal*I site 8 bp from the 3′ end. The 3′ half of *REB1* was formed by using two oligonucleotides, with *Sal*I linkers, starting at position 1914 or 1956. In all cases, PCR2 was used as a template. After purification, the PCR products were cut with *Nde*I and *Sal*I (for the 5′ products) or with *Sal*I and *Bam*HI (for the 3′ products) and ligated with the pET11a vector in all four

combinations to produce PCR10 to PCR13 (Fig. 1A). The constructs were sequenced at the junctions for verification.

Each resulting plasmid was transformed into *E. coli* BL21(DE3) (47). Expression of the recombinant protein was induced by incubation of mid-log-phase cultures with 0.4 mM isopropylthiogalactopyranoside (IPTG) for 3 h. The cells were then centrifuged and resuspended at 1/20 volume in a solution of 10 mM Tris HCl (pH 8)–1 mM EDTA. The cells were initially disrupted by a rapid freeze-thaw and then sonicated at 4°C following the addition of 1 mM phenylmethylsulfonyl fluoride. The sample was centrifuged at 13,000 rpm in an Eppendorf Microfuge for 15 min. To the soluble fraction was added 3 volumes of saturated $(NH_4)_2SO_4$. After 30 min on ice, the sample was centrifuged and the pellet was resuspended in 10 mM Tris–1 mM EDTA (pH 7.0). These samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and to gel retardation assays (Fig. 1 and 7).

Preparation of crude yeast extract. Mid-log-phase cultures (10^7 cells per ml) of *S. cerevisiae* or *K. lactis* grown in YPD medium were harvested. The cells were washed once with H_2O and then with homogenization buffer (37). The cells were resuspended at 1/50 the original volume in homogenization buffer and disrupted by vortexing with glass beads. The homogenate was subjected to centrifugation at 13,000 rpm in an Eppendorf centrifuge for 10 min. The supernatant following centrifugation was used for SDS-PAGE and DNA binding assays.

Probes for Reb1p binding. Complementary oligonucleotides (38)

JW107 5′-GATCTACTGGGTTACCCGGGGCACCTG-3′
JW106 3′- ATGACCCAATGGGCCCCGTGGACCTAG-5′

containing the Reb1p-binding site from the rRNA promoter (underlined) were radiolabeled with [^{32}P]ATP by using polynucleotide kinase. The oligonucleotides were annealed and purified from the free nucleotide. This probe was used in gel retardation assays. For Southwestern (DNA-protein) blot analysis, the double-stranded, radiolabeled oligonucleotide was ligated to form oligomers as described previously (29).

For the Cu-phenanthroline protection assays, a 312-bp *Hin*pI-*Eco*RI fragment from the rRNA spacer containing the sequences from −268 to +44 with respect to the 35S rRNA transcription initiation site was cloned into the *Sma*I site within the polylinker of the *E. coli* vector pUC19 and named pUC-rRTI-2. A 345-bp *Eco*RI-*Hin*dIII fragment containing these sequences was labeled with [α-^{32}P]dATP at either end, separately, using the Klenow fragment of DNA polymerase.

As a probe for the *K. lactis* transcription initiation region (−302 to +38), a 341-bp DNA was synthesized by PCR using pGEM3Z-Kl.5 as a template and was end labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Gel retardation assays. Each reaction mixture contained the protein sample, 1 μg of poly(dI-dC) (Pharmacia), 1,500 cpm (Cerenkov) of the ^{32}P-radiolabeled oligonucleotide probe in 20 mM Tris HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 500 μg of gelatin per ml, and 10% glycerol. The samples were incubated on ice for 20 min. Samples were then loaded onto a TBE gel (50 mM Tris base, 50 mM boric acid, 1 mM Na EDTA, adjusted to pH 7.5) containing 5% acrylamide and electrophoresed at 100 V for 1.5 h in TBE. The gels were dried and autoradiographed.

Gel retardation assays of in vitro-translated *REB1* proteins were performed as described above except that 1 ng of unlabeled oligonucleotides JW106 and JW107 was used instead of the radiolabeled probe.

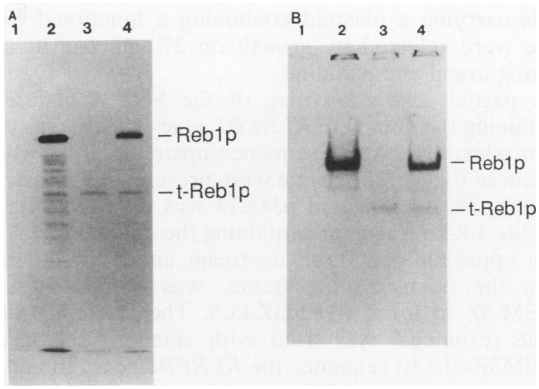

FIG. 2. Evidence that Reb1p appears to bind DNA as a monomer. Reb1p and a t-Reb1p were synthesized, either separately or together, by in vitro translation in a reticulocyte extract as described in Materials and Methods. (A) SDS-PAGE analysis of the translation products, using no mRNA (lane 1), *REB1* mRNA (lane 2), *t-REB1* mRNA (lane 3), and a mixture of the two mRNAs (lane 4). The major products, Reb1p and t-Reb1p, are indicated. (B) Gel retardation assays with the translation products of panel A (same lane designations) and oligonucleotides JW107 and JW106. Note that any protein not binding to the oligonucleotide will not migrate into this gel.

