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REB1, a Yeast DNA-Binding Protein with Many Targets, Is Essential for Cell Growth and Bears Some Resemblance to the Oncogene *myb*

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REB1 is a DNA-binding protein that recognizes sites within both the enhancer and the promoter of rRNA transcription as well as upstream of many genes transcribed by RNA polymerase II. We report here the cloning of the gene for REB1 by screening a yeast genomic λgt11 library with specific oligonucleotides containing the REB1 binding site consensus sequence. The *REB1* gene was sequenced, revealing an open reading frame encoding 809 amino acids. The predicted protein was highly hydrophilic, with numerous OH-containing amino acids and glutamines, features common to many of the general DNA-binding proteins of *Saccharomyces cerevisiae*, such as ABF1, RAP1, GCN4, and HSF1. There was some homology between a portion of REB1 and the DNA-binding domain of the oncogene *myb*. *REB1* is an essential gene that maps on chromosome II. However, the physiological role that it plays in the cell has yet to be established.

In the yeast *Saccharomyces cerevisiae*, about 200 copies of the rRNA genes are arranged in a single tandem array that is transcribed by RNA polymerase I. Each repeat contains a 7-kilobase (kb) transcription unit whose product is processed to form the 25S, 18S, and 5.8S RNAs of the mature ribosome. Adjacent transcription units are separated by a 2.5-kb spacer. Within this spacer lies an enhancer specific for RNA polymerase I. This enhancer is active in either orientation, and unlike RNA polymerase II enhancers in *S. cerevisiae*, it stimulates transcription when placed either upstream or downstream of the rRNA gene (6, 17).

The enhancer lies 2 kb upstream of the transcription initiation site and only 100 base pairs from the end of the 35S precursor rRNA. Indeed, current evidence suggests that the end of 35S RNA arises from a processing event and that transcription continues into the enhancer and may terminate within it (19, 27). These considerations suggested that there might be coupling between termination and initiation (7, 17, 19).

On the assumption that protein factors are involved both in termination and in the activation of transcription, we identified two proteins, termed REB1 and REB2, that bind within the yeast rRNA enhancer (28). REB1 has been purified to homogeneity as a single polypeptide with an apparent M_r of 125,000 (B. E. Morrow, Q. Ju, and J. R. Warner, unpublished data). REB1 binds not only to the enhancer but also to a similar sequence about 200 base pairs upstream of the 35S RNA transcription initiation site. Nevertheless, the function of REB1 in rRNA transcription is not clear, since deletion of the REB1 binding sequences from a test gene had little effect on its transcription (22).

It now seems likely that REB1 may play a more general role in the organization of the cell's DNA. By the criteria of oligonucleotide competition, high-resolution footprint analysis and migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (28; Morrow et al., unpublished), REB1 is identical to the protein studied by others under the names Y (8), GRF2 (4), and QBP (3). The REB1 consensus binding site CCGGGTA (28) or CGGGTRR (where R is any purine) (4) is found upstream of a number of genes, e.g., *ACT1*, *RAP1*, *TRP5*, *SIN3*, *SWI5*, and *TOP1* (4, 43; Morrow et al., unpublished), as well as in the centromere, *CEN4*, and the subtelomeric X and Y regions (4). In each case some level of binding of REB1 was observed (4, 27, 43). REB1 was shown to bind within the *GAL1-GAL10* upstream activating sequence (UAS), leading to a localized exclusion of nucleosomes (8). Although this site is not essential for the Gal4-dependent transcription of *GAL1* and *GAL10* (44), it may be involved in Gal4-independent transcription (9). In certain circumstances and in a highly position-dependent manner, the REB1 binding site can act to stimulate transcription (3, 4). In other circumstances it can inhibit transcription (43).

In order to understand the function of this abundant protein with widespread binding sites, we have cloned the gene for REB1 by screening a yeast λgt11 library with a double-stranded oligonucleotide to which REB1 binds specifically (37, 42). REB1 is an essential gene. The amino acid sequence deduced from the open reading frame contains 809 amino acids with a high density of hydrophilic residues. It has some homology to the DNA-binding domain of the oncogene *myb* (20, 21).

MATERIALS AND METHODS

Strains, media, and libraries. The *S. cerevisiae* strain used for most of this study was W303 (*Mata*/α *ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1 can1-100*) (38) and its haploid derivatives. BJ926 (*Mata*/α *trp*/+ +/*his prc1-126*/*prc1-126 pep4-3*/*pep4-3 prb1-1133*/*prb1-1133 can1*/*can1*), obtained from the Yeast Genetic Stock Center, Berkeley, Calif., is a protease-deficient strain (18) used for preparing REB1 protein.

Escherichia coli strains Y1090 [*lacU169 proA*$^+$ *lon araD139 strA supF* (*trpC22*::Tn*10*) (pMC9)] and Y1089 [*lacU169 proA*$^+$ *lon araD139 strA hflA* (*chr*::Tn*10*) (pMV10)] were used as λgt11 host strains. Y1090 was used to screen λgt11 recombinants, and Y1089 was used to generate lysogens for the analysis of *lacZ-REB1* fusion protein (16).

Preparation of the oligonucleotide probe. A 200-ng amount

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of each of two complementary oligonucleotides, JW107 and JW106

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JW107 GATCTACTGGGTTACCCGGGGCACCTG
JW106      ATGACCCAATGGGCCCCGTGGACCTAG
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were separately phosphorylated with [γ-^{32}P]ATP by using polynucleotide kinase. Labeled oligonucleotides were mixed and annealed by heating at 85°C for 2 min, 65°C bath for 15 min, 37°C for 15 min, and finally at room temperature for 15 min. Annealed oligonucleotides were ligated overnight at 12°C and then passed through a Sepharose P-60 column to remove unincorporated [γ-^{32}P]ATP. Labeled DNA fractions were used directly as probes.

Screening the λgt11 library. The λgt11 library of sheared *S. cerevisiae* S288c DNA (14) was a generous gift of M. Snyder (Yale University) and was screened essentially as described before (37, 42). Duplicate nitrocellulose replicas were made form each plate and dried for 15 min at room temperature. All subsequent steps were performed at 4°C. Each dried filter was immersed in 6 M guanidine hydrochloride (GuHCl) in binding buffer (25 mM Tris hydrochloride [Tris-HCl, pH 8.0], 0.13 mM EDTA, 0.6 mM dithiothreitol [DTT], 87 mM KCl, 6.2 mM $MgCl_2$, 0.6 mM $CaCl_2$, 7% glycerol) (13) and gently shaken for 5 min. The bound protein was then renatured by immersing the filter in a series of decreasing concentrations of GuHCl (3.0, 1.5, 0.75, 0.38, 0.19, and 0 M), each for 5 min. After renaturation, the filter was blocked in 5% instant nonfat dry milk (Carnation) in binding buffer for 30 min. The blot was incubated with the concatenated oligonucleotide (10^6 dpm/ml) in 0.25% nonfat dry milk in binding buffer for 16 h with gentle shaking. The filter was then washed twice with 0.25% nonfat dry milk in binding buffer and autoradiographed.