Constructs for in vitro translation. A 3.5-kb *Bam*HI-*Sal*I fragment from pBM272-41, containing the *REB1* coding region, was inserted into the *Bam*HI-*Sal*I site of pGEM-3Z to form *REB1-1*.

To generate an N-terminally truncated Reb1p (t-Reb1p), oligonucleotides 5′-AATTCTCCATGGTGCCACCATGG AG and 5′-AATTCTCCATGGTGGCACCATGGAG were annealed, leaving *Eco*RI overhangs. This double-stranded oligonucleotide was ligated to a 2.1-kb *Eco*RI fragment containing the C-terminal two-thirds of the *REB1* coding region. This oligonucleotide provides an ATG in frame with the rest of the *REB1* coding region to allow for translation of the open reading frame. This ligation product was then cloned into the *Eco*RI site of the pGEM-3Z polylinker to form *t-REB1*.

In vitro transcription and translation. The templates were linearized with *Sal*I to produce runoff transcripts containing *REB1* sequences only. Both *REB1* and *t-REB1* mRNAs were synthesized separately, using T7 RNA polymerase. Capped RNA transcripts were prepared as described in the Promega guide.

The RNA (1 μg) from *REB1*, *t-REB1*, or both was translated by using a reticulocyte lysate translation mixture (Bethesda Research Laboratories) plus 10 μCi of [^{35}S]methionine (NET009A; NEN).

Cloning and sequencing of *Kl-REB1* gene. The *K. lactis* gene for Reb1p was cloned by genetic complementation in *S. cerevisiae* by using a modified plasmid shuffle technique. In *S. cerevisiae* haploid strain J342, the *LEU2* gene replaced the major part of the *REB1* gene. Reb1p was supplied from pBM272-41, a *HIS3*-containing plasmid with the *REB1* gene under the control of the *GAL1* promoter. Colonies could form on minimal medium containing 2% galactose but not on medium containing 2% glucose. An amplified genomic recombinant DNA library from 9-kb *Sau*3A fragments from *K. lactis* (strain NRRL Y1140), cloned into the multicopy shuttle vector pAB24 (kindly provided by R. Dickson, University of Kentucky), was used to transform strain J342.

Cells carrying a plasmid containing a functional *Kl-REB1* gene were selected by growth on 2% glucose in medium lacking uracil and histidine.

A partial restriction map of the 8-kb *K. lactis* insert containing the complete *Kl-REB1* gene (1.8 kb) was defined. Approximately 2 kb of sequence upstream and 4 kb downstream of the *Kl-REB1* gene were present within the plasmid named p342.14. Plasmid p342.14 was digested with *Sma*I, and the 4.8-kb fragment containing the entire *Kl-REB1* gene, with approximately 1 kb upstream and 2 kb downstream from the open reading frame, was cloned into vector pGEM-3Z to form pGEM3Z-kl.5. The Erase-a-Base technique (Promega) was used with smaller subclones from pGEM3Z-kl.5 to sequence the *Kl-REB1* gene. In addition, a series of oligonucleotides was used to complete the sequence on both strands. The DNA was purified by the cetyltrimethylammonium bromide (CTAB) method (9) and sequenced (Sequenase kit; U.S. Biochemical).

Nucleotide sequence accession number. The *Kl-REB1* sequence has been submitted to GenBank under accession number L03789.

RESULTS

The DNA-binding domain of Reb1p. To define the DNA-binding domain of Reb1p, we constructed a series of 5′ and 3′ deletions of the *REB1* coding region (Fig. 1A), which was then introduced into a bacterial expression vector (47). Expression of the truncated Reb1p proteins was analyzed by SDS-PAGE (Fig. 1B). Although the predicted molecular weight of Reb1p is 92,058, the protein in both yeast (29; data not shown) and bacterial (Fig. 1B, lane 2) extracts migrates anomalously at the position of approximately 125,000 Da (38).

Each of the protein samples shown in Fig. 1B was used in a band shift assay, using as a probe a double-stranded oligonucleotide containing the Reb1p-binding site found just upstream of the rRNA promoter. Comparison of the abundance of the expressed protein (arrowheads in Fig. 1B) with the strength of the band shift (Fig. 1C) permits an estimate of the DNA-binding ability of the protein. The full-size product (PCR1) forms a complex that migrates just slightly faster than the complex with purified yeast Reb1p (not shown), perhaps as a result of phosphorylation of the latter. Such phosphorylation is evidently not required for binding to DNA. Proteins PCR2 to PCR6 represent truncations from the N-terminal end. Deletion of 201 amino acids (PCR2) or even 380 amino acids (PCR3) has little effect on the binding to DNA. Deletion of 18 additional amino acids (PCR4) causes a reduction of binding activity; deletion of 12 more (PCR5) leads to barely detectable binding, and any further deletion (PCR6) abolishes binding.