Preparation of crude lysogen extract. *E. coli* Y1089 was infected with QJ1 phage (5 PFU/cell). Lysogenic cells were detected by their temperature sensitivity. A 10-ml culture of lysogenic cells at an OD_{600} of 0.5 was diluted with an equal volume of LB at 55°C and incubated at 42°C for 20 min to induce expression of the prophage. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 10 mM to induce the synthesis of the LacZ-REB1 fusion protein. The culture was then incubated for 1 h at 37°C with vigorous shaking. The culture was centrifuged, and the cells were suspended in lysis buffer (100 mM Tris-HCl [pH 7.4], 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The cells were disrupted by three cycles of rapid freeze-thaw, debris was removed by low-speed centrifugation, and the supernatant was dialyzed against dialysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 1 mM EDTA, 0.6 mM DTT, 10% glycerol, 0.05% Nonidet P-40) at 4°C. The dialyzed extract was stored at −70°C. Portions were used either directly in a gel retardation assay or for further purification.

DNA sequence analysis. The *Eco*RI fragment from QJ1 was subcloned into pGEM-Blue; the recombinant plasmid was cut with *Bam*HI and *Sph*I and treated with exonuclease III (Erase-a-base; Promega Biotech) to make a series of deletions that were subsequently cloned and used for DNA sequencing. After one strand had been sequenced, a series of oligonucleotides were used to sequence the other strand.

Yeast genomic PCR reaction used for sequence analysis. An asymmetric polymerase chain reaction (PCR) procedure was used to synthesize single-stranded DNA from yeast genomic DNA for sequencing (14). Oligonucleotides JW209 (nucleotides 392 to 411 of Fig. 7) (500 ng) and JW169 (complementary to nucleotides 934 to 915 of Fig. 7) (100 ng) were used as primers for 30 cycles of a PCR starting with genomic DNA as a template. After phenol extraction, the mixture was spin-dialyzed to remove dNTPs and oligonucleotides with a Centricon-30 microconcentrator. Single-stranded DNA was precipitated with ethanol and used directly for sequencing.

Disruption of *REB1*. An *Eco*RI-*Kpn*I fragment (A; see Fig. 2) from the *REB1* gene was cloned into pGEM-Blue. The recombinant plasmid was cut with *Bgl*II and treated with Klenow fragment to make blunt ends. A 1.16-kb *Hin*dIII fragment containing the *URA3* gene was treated with Klenow fragment and ligated into the *Bgl*II site. The new *Eco*RI-*Kpn*I fragment containing the *URA3* gene was excised from the plasmid and used to transform W303 (a/α) (33). The transformants were selected on medium lacking uracil. A Southern blot of genomic DNA was probed with the nick-translated *Eco*RI-*Kpn*I fragment to verify the disruption of *REB1*. One such transformant was sporulated, and tetrads were dissected.

Primer extension. Total RNA was made from strain W303a. Polyadenylated [poly(A)$^+$] RNA was purified with oligo(dT)-cellulose. Poly(A)$^+$ RNA (10 μg) was lyophilized and dissolved in 10 μl of annealing buffer (0.1 M Tris-HCl [pH 8.3], 0.14 M KCl) containing 20 ng of ^{32}P-labeled oligonucleotide JW233 (TCGTTTCTATTACTGTCATC; complementary to nucleotides 209 to 190 of Fig. 6). The mixture was denatured at 90°C for 3 min and annealed at 41°C for 5 min. Extension buffer (0.1 M Tris-HCl [pH 8.3], 0.14 M KCl, 15 mM DTT, 0.45 mM dNTPs) (12 μl) and 20 U (1 μl) of reverse transcriptase were added, and incubation was continued at 41°C for 2 h. The reaction was stopped by adding 2.1 μl of 3 M sodium acetate and 52 μl of ethanol, and the mix was left on dry ice for 20 min. The DNA-RNA hybrids were collected by centrifugation and dissolved in 4 μl of RNase (1 μg/μl). After incubation at 37°C for 20 min, 2.5 μl of 95% formamide containing 10 mM EDTA was added, and the sample was heated at 70°C for 2 min and loaded on a 6% sequencing gel.

Yeast genomic PCR used for gel retardation and footprint assay. Since there are no restriction sites available for constructing a probe containing the two REB1 binding sites upstream of the *REB1* gene, a PCR probe was made for gel retardation and footprint assays. Oligonucleotides JW202 (TACGTAAACTTTCAATACAT; nucleotides −360 to −341, 1 μg) and JW243 (ATAACCTTGCTTGTGATGAT; complementary to nucleotides −71 to −90, 1 μg) were labeled with [γ-^{32}P]ATP by using polynucleotide kinase. The labeled oligonucleotides were used as primers for 30 cycles of PCR starting with W303a genomic DNA as a template. The reaction mixture was purified through a Sepharose P-60 column and used directly for the gel retardation assay (1,500 cpm, Cerenkov). Only one of the oligonucleotide primers was ^{32}P labeled when used in footprint assays.

Nucleotide sequence accession number. The nucleotide sequences determined in this study have been submitted to GenBank under accession no. M36598.

RESULTS

Cloning of *REB1* from a yeast λgt11 expression library. We have cloned the *REB1* gene by virtue of its high-affinity binding to a specific DNA sequence within the rDNA promoter region. A λgt11 expression library of sheared *S. cerevisiae* DNA was screened, following a GuHCl denaturation-renaturation protocol, with a concatamerized oligonucleotide probe to the REB1 binding site near the rRNA promoter (37, 42) (see Materials and Methods). Duplicated plaque lifts were used to avoid artifacts. Out of 60,000

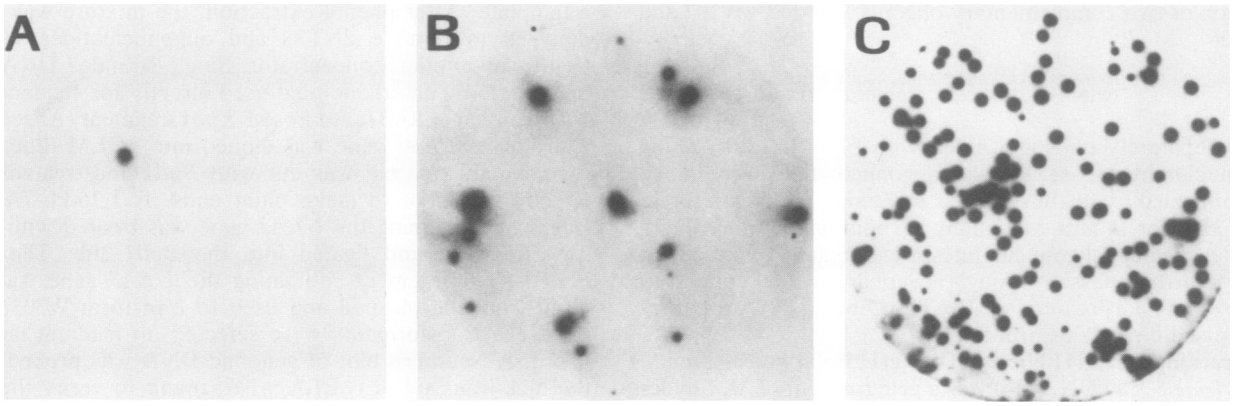

FIG. 1. Cloning of *REB1* from a λgt11 library. Bacterial cells (Y1090) were infected with phage from a yeast genomic λgt11 library (16) and plated onto 9-cm petri dish plates at 1,000 PFU/plate. Duplicate nitrocellulose replicas were made from each plate, cycled through GuHCl, and probed with a labeled, concatenated oligonucleotide containing the REB1 binding site (10^6 cpm/ml). The filter was washed and autoradiographed. A positive plaque obtained from the first screening (A) was purified to homogeneity in two steps (B and C).

plaques screened, 4 were found to be clearly positive on duplicate filters (Fig. 1A) and were purified to homogeneity (Fig. 1B and C).

Restriction enzyme analysis and cross-hybridization tests on DNA from the four plaque-purified phages showed that each had the identical 4.3-kb *Eco*RI insert. This phage was named QJ1 (Fig. 2).

To produce a fusion protein, *E. coli* Y1089 was lysogenized with QJ1. A culture of the lysogen was induced to synthesize fusion protein that was then extracted as described in Materials and Methods. Gel retardation assays performed with the crude extract showed that the fusion protein could form a complex with a probe containing the rDNA enhancer region (Fig. 3, lane 6). The double-stranded oligonucleotide representing the REB1 binding site competed for specific binding (lane 7). Deletion of the core sequence CGGGTA from the REB1 binding site abolished binding (lane 8). As is the case with authentic REB1 (28), the fusion protein had substantially higher affinity for the rDNA promoter site than for the enhancer site (data not shown). A footprint of the fusion protein bound to the promoter region was identical to that of purified REB1 (Fig. 4). Approximately 20 nucleotides on each strand were protected.

Both the gel retardation and footprint assays showed that the fusion protein and the yeast REB1 protein bound specifically to the same DNA sequence. Therefore, the 4.3-kb insert must contain the region encoding the DNA-binding domain of REB1.

Characterization of the *REB1* clone. To identify the potential *REB1* coding sequences within the insert, a partial restriction map was constructed to orient the 4.3-kb insert in the recombinant phage. Portions of the insert were used to probe a Northern (RNA) blot (Fig. 5). Although several bands were observed when the entire insert was used as a probe (lane T), the A and B fragments adjacent to the *lacZ* fusion region detected only a single band of about 3 kb (lanes A and B), close to the size predicted for an mRNA coding for the REB1 protein that migrates on an SDS gel as a protein of about 125,000 daltons (Da) (Morrow et al., unpublished). Use of SP6 and T7 transcription probes of the A fragment confirmed that the orientation of transcription in *S. cerevisiae* from left to right, as oriented in Fig. 2. In the recombi-

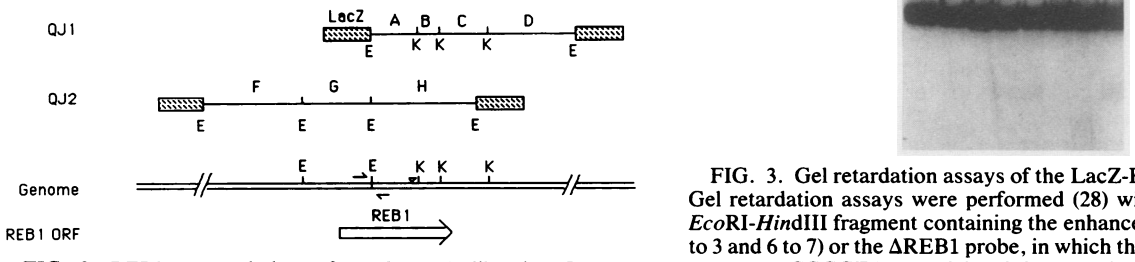

FIG. 2. *REB1* gene and clones from the λgt11 libraries. QJ1 was the first clone isolated; QJ2 was the second (see text). The lines represent insertions, and hatched bars represent lambda phage DNA. Relevant restriction enzyme sites are indicated: E, *Eco*RI; K, *Kpn*I. Genome and REB1 ORF show the location of the REB1 open reading frame on chromosome II. The locations of the oligonucleotides used for the asymmetric genomic PCR are indicated by two arrows. The *Bgl*II site used for gene disruption is shown as an inverted triangle.

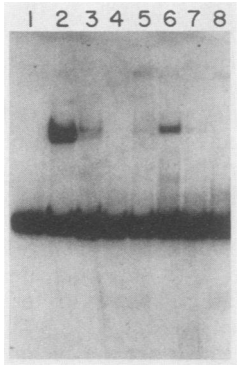

FIG. 3. Gel retardation assays of the LacZ-REB1 fusion protein. Gel retardation assays were performed (28) with either the 190-bp *Eco*RI-*Hin*dIII fragment containing the enhancer REB1 site (lanes 1 to 3 and 6 to 7) or the ΔREB1 probe, in which the core REB1 binding sequence, CGGGTA, was deleted (lanes 4, 5, and 8). REB1 protein was partially purified by DEAE-Sepharose chromatography (28). The crude extract of IPTG-induced QJ1 lysogen was used as the LacZ-REB1 fusion protein. A double-stranded oligonucleotide containing the REB1 site within the rRNA promoter (JW106 and JW107) was used as a competitor. Lane 1, No protein; lane 2, REB1 protein; lane 3, REB1 protein plus competitor; lane 4, no protein; lane 5, REB1 protein; lane 6, LacZ-REB1 protein; lane 7, LacZ-REB1 protein plus competitor; lane 8, LacZ-REB1 protein.