Deletion of 67 amino acids from the C terminus (PCR7) causes a moderate reduction in DNA binding, while more extensive deletions (PCR8 and PCR9) abolish binding. Thus, the region required for optimal binding to DNA is nearly the entire the C-terminal half of Reb1p. This region includes an approximately 105-amino-acid region that bears substantial homology to the oncoprotein Myb (29). However, the complete binding region is clearly far larger than the Myb domain.

Reb1p appears to bind as a monomer. The high-affinity binding observed for t-Reb1p suggested that the method devised by Hope and Struhl (24) could be used to ascertain whether dimerization of Reb1p is essential for binding to DNA. mRNAs corresponding to the full-length protein and to amino acids 201 to 809, the shorter version that retains strong binding, were translated in a reticulocyte extract (Fig. 2A) and then used in a band shift assay (Fig. 2B). It is apparent that products of each mRNA could bind DNA (Fig. 2A and B, lanes 2 and 3). When the mRNAs were mixed and translated together (Fig. 2A, lane 4) and the translation products were used in a band shift assay, bands of intermediate size were not observed (Fig. 2B, lane 4), nor was there a decrease of the band corresponding to the smaller protein, as might be expected if the large and small versions could interact to bind the DNA probe. This result suggests that Reb1p binds as a monomer.

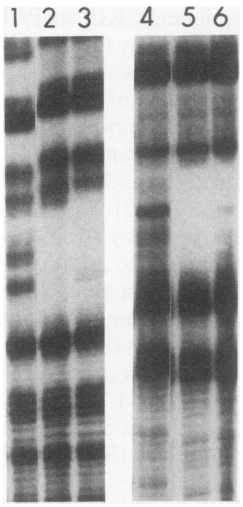

FIG. 3. Footprint analysis of Reb1p and Kl-Reb1p. Indirect Cu-phenanthroline protection assays were performed with partially purified Reb1p (through the heparin-Sepharose step [38]) from either *S. cerevisiae* (lanes 2 and 5) or *K. lactis* (lanes 3 and 6). The assays were performed with a 350-bp DNA fragment containing the Reb1p-binding site near the *S. cerevisiae* rRNA promoter region. The probe was labeled separately on one strand with Klenow fragment (lanes 1 to 3) and on the other with polynucleotide kinase (lanes 4 to 6). Lanes 1 and 4 contain uncomplexed DNA.

***K. lactis* contains a Reb1p homolog.** One approach to defining further the important structural regions of Reb1p is to compare sequences of protein homologs from related organisms. Conserved regions are likely to be regions of functional importance. The budding yeast *K. lactis* is related, but not closely, to *S. cerevisiae*. Comparison of their 18S rDNA sequences suggests that they have been separated for $>10^8$ years (3, 50), yet their intron-exon signals are the same (10). At the time this work was started, there was one example of a *K. lactis* DNA-binding protein that would function in *S. cerevisiae*, Lac9p, analogous to Gal4p of *S. cerevisiae*. Comparison of their sequences indicated that there are both conserved and divergent regions (35, 45, 53). More recently, genes for two additional DNA-binding proteins, Hsp1 (27) and Abf1p (19), have been isolated from *K. lactis*.

An extract of *K. lactis* contains a protein that yields a footprint identical to that of *S. cerevisiae* Reb1p (Fig. 3). Southwestern blot analysis also identified a *K. lactis* homolog to Reb1p that, surprisingly, appears to be only 75% the size of the *S. cerevisiae* protein (Fig. 4, lanes 1 and 2).

The *K. lactis REB1* homolog cloned by genetic complementation. Since *REB1* is essential for the growth of *S. cerevisiae* (29), we attempted to clone the *K. lactis REB1* gene by

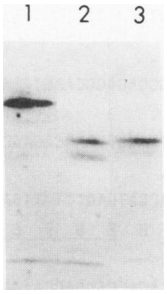

FIG. 4. Southwestern blot analysis of Reb1p and Kl-Reb1p. Southwestern blots were prepared with equal amounts of crude extracts from *S. cerevisiae* W303a (lane 1), *K. lactis* (lane 2), and *S. cerevisiae* J343 (lane 3). The radiolabeled, annealed, and polymerized oligonucleotides JW107 and JW106 were used as a probe (29).