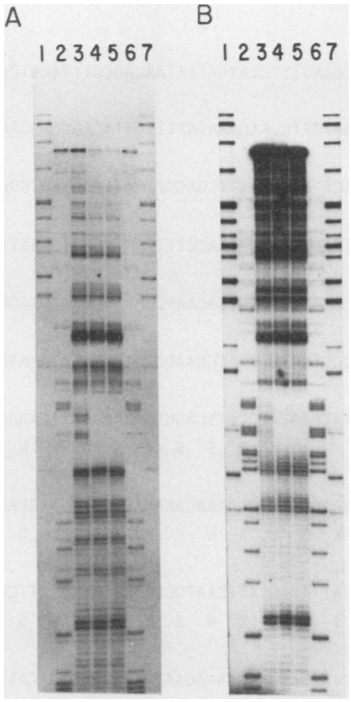

FIG. 4. Footprint analysis of the LacZ-REB1 fusion protein. Large-scale gel retardation assays were performed with a 350-bp *Eco*RI-*Hin*dIII probe containing the REB1 binding site from the rRNA promoter region. The probe was labeled separately on each strand either by filling in with Klenow fragment (A) or by kinase treatment (B). Either partially purified REB1 (DEAE-Sepharose chromatography) (lane 4) or crude QJ1 lysogen extract (lane 5) was incubated with the DNA probe (100,000 cpm, Cerenkov) and applied to native gels. The uncomplexed DNA (lane 3) and protein DNA complexes (lanes 4 and 5) were isolated following chemical cleavage of DNA with 1,10-phenanthroline–copper ion. Lanes 1 and 7 contain a radiolabeled DNA standard. The Maxam-Gilbert G-reaction sequencing ladder is shown in lanes 2 and 6.

nant phage QJ1, transcription from the *lacZ* promoter would be transcribed to produce a LacZ-REB1 fusion protein encoded by sequences in the lambda A, B, and C fragments.

The cloned A, B, and C fragments were sequenced. A single open reading frame, without an initiation codon, started at the left end of fragment A and ended in the middle of fragment C.

The entire *REB1* gene was cloned from a second genomic λgt11 library (obtained from Clontech, Palo Alto, Calif.) that was screened with an oligonucleotide within the A fragment, located 100 nucleotides downstream of the *Eco*RI site (Fig. 2). Positive candidates were checked with a second oligonucleotide from the A fragment. One plaque, termed QJ2, was positive with both oligonucleotides.

DNA isolated from QJ2 was mapped as shown in Fig. 2. Sequencing of fragment H showed that it had the sequences of fragment A from QJ1. Sequencing of fragment G revealed a single open reading frame crossing the *Eco*RI site to link up with that observed within fragment A.

Since the original library was constructed so that none of the endogenous *Eco*RI sites should have been found at the end of an insert (16), the possibility remained that the juxtaposition of fragments G and H in QJ2 could have arisen during the cloning procedure. That the arrangement as shown actually occurs within the yeast genome was established by carrying out PCR on genomic DNA with an

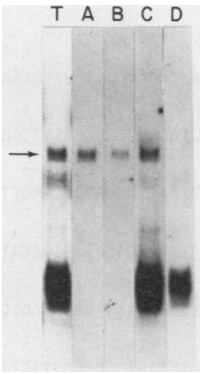

FIG. 5. Characterization of the REB1 clone. Poly(A)$^+$ RNA was made from strain W303a by using a oligo(dT)-cellulose column. Poly(A)$^+$ RNA (3 μg) was loaded onto each lane of a denaturing agarose gel, blotted to nitrocellulose, and probed with a fragment from nick-translated phage. Lane T was probed with the entire 4.3-kb *Eco*RI-*Eco*RI fragment. Lanes A, B, C, and D were probed with each of the four *Eco*RI-*Kpn*I fragments A, B, C, and D, respectively (Fig. 2). The arrow indicates the mRNA of *REB1*.

oligonucleotide from each side of the *Eco*RI site as primers (Fig. 2). A product of the predicted size was obtained; its sequence confirmed the arrangement shown in Fig. 2.

***REB1* is an essential gene.** *REB1* was disrupted by using the gene replacement technique of Rothstein (33). A 1.16-kb *URA3* fragment was inserted into the *Bgl*II site (Fig. 2), and an *Eco*RI-*Kpn*I fragment containing the *URA3* insert was used to transform the diploid strain W303 (**a**/α). Southern analysis of Ura$^+$ transformants showed that one of the two *REB1* genes had been disrupted. Tetrad analysis of the spores derived from the transformant showed 2:2 segregation of lethality (Fig. 6A). The two viable spores were Ura$^-$ and thus had a functional *REB1* gene. Microscopic examination showed that spores in which the *REB1* gene was disrupted could undergo only two to three divisions. No gross morphological abnormalities were visible in these *REB1*-disrupted cells.

High-stringency Southern hybridization analysis showed

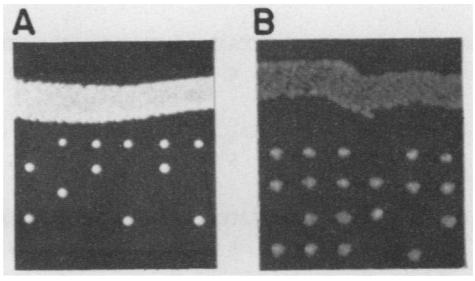

FIG. 6. *REB1* gene disruption and its complementation by a cloned *REB1* gene. (A) A 1.16-kb *URA3* gene was cloned into the *Bgl*II site of the A fragment of QJ1 (Fig. 2). The new *Eco*RI-*Kpn*I fragment was used to transform W303 (**a**/α). The Ura$^+$ transformants were confirmed by Southern analysis. The *REB1*-disrupted diploid was sporulated and used for tetrad analysis. (B) A 3.6-kb *Eco*RI fragment containing fragments G and H (Fig. 2) was subcloned into the *Eco*RI site of the centromere plasmid pRS313. This recombinant plasmid was used to transform W303 (**a**/α) in which one copy of *REB1* had been disrupted by the *HIS3* gene. The His$^+$ Ura$^+$ transformants were sporulated and used for tetrad analysis. Note that the three spore tetrads were due to the failure of the centromere plasmid to appear in all four spores.