genetic complementation. In strain J342 of *S. cerevisiae*, the genomic *REB1* was replaced by *LEU2*; growth depends on the presence of a plasmid with the complete *REB1* gene under control of the *GAL1* promoter. Strain J342 can grow in medium containing galactose but not glucose. A *K. lactis* genomic library of 9-kb partial *Sau*3A fragments in a plasmid carrying the *S. cerevisiae* 2μm origin of replication and the *URA3* marker was transformed into strain J342. A clone that complemented the Reb1p deficiency was selected by growth in glucose. Approximately 0.03% of the Ura^+ transformants grew in glucose, equivalent to one colony per two to three times the *K. lactis* genome. To confirm that the *K. lactis* *REB1* gene had been cloned, the plasmid containing the putative *K. lactis* *REB1* gene was isolated from the transformed *S. cerevisiae* strain and used to retransform a diploid *S. cerevisiae* strain with the genotype *REB1/reb1::LEU2*. Following sporulation, tetrad analysis yielded four viable spores, two of which carried both the *LEU2* and *URA3* markers. Since the genomic *REB1* gene was disrupted in such cells, the plasmid must contain a *K. lactis* gene that is able to complement the essential function of *REB1*. Strain J343 was established from a Leu^+ Ura^+ spore that contains the *K. lactis* homolog of *REB1*, termed *Kl-REB1*.

Southwestern analysis confirmed that J343 contained a DNA-binding protein with Reb1p-binding specificity that had the same apparent molecular weight as did the Reb1p of *K. lactis* (Fig. 4, lane 3). Southwestern blot analysis indicates that there are nearly equivalent Reb1p DNA-binding activities present in the extracts from wild-type *S. cerevisiae* and from strain J343, containing the cloned *K. lactis* gene. Furthermore, the generation time of strain J343 is only 25% greater than that of the wild-type strain, W303a. Thus, during evolution, the *K. lactis* protein has retained not only the DNA-binding activity but also the functional elements of Reb1p.

Sequence analysis of *Kl-REB1*. *Kl-REB1* was subcloned and sequenced (Fig. 5). The gene is predicted to encode a protein (Kl-Reb1p) of 595 amino acids with a molecular mass of 67,975 Da. Comparison of the sequence with that of *S. cerevisiae* *REB1* (29; GenBank accession number M36598) confirms that while Kl-Reb1p is 25% shorter than *S. cerevisiae* Reb1p, as was suggested by Southwestern blot analysis (Fig. 4), there is 40% identity in the amino acid sequences (underlined in Fig. 5). The identity is particularly evident in the C-terminal portion of the proteins.

A dot plot can detect regions of similarity that are not necessarily apparent from a one-to-one comparison of two amino acid sequences. The amino acid sequences of Reb1p and Kl-Reb1p were compared in the dot plot of Fig. 6. It is evident that most of the *K. lactis* sequences are represented within the *S. cerevisiae* protein. There are, however, several interruptions. Some of these, e.g., between residues 180 and 225 of the *K. lactis* protein, represent evolutionary divergence. Others, e.g., approximately at residues 40 and 420, represent deletions (or insertions into the *S. cerevisiae* gene).

The Reb1p DNA-binding domain contains two long stretches of homology to Kl-Reb1p, as indicated in Fig. 5 and 6. The first, of 120 residues (residues 295 to 415) of the *K. lactis* protein, includes the Myb motif. The second is from residue 461 to about 529. While the two regions are only about 40 amino acids apart in the *K. lactis* protein, they are nearly 140 amino acids apart in the *S. cerevisiae* protein. This finding suggests that during evolution there has been an insertion, or deletion, of more than 100 residues. The lack of conservation in this region suggests that the binding site of Reb1p may be bipartite, including the Myb-like region and the more C-terminal region, separated by a dispensable region.

The Reb1p DNA-binding domain is bipartite. To determine whether the DNA-binding domain of Reb1p is indeed bipartite, we constructed internal deletions between the two conserved regions (PCR10 to PCR13; Fig. 1A). Several were used, as we did not know how any specific deletion would affect the folding of the protein. After expression in *E. coli*, extracts were analyzed by SDS-PAGE (Fig. 7A) and in a gel retardation assay for DNA-binding activity. As shown in Fig. 7B, each had DNA-binding activity, although to a somewhat diminished degree compared with the wild-type protein. We conclude, then, that the DNA-binding region of Reb1p is bipartite, with the minimum binding domain encompassing approximately residues 400 to 566 and residues 657 to 742.

An upstream Reb1p-binding site is conserved. An interesting feature of the *REB1* gene of *S. cerevisiae* is the presence of two Reb1p-binding sites upstream from its transcription initiation site: TTACCCGT and CCGGGTGG, at positions −234 and −261, respectively, from the AUG. Specific binding to each site was demonstrated by gel retardation analysis (29).

Although there is no apparent sequence homology between the upstream regions of *REB1* and *Kl-REB1*, searching this sequence for the Reb1p-binding consensus sequence, CCGGGTAA, revealed a potential binding site, TACCCGG, at position −245 (Fig. 5). Gel retardation analysis of a fragment of DNA containing this region is shown in Fig. 8. A single protein-DNA complex was observed with use of purified *S. cerevisiae* Reb1p (Fig. 8, lanes 1 to 3) and PCR1 (Fig. 8, lanes 7 to 9). Binding to the *Kl-REB1* upstream site was competed for by the double-stranded oligonucleotides JW107 and JW106, containing a strong Reb1p-binding site (Fig. 8, lanes 4 to 6 and 10 to 12). Thus, the presence of a Reb1p-binding site upstream of the *REB1* gene has been conserved through evolution.