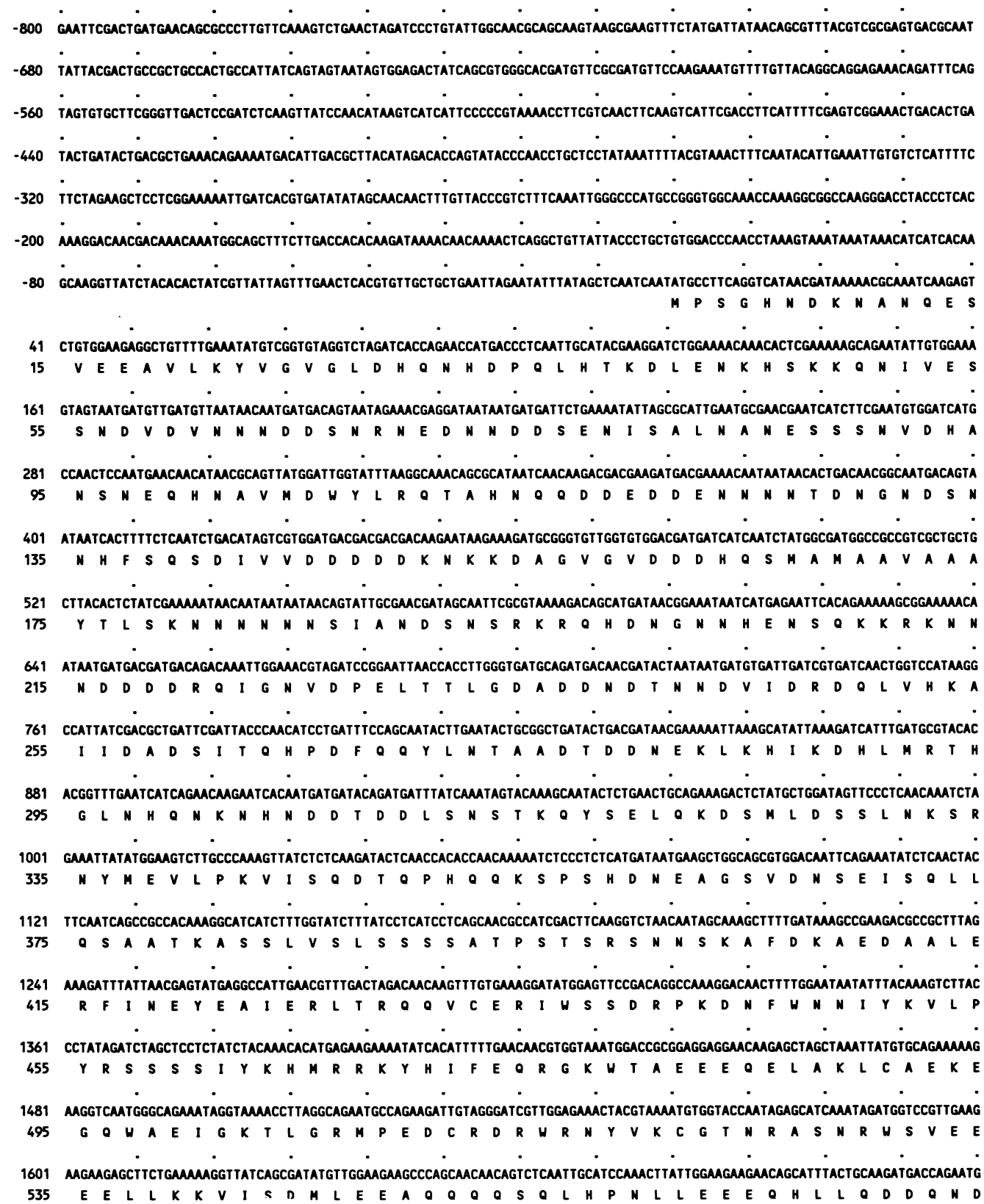
-800 GAATTCGACTGATGAACAGCGCCCTTGTTCAAAGTCTGAACTAGATCCCTGTATTGGCAACGCAGCAAGTAAGCGAAGTTTCTATGATTATAACAGCGTTTACGTCGCGAGTGACGCAAT
-680 TATTACGACTGCCGCTGCCACTGCCATTATCAGTAGTAATAGTGGAGACTATCAGCGTGGGCACGATGTTCGCGATGTTCCAAGAAATGTTTTGTTACAGGCAGGAGAAACAGATTTCAG
-560 TAGTGTGCTTCGGGTTGACTCCGATCTCAAGTTATCCAACATAAGTCATCATTCCCCCGTAAAACCTTCGTCAACTTCAAGTCATTCGACCTTCATTTTCGAGTCGGAAACTGACACTGA
-440 TACTGATACTGACGCTGAAACAGAAAATGACATTGACGCTTACATAGACACCAGTATACCCAACCTGCTCCTATAAATTTTACGTAAACTTTCAATACATTGAAATTGTGTCTCATTTTC
-320 TTCTAGAAGCTCCTCGGAAAAATTGATCACGTGATATATAGCAACAACTTTGTTACCCGTCTTTCAAATTGGGCCCATGCCGGGTGGCAAACCAAAGGCGGCCAAGGGACCTACCCTCAC
-200 AAAGGACAACGACAAACAAATGGCAGCTTTCTTGACCACACAAGATAAAACAACAAAACTCAGGCTGTTATTACCCTGCTGTGGACCCAACCTAAAGTAAATAAATAAACATCATCACAA
-80 GCAAGGTTATCTACACACTATCGTTATTAGTTTGAACTCACGTGTTGCTGCTGAATTAGAATATTTATAGCTCAATCAATATGCCTTCAGGTCATAACGATAAAAACGCAAATCAAGAGT
M P S G H N D K N A N Q E S
41 CTGTGGAAGAGGCTGTTTTGAAATATGTCGGTGTAGGTCTAGATCACCAGAACCATGACCCTCAATTGCATACGAAGGATCTGGAAAACAAACACTCGAAAAAGCAGAATATTGTGGAAA
15 V E E A V L K Y V G V G L D H Q N H D P Q L H T K D L E N K H S K K Q N I V E S
161 GTAGTAATGATGTTGATGTTAATAACAATGATGACAGTAATAGAAACGAGGATAATAATGATGATTCTGAAAATATTAGCGCATTGAATGCGAACGAATCATCTTCGAATGTGGATCATG
55 S N D V D V N N N D D S N R N E D N N D D S E N I S A L N A N E S S S N V D H A
281 CCAACTCCAATGAACAACATAACGCAGTTATGGATTGGTATTTAAGGCAAACAGCGCATAATCAACAAGACGACGAAGATGACGAAAACAATAATAACACTGACAACGGCAATGACAGTA
95 N S N E Q H N A V M D W Y L R Q T A H N Q Q D D E D D E N N N N T D N G N D S N
401 ATAATCACTTTTCTCAATCTGACATAGTCGTGGATGACGACGACGACAAGAATAAGAAAGATGCGGGTGTTGGTGTGGACGATGATCATCAATCTATGGCGATGGCCGCCGTCGCTGCTG
135 N H F S Q S D I V V D D D D D K N K K D A G V G V D D D H Q S M A M A A V A A A
521 CTTACACTCTATCGAAAAATAACAATAATAATAACAGTATTGCGAACGATAGCAATTCGCGTAAAAGACAGCATGATAACGGAAATAATCATGAGAATTCACAGAAAAAGCGGAAAAACA
175 Y T L S K N N N N N N S I A N D S N S R K R Q H D N G N N H E N S Q K K R K N N
641 ATAATGATGACGATGACAGACAAATTGGAAACGTAGATCCGGAATTAACCACCTTGGGTGATGCAGATGACAACGATACTAATAATGATGTGATTGATCGTGATCAACTGGTCCATAAGG
215 N D D D D R Q I G N V D P E L T T L G D A D D N D T N N D V I D R D Q L V H K A
761 CCATTATCGACGCTGATTCGATTACCCAACATCCTGATTTCCAGCAATACTTGAATACTGCGGCTGATACTGACGATAACGAAAAATTAAAGCATATTAAAGATCATTTGATGCGTACAC
255 I I D A D S I T Q H P D F Q Q Y L N T A A D T D D N E K L K H I K D H L M R T H
881 ACGGTTTGAATCATCAGAACAAGAATCACAATGATGATACAGATGATTTATCAAATAGTACAAAGCAATACTCTGAACTGCAGAAAGACTCTATGCTGGATAGTTCCCTCAACAAATCTA
295 G L N H Q N K N H N D D T D D L S N S T K Q Y S E L Q K D S M L D S S L N K S R
1001 GAAATTATATGGAAGTCTTGCCCAAAGTTATCTCTCAAGATACTCAACCACACCAACAAAAATCTCCCTCTCATGATAATGAAGCTGGCAGCGTGGACAATTCAGAAATATCTCAACTAC
335 N Y M E V L P K V I S Q D T Q P H Q Q K S P S H D N E A G S V D N S E I S Q L L
1121 TTCAATCAGCCGCCACAAAGGCATCATCTTTGGTATCTTTATCCTCATCCTCAGCAACGCCATCGACTTCAAGGTCTAACAATAGCAAAGCTTTTGATAAAGCCGAAGACGCCGCTTTAG
375 Q S A A T K A S S L V S L S S S S A T P S T S R S N N S K A F D K A E D A A L E
1241 AAAGATTTATTAACGAGTATGAGGCCATTGAACGTTTGACTAGACAACAAGTTTGTGAAAGGATATGGAGTTCCGACAGGCCAAAGGACAACTTTTGGAATAATATTTACAAAGTCTTAC
415 R F I N E Y E A I E R L T R Q Q V C E R I W S S D R P K D N F W N N I Y K V L P
1361 CCTATAGATCTAGCTCCTCTATCTACAAACACATGAGAAGAAAATATCACATTTTTGAACAACGTGGTAAATGGACCGCGGAGGAGGAACAAGAGCTAGCTAAATTATGTGCAGAAAAAG
455 Y R S S S S I Y K H M R R K Y H I F E Q R G K W T A E E E Q E L A K L C A E K E
1481 AAGGTCAATGGGCAGAAATAGGTAAAACCTTAGGCAGAATGCCAGAAGATTGTAGGGATCGTTGGAGAAACTACGTAAAATGTGGTACCAATAGAGCATCAAATAGATGGTCCGTTGAAG
495 G Q W A E I G K T L G R M P E D C R D R W R N Y V K C G T N R A S N R W S V E E
1601 AAGAAGAGCTTCTGAAAAAGGTTATCAGCGATATGTTGGAAGAAGCCCAGCAACAACAGTCTCAATTGCATCCAAACTTATTGGAAGAAGAACAGCATTTACTGCAAGATGACCAGAATG
535 E E L L K K V I S D M L E E A Q Q Q Q S Q L H P N L L E E E Q H L L Q D D Q N D