DISCUSSION

Reb1p from *K. lactis* is functionally homologous to the Reb1p of *S. cerevisiae* by at least two criteria: it yields essentially the same footprint on binding to DNA, and it supports growth of *S. cerevisiae* cells at nearly the same rate. However, the *K. lactis* protein is 25% smaller than the *S. cerevisiae* protein (Fig. 4) and overall has only 40% sequence identity to it (Fig. 5).

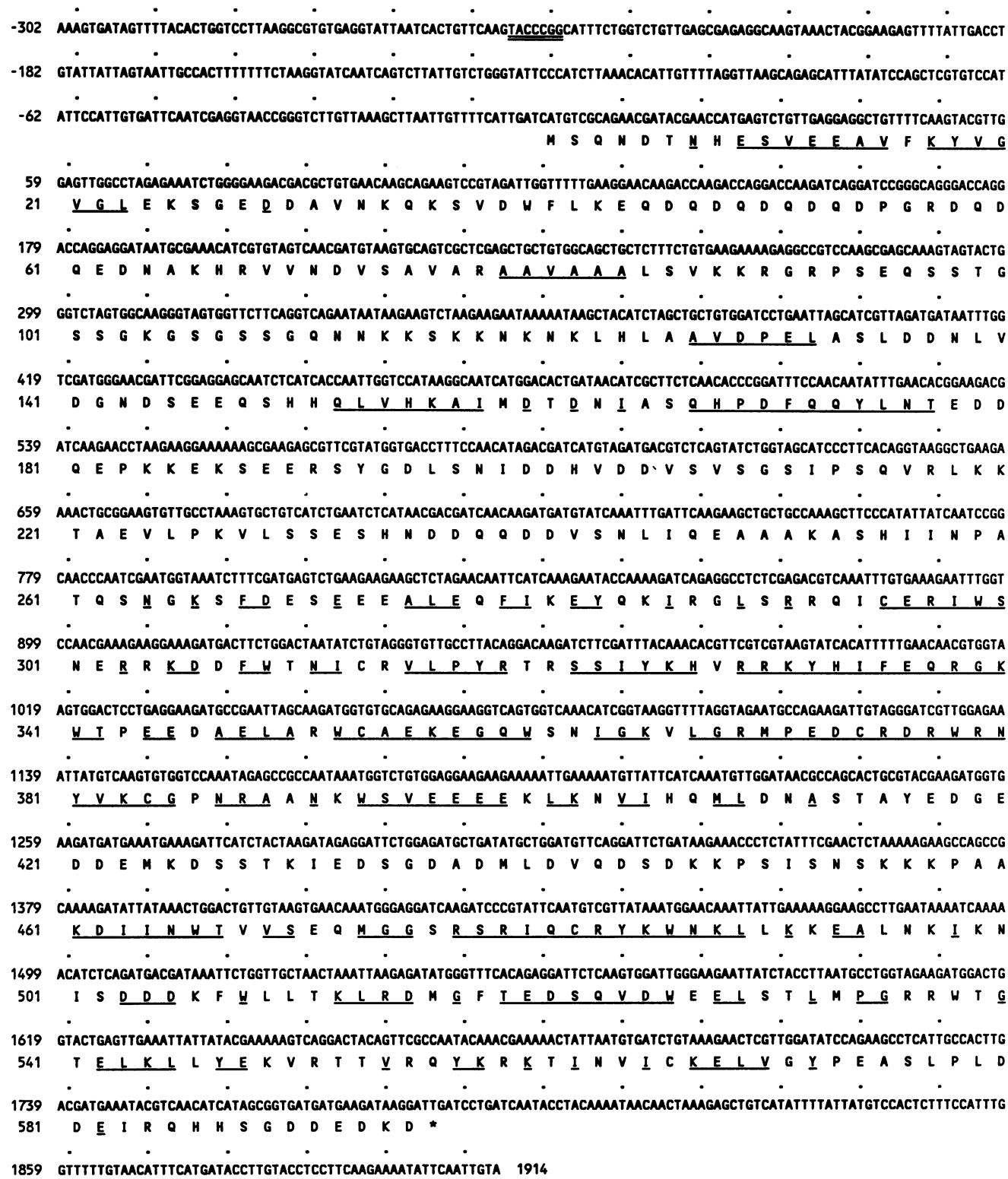

FIG. 5. Sequence of *Kl-REB1*. The *REB1* gene cloned from *K. lactis* was sequenced, and an open reading frame was ascertained. The predicted amino acid sequence is shown. Underlined residues are identical when Kl-Reb1p and Reb1p are aligned.