FIG. 7. Sequence of *REB1* and of its open reading frame. The sequence of *REB1* was determined on both strands by the dideoxy method as described in the text. The predicted amino acid sequence is shown. The 5′ end of the transcript is at nucleotide −156.

that *REB1* is a single-copy gene. A blot of electrophoretically separated chromosomes of *S. cerevisiae* was probed with a ^{32}P-labeled fragment of *REB1*. A single band corresponding to chromosome II cross-hybridized (data not shown).

Cloned *REB1* gene is functional. In order to confirm that the *REB1* gene as isolated and sequenced is a functional gene, a 3.6-kb piece of DNA containing the G and H fragments from QJ2 (Fig. 2) was subcloned into the *Eco*RI site of a yeast centromere plasmid, pRS313 (36), carrying the *URA3* gene. The recombinant plasmid was used to transform W303 (**a**/α) in which one copy of *REB1* had been disrupted by the *HIS3* gene. On sporulation, the transformant yielded four viable spores (Fig. 6B). The existence of viable Ura$^+$ His$^+$ spores demonstrated that the cloned *REB1* gene could complement a disrupted *REB1* gene.

Characterization of the *REB1* sequence. The sequence of the *REB1* gene is shown in Fig. 7. There was a single open reading frame encoding a protein of 809 amino acids. The amino acid sequence deduced from the DNA sequence was consistent with the result of N-terminal sequence analysis of purified REB1 protein. If there is no posttranslational processing, the molecular weight of the REB1 protein deduced from DNA sequence is 92,058. Purified REB1, however, migrated on an SDS-polyacryamide gel as if it were 125,000 Da. This discrepancy seems to be characteristic of yeast DNA-binding proteins, e.g., GCN4 (12), RAP1 (35), ABF1 (11, 31), and HSF1 (39).