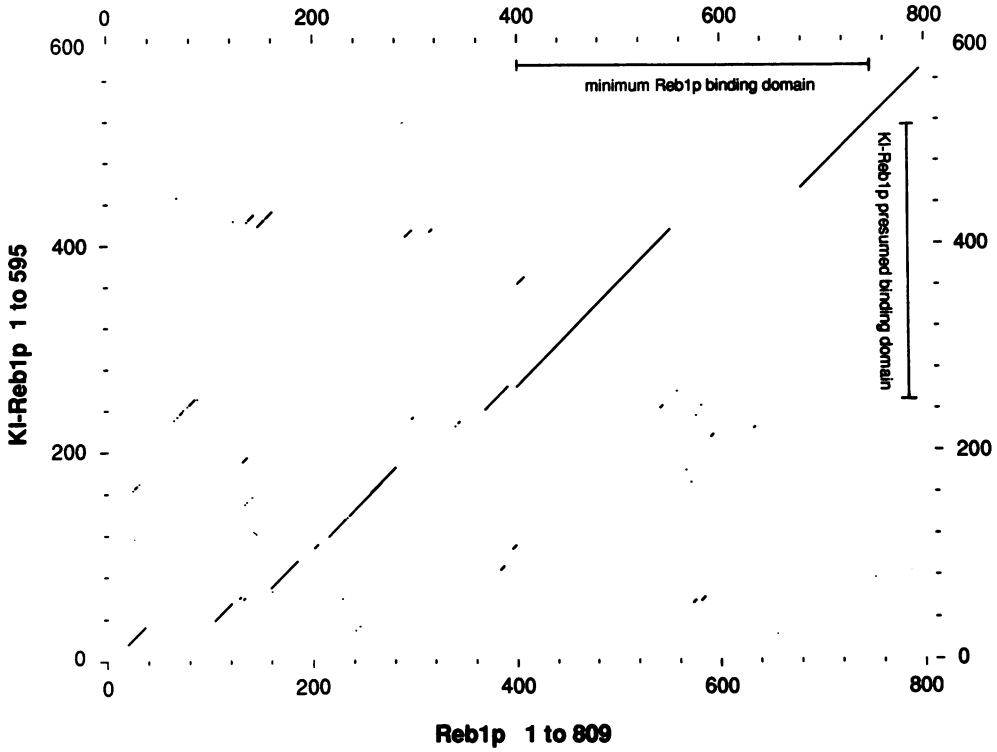

FIG. 6. Dot plot analysis of Kl-Reb1p and Reb1p. The amino acid sequence of Kl-Reb1p was compared with that of Reb1p, using the Genetics Computer Group COMPARE program (11), with the following parameters: window = 30 and stringency = 17. Regions of homology are represented by adjacent dots that connect to a line. The insertion (or deletion) of residues between homologous regions is apparent from the horizontal offset between two adjacent diagonals.

Despite these differences, the dot plot of Fig. 6 shows that large regions of *K. lactis* Reb1p are represented by similar sequences within the *S. cerevisiae* protein. The difference in size between the two proteins is due largely to insertions within the *S. cerevisiae* protein (or deletions in the *K. lactis* protein) at several sites, e.g., residues 40 to 100, 130 to 170, 270 to 369, and 550 to 680.

In addition to the homologies in the DNA-binding region, described below, there are two highly conserved regions near the N terminus of the protein (Fig. 5): from residues 9 to 22, there are 14 of 15 identities; from residues 152 to 177, there are 21 of 26 identities. The evolutionary conservation of these regions suggest that they play an important role in the function of Reb1p, perhaps by interacting with other proteins.

It is often possible to identify a short sequence of a protein that forms a discrete domain entirely responsible for the binding to DNA (16, 17, 22, 28, 31). Proteins containing specific structural motifs such as a helix-turn-helix, a Zn-binding domain, or a leucine zipper tend to have small DNA-binding domains of less than 100 amino acids (22). For Reb1p, however, the DNA-binding domain consists of the 430-amino-acid C-terminal portion of the protein. Removal of the N-terminal 18 amino acids or the C-terminal 67 amino acids of this region reduced binding activity by half. Further deletions from either end of this 344-amino-acid region destroyed binding activity. Reb1p appears to bind as a monomer (Fig. 2), implying that the extent of the binding domain is not due to a need for a dimerization site.

Within the DNA-binding region of Reb1p are two stretches of amino acids that are highly conserved between the homologs in the two yeast strains. The first, of about 105 amino acids, has 77% identity to Kl-Reb1p and 30% identity to the DNA-binding domain of Myb (43, 44). In particular, Reb1p has a region of repeating tryptophan residues that are characteristic of the binding region of Myb. Figure 9 shows an alignment of the Reb1p's of the two yeasts with Myb and with Bas1p, another protein of *S. cerevisiae* that has homology to Myb (49).

Myb is characterized by three repeats of 52 amino acids, each containing three regularly spaced tryptophan residues (1, 15, 43, 44). The binding of Myb to DNA requires only repeats 2 and 3 (25) (Fig. 9), residues 90 to 194. It has been proposed that each repeat includes a helix-turn-helix motif (16, 18, 43) or a helix-turn-helix-turn-helix motif (30) and that these motifs combine to form an extensive DNA recognition site (18). Mutational analysis suggests that the hydrophobic, rather than the aromatic, nature of the tryptophans is critical, perhaps in stabilizing a helix-loop-helix structure (43).

Reb1p, when aligned with Myb, has only one and a half of those repeats (residues 476 to about 549 of the *S. cerevisiae* Reb1p; residues 340 to 412 of the *K. lactis* protein), perhaps accounting for the lack of binding of this region alone. Bas1p, in contrast, has two full repeats (residues 114 to 210) (49). It is interesting that a *Drosophila* transcription factor, adf-1, has a region homologous to just one of the Myb-like repeats of Reb1p (13). Its DNA-binding domain has not yet been determined.

The second long stretch of homology between the two yeast proteins, residues 461 to 570 of *K. lactis* Reb1 (Fig. 5; residues 687 to 762 of *S. cerevisiae* Reb1p) bears some resemblance to Myb as well. There is a set of four regularly

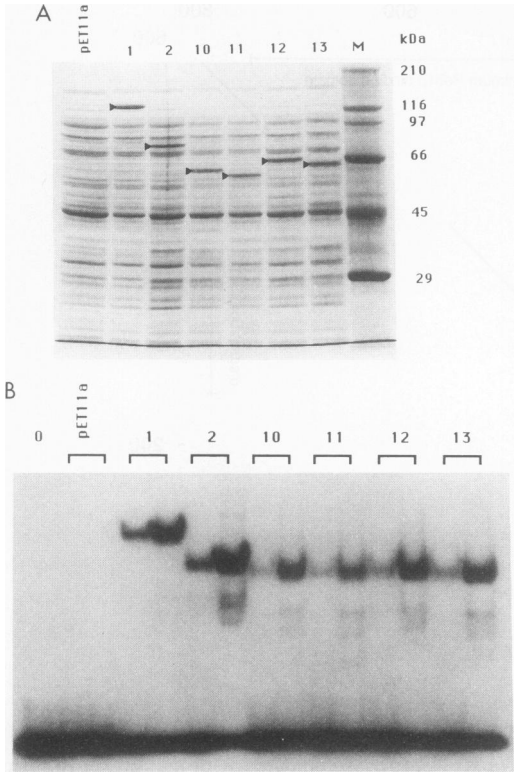

FIG. 7. (A) Expression of Reb1p with internal deletions in *E. coli*. The expression of PCR1, PCR2, and PCR10 to PCR13 (Fig. 1A) was analyzed as in Fig. 1B. Thirty-five microliters of sample was used in each lane. Lane M, molecular size markers. (B) Gel retardation analysis of Reb1p with internal deletions. Gel retardation analysis, as in Fig. 1C, was carried out with the samples of panel A. One (left-hand lanes) and 4 (right-hand lanes) μl of each sample were assayed. In lane 0, no extract was added.

spaced tryptophans 18 to 22 residues apart, although the intervening amino acids are much less conserved.

We propose that the remainder of the DNA-binding site is formed from this Myb-like region. The binding site of Reb1p

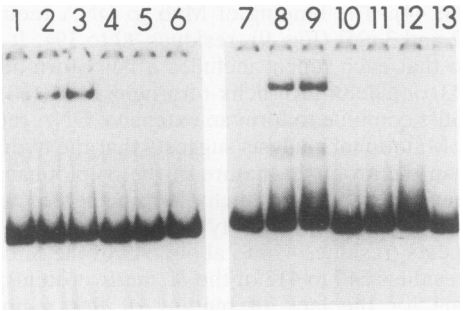

FIG. 8. Gel retardation analysis of the Reb1p-binding site upstream from the *Kl-REB1* gene. A 341-bp radiolabeled PCR-synthesized DNA fragment containing the region from −302 to +38 relative to the translation initiation site of Kl-Reb1p was incubated with increasing concentrations of either purified *S. cerevisiae* Reb1p (lanes 1 to 3) or PCR1 protein (lanes 7 to 9). Lanes 4 to 6 and 10 to 12 are identical to lanes 1 to 3 and 7 to 9, respectively, except that a specific competitor for Reb1p binding (100 ng of annealed double-stranded oligonucleotides JW107 and JW106) (38) was added to each reaction. Lane 13 contains only the radiolabeled probe as a control.

is large, then, because it is composed of two distinct regions, each with the characteristics of the Myb domain. These are separated in the *K. lactis* protein by about 40 amino acids and in the *S. cerevisiae* protein by nearly 140 amino acids. Because the sequence separating the two regions is the most divergent part of the protein, we suggest that it is distinct from the DNA-binding elements and is essentially irrelevant both to DNA binding and to the protein's function. Since Reb1p protects only 15 to 20 nucleotides in a chemical or enzymatic footprint assay (38) (Fig. 3), we conclude that the two parts of the binding site are adjacent in the three-dimensional structure of the protein.