REB1 contained none of the motifs common to many DNA-binding proteins, such as a zinc finger (32) or a leucine zipper (23). It was unusual in its hydrophilicity and its charge, with 12.7% basic residues (Arg plus Lys) and 17.8% acidic residues (Asp plus Glu), as well as 13.8% Ser and Thr and 11.4% Gln. As expected in a protein with such a charge density, there were regions of high negative charge, e.g., residues 117 to 149 and 533 to 584, that could play a role in the activation of polymerase (12, 26).

The sequence in Fig. 7 also reveals the carboxy-terminal 114 amino acids of an open reading frame that started in the upstream DNA sequences. It had no apparent homology to known genes.

REB1 has homology to the Myb proteins. A search of the GenBank and NBRF data banks found no proteins with a high degree of homology to REB1. However, the search did reveal that REB1 has substantial similarity to the DNA-binding domain of the Myb proteins (Fig. 8) (20, 21). It has been suggested that the periodic tryptophans in Myb form its DNA-binding site (1). It is intriguing that the homology between REB1 and Myb includes this motif. Another yeast DNA-binding protein, BAS1, also has substantial homology to Myb (41). However, aside from the regions of regular tryptophans, there is essentially no sequence homology between BAS1 and REB1.

5′ end of the *REB1* transcript. Primer extension was used to determine the 5′ end of the *REB1* mRNA. By using poly(A)$^+$ RNA as a template and an oligonucleotide 187 nucleotides downstream of the translation start codon (Fig. 7) as a primer, cDNA was synthesized by reverse transcriptase, and the product was compared with a sequencing reaction with the same primer (Fig. 9). The 5′ end of *REB1* mRNA was at nucleotide −156 (Fig. 7). The first AUG in this long 5′ leader initiated the *REB1* open reading frame.

REB1 binding sites within the *REB1* gene. Examination of the sequences upstream of the transcription unit of *REB1* revealed two potential REB1 binding sites, TGTTACCCGT at −270 to −260 and CCGGGTGGAT at −241 to −233. Gel retardation analysis with pure REB1 protein revealed two protein-DNA complexes (Fig. 10, lanes 5 to 8), suggesting that there are two sites within the fragment −71 to −360. After treatment of that fragment with *Apa*I, which cuts between the two putative binding sites, both subfragments were retarded by pure REB1 protein (Fig. 10, lanes 9 to 12). These observations suggest that REB1 may be involved in regulating its own synthesis.

In addition, at nucleotides −318 to −305 there is a putative heat shock factor-binding site, CTAGAAGCTCCTCG (38).

```
          430        440        450        460        470        480
REB1   TRQQVCERIWSSDRPKDNFWNNIYKVLPYRSSSSIYKHMRRKYHIFEQRGKWTAEEEQEL
                                                      :| || ||:| :
MYB    MGAPLNCPMKSMPLFPSSAQTGITPPRQNRTDVQCQHRWQKVLNPELIKGPWTKEEDQRV
              10         20         30         40         50         60

          490        500        510        520        530        540
REB1   AKLCAE-KEGQWAEIGKTL-GRMPEDCRDRWRNYVKCGTNRASNRWSVEEEELLKKVISD
         | :     :|: |:| | ||:  :||:||:|::: :::::|  |: ||: :: ::
MYB    IELVQKYGPKRWSVIAKHLKGRIGKQCRERWHNHLNPEVKKTS--WTEEEDRIIYQA-HK
              70         80         90        100        110

          550        560        570        580        590        600
REB1   MLEEAQQQQSQLHPNLLEEEQHLLQDDQNDHRNNDED--DDDTASAAAARAVVFKNNNNF
       :|::  :: ::| |:  ::: :   ::   :: ::|:  :: : :::::  |: |::||::
MYB    RLGNRWAEIAKLLPGRTDNAIKNHWNSTMRRKVEQEGYLQEPSKASQTPVATSFQKNNHL
       120        130        140        150        160        170

            610        620        630        640        650        660
REB1   FNKSSKMMTMLLRSCAAASSSLGENKDEDKPHESLGIQLDDNSQNSMVPAPSATSTHSKS

MYB    MGFGHASPPSQLSPSGQSSVNSEYPYYHIAEAQNISSHVPYPVALHVNIVNVPQPAAAAI
       180        190        200        210        220        230
```

FIG. 8. Homology between REB1 and Myb. The sequence of REB1 was compared with the NBRF protein data bank by using the FASTA program (30). A region of REB1 showed substantial homology with all the Myb proteins in the data bank. The computer-optimized alignment of REB1 with the mouse-transforming Myb oncogene (34) (NBRF identifier TVMSMA) is shown, with the recurrent tryptophans emphasized. Note that more extensive overlap of the tryptophan regions could be obtained by shifting the Myb sequence to the left, at the cost, however, of less perfect alignment of the other amino acids. A vertical line indicates identical amino acids; a colon indicates amino acids identified by the PAM250 matrix (5) as having a relatively high probability of replacement of each other. In some cases, e.g., R:K, G:D, and L:I, the structural similarity is obvious. In other cases, the probability has been determined empirically.

DISCUSSION

We have recently purified (Morrow et al., unpublished) and now cloned the gene encoding REB1, a specific DNA-binding protein in *S. cerevisiae*. Although REB1 was initially identified as an rDNA enhancer-binding protein (28), it has since been shown to bind to the rRNA promoter region (28), to the *GAL1-GAL10* UAS (4, 8; Morrow et al., unpublished), and upstream of *ACT1*, *RAP1* (4), *SIN3*, *SWI5* (43), and, indeed, *REB1* itself (Fig. 10). Thus, REB1 sites are within transcriptional control regions of genes transcribed by both RNA polymerase I and II. In addition, REB1 can bind in the centromere, *CEN4*, and within the subtelomeric X and Y regions (4), not unlike other ubiquitous DNA-binding proteins in *S. cerevisiae*, such as ABF1 and RAP1.

The sequence of REB1 (Fig. 7) is remarkable for its high concentration of charged amino acids, of serine and threonine, and of glutamine. It is interesting that a number of the other DNA-binding proteins, such as RAP1 (35), ABF1 (11, 31), and HSF1 (39), share this property, as well as being the same general size, 800 ± 50 amino acids. Nevertheless, these proteins bear little resemblance to each other when the sequences are compared. It is not easy to see how they could have evolved from a single primordial DNA-binding protein. In particular, there seems to be no characteristic motif for the DNA-binding regions of the different proteins.