Another example of a bipartite DNA-binding domain is the POU region of the mammalian transcription factor Oct-1 (2, 48). In this case, within a stretch of about 150 amino acids are a POU-specific domain and a POU-homeo domain, both essential for strong, specific binding, separated by a 15- to 27-amino-acid stretch that is highly variable but nevertheless contributes to the differential binding to individual DNA sequences. Reb1p differs in that the nonconserved stretch is far larger and does not appear to influence the function of the protein.

The sequence for which Reb1p has the highest affinity is CCGGGTAA, found near the rRNA promoter. Binding with lower affinities occurs at a number of other locations within the genome (7, 38), leading to a minimum consensus sequence of CGGGTA. The chicken v-Myb recognition sequence is TCCGTTA (5), while that of Bas1p of *S. cerevisiae* is TCCGGTA (49). Thus, they are similar but not identical. In our hands, Reb1p did not bind to double-stranded oligonucleotides containing either the Myb or the Bas1p recognition sequence, nor did Myb (kindly provided by P. Reddy, Wistar Institute) bind to the Reb1p recognition site (data not shown). These results suggest that the conserved Myb motif is overlaid by specific amino acid-nucleotide interactions.

We presume that there are functional elements of Reb1p in addition to the DNA-binding region and that these are similar in the two yeast proteins. We have found that a truncated Reb1p of *S. cerevisiae*, missing the first 201 amino acids (equivalent to PCR2; Fig. 1A) will not support the growth of cells whose genomic *REB1* gene has been destroyed or of cells whose genomic *REB1* gene is temperature sensitive (unpublished data). Although there is significant sequence divergence between the 5′ halves of the Reb1p's of the two yeast species, there are short stretches of homology between the two proteins, particularly residues 7 to 23 and 152 to 177 (Fig. 5 and 6). These conserved regions may be important for the biological activity of Reb1p.

Two other abundant DNA-binding proteins of *S. cerevisiae*, Reb1p (23) and Abf1p (12, 21), also have lengthy DNA-binding domains. Abf1p (21, 41), Rap1p (26, 46), and Reb1p (7, 29, 38) all bind to numerous sites within the genome and may play multiple roles in the organization and regulation of transcription of the genome. Whether the extended DNA-binding domain is related to the multiple functions of these proteins remains to be seen.

It is interesting that there are now three proteins, Abf1p, Hsp1p, and Reb1p, whose *K. lactis* counterparts are functional in *S. cerevisiae* even though they are substantially shorter. Are the *S. cerevisiae* proteins growing or the *K. lactis* proteins shrinking, and why?

The value of comparing the genes of distantly related organisms is not limited to the coding region. Although the noncoding sequences of *REB1* and *Kl-REB1* have diverged almost completely, a Reb1p-binding sequence is present approximately 250 bp upstream from the translation initia-

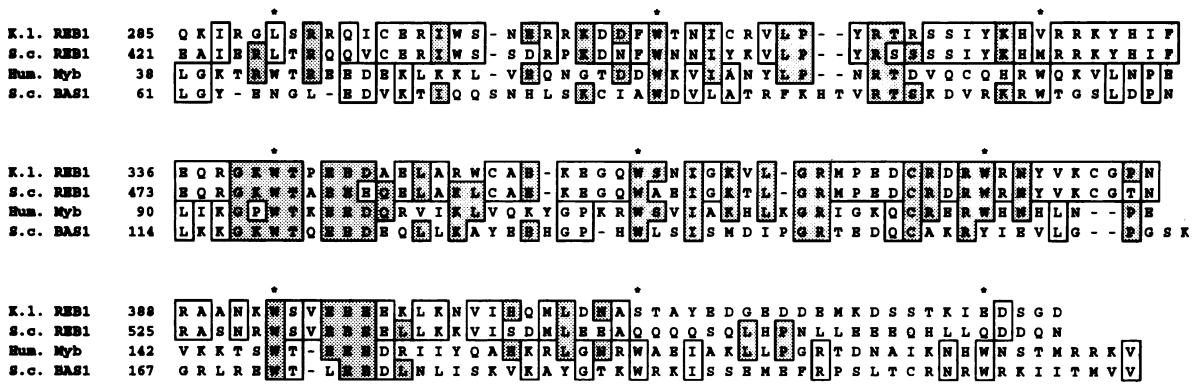

FIG. 9. Amino acid comparison of Kl-Reb1p, Reb1p, human Myb, and Bas1p in the region of homology to the Myb DNA-binding domain. The amino acid sequences derived from *Kl-REB1*, *REB1* (29), human *myb* (43), and *BAS1* (49) were compared and aligned for maximum homology. Each line portrays one of the Myb repeats. The tryptophan residues (W) of Myb are indicated with asterisks. Numbers at the beginning of the lines are amino acid positions. (Note that the alignment between Reb1p and Myb, constructed here by eye, is offset from, but more favorable than, the alignment constructed by computer in reference 11.)

tion site of the *REB1* genes in both organisms. The binding site for Reb1p could serve as a mechanism for autoregulation of the expression of the *REB1* or *Kl-REB1* gene. Experiments are under way to determine whether this is so.

ACKNOWLEDGMENTS

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