Although the DNA-binding region of REB1 has been localized only to the carboxy two-thirds of the molecule, a reasonable assumption is that it resides in the region that resembles the DNA-binding region of Myb, with its motif of

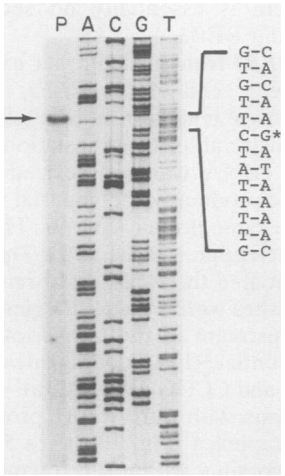

FIG. 9. Primer extension analysis of *REB1* mRNA. Poly(A)$^+$ RNA (10 μg) and ^{32}P-labeled oligonucleotide JW233 (complementary to nucleotides +209 to +190 in Fig. 7) (20 μg) were used in a primer extension reaction. The product (8,000 cpm, Cerenkov) was analyzed on a 6% sequencing gel (lane P). The noncoding strand of the cloned *REB1* gene was sequenced by using the same oligonucleotide (lanes ACGT). The arrow shows the main product of primer extension, at nucleotide −156 of Fig. 7, indicated as G*.

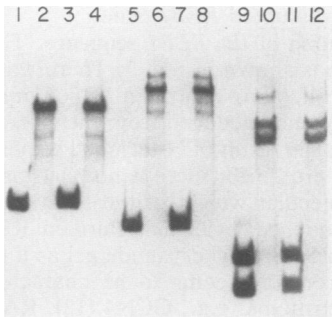

FIG. 10. Binding of REB1 upstream of the *REB1* gene. A 350-bp labeled *Eco*RI-*Hin*dIII fragment containing the *REB1* site of the promoter region was used in lanes 1 to 4. Alternatively, a 290-bp labeled PCR product (nucleotides −360 to −71 of Fig. 7) containing the two putative REB1 binding sites was used (lanes 5 to 8). Finally, this PCR product was cut with *Apa*I to yield two fragments (lanes 9 to 12). These labeled DNA fragments were used as probes for gel retardation assays with purified REB1 (lanes 2 to 4, 6 to 8, and 10 to 12). Lanes 1, 5, and 9 do not contain protein. Binding of REB1 was competed with by the double-stranded oligonucleotide containing the REB1 site that was used in the original screen (JW106 and JW107; see text) (lanes 3, 7, and 11) but not by an unrelated double-stranded oligonucleotide (lanes 4, 8, and 12).

recurring tryptophans (1). A quite distinct yeast protein that resembles the tryptophan motif of Myb is BAS1, which is involved in the constitutive low-level transcription of certain genes involved in amino acid synthesis, although it may have other functions as well (41). The *ets* oncogene family also encode a conserved set of recurrent tryptophans that has been proposed as a DNA-binding domain (10), but the other amino acids show no resemblance to the REB1 sequence. None of the other characteristic motifs of DNA-binding proteins appear in REB1, i.e., there is no leucine zipper region (23); there is no zinc finger region (32); and there does not appear to be a conventional helix-loop-helix with its associated basic region (29), although there are numerous predicted helices, some of which have a limited degree of amphipathic character. Nor does the sequence of REB1 bear any resemblance to that of hUBF, the protein factor that binds to the enhancer region upstream of the human rRNA genes (15).

We have shown (Fig. 6) that *REB1* is essential for cell growth, yet we do not know its specific physiological function. Although REB1 binds independently to two sites within the rDNA repeat both in vitro (28) and in vivo (22, 24), experiments with test genes suggest that these binding sites do not contribute to rRNA transcription (22). While some evidence suggests that sequences very close to the REB1 site in the rDNA enhancer element are involved in the termination of rRNA transcription, other evidence does not support this conclusion (22). If the role of the REB1 site in the enhancer involves termination, the REB1 site in the promoter may be a "fail-safe" termination site to prevent the disruption of the initiation complex by the passage of a rogue polymerase molecule that escaped termination after transcribing the upstream gene. Alternatively, it is possible that REB1 plays some role in organizing the rDNA repeats in the chromosome (17) that is not apparent from experiments on test genes.

The role of REB1 in transcription by RNA polymerase II is no more clear. Although there are REB1 binding sites upstream of many genes, in no case have they been shown to be an essential part of an upstream activating or inhibiting site. The most thoroughly studied has been the REB1 site in the *GAL1-GAL10* UAS. In binding there, REB1 has a significant effect on the structure of the surrounding chromatin (8), yet its binding sequence can be deleted with little effect on the Gal4-dependent induction of transcription by galactose (44). In more recent experiments, however, Finley and West (9) have identified an element, termed GAE_1, that by itself can cause transcription at the *GAL1* promoter if certain negatively acting "operator" regions are removed. GAE_1 contains the sequence to which REB1 binds, but it has not been determined whether the binding of REB1 is in fact responsible for the activity of GAE_1.

In a number of artificial constructs, the presence of a REB1 binding site was able either to potentiate (3, 4) or to inhibit (43) transcription from nearby promoters. The precise location was important, however, in a way that is uncharacteristic of the usual upstream activating or inhibiting elements. Wang et al. (43) have found that REB1 binding activity is reduced in strains carrying mutations in the gene *SIN3*, particularly after growth in galactose. Again, the biological significance of this observation is unclear. In any case, the availability of the cloned gene will enable us to ask more precise questions about function by observing the response of the cell to the withdrawal of REB1, by generating temperature-sensitive mutations in *REB1*, and by identifying mutant genes that are able to suppress the lethal effects of the deletion of *REB1*.

The REB1-LacZ fusion protein described in Fig. 3 and 4 was purified to homogeneity and used to raise antiserum in rabbits. Immunoprecipitation with this serum showed that the REB1 protein is phosphorylated on certain serine residues in vivo (Morrow et al., unpublished). Other DNA-binding proteins, e.g., HSF1, are also phosphorylated (39); in the case of HSF1, heat shock causes numerous changes in phosphorylation, without, however, having much effect on the DNA-binding properties of the molecule (37). We have not yet determined whether environmental conditions affect the phosphorylation or activity of REB1. It is interesting that c-*myb* can be phosphorylated by casein kinase II and that phosphorylation causes it to lose its DNA-binding activity (25). Most of the oncogenic mutants of *myb* have lost the phosphorylation site. Casein kinase II has also been identified as important in the regulation of rRNA transcription following mitogenic stimulation due to its phosphorylation of RNA polymerase I, nucleolin, etc. (2).

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