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A REB1-Binding Site Is Required for GCN4-Independent *ILV1* Basal Level Transcription and Can Be Functionally Replaced by an ABF1-Binding Site

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The *ILV1* gene of *Saccharomyces cerevisiae* encodes the first committed step in isoleucine biosynthesis and is regulated by general control of amino acid biosynthesis. Deletion analysis of the *ILV1* promoter revealed a GC-rich element important for the basal level expression. This *cis*-acting element, called $ILV1_{BAS}$, is functional independently of whether GCN4 protein is present. Furthermore, unlike the situation at *HIS4*, the magnitude of GCN4-mediated derepression is independent of $ILV1_{BAS}$. The element has homology to the consensus REB1-binding sequence CGGGTARNNR. Gel retardation assays showed that REB1 binds specifically to this element. We show that REB1-binding sites normally situated in the *SIN3* promoter and in the 35S rRNA promoter can substitute for the *ILV1* REB1 site. Furthermore, a *SIN3* REB1 site containing a point mutation that abolishes REB1 binding does not support *ILV1* basal level expression, suggesting that binding of REB1 is important for the control of *ILV1* basal level expression. Interestingly, an ABF1-binding site can also functionally replace the *ILV1* REB1-binding site. A mutated ABF1 site that displays a very low affinity for ABF1 does not functionally replace the *ILV1* REB1 site. This suggests that ABF1 and REB1 may have related functions within the cell. Although the REB1-binding site is required for the *ILV1* basal level expression, the site on its own stimulates transcription only slightly when combined with the *CYC1* downstream promoter elements, indicating that another *ILV1* promoter element functions in combination with the REB1 site to control high basal level expression.

The regulation of the yeast genes encoding enzymes involved in amino acid biosynthesis is very different from the regulation of the corresponding genes in bacteria. When amino acids are present in the growth medium, bacteria turn off the transcription of the genes for their amino acid biosynthetic enzymes. Under similar growth conditions, yeast cells maintain a significant level of transcription from amino acid biosynthetic genes, often referred to as the basal level.

Two transcription factors, BAS1 and BAS2, are required for *HIS4* basal level expression (2, 31). Both are regulatory proteins with global functions. *BAS2* is identical to *PHO2* and was first identified as a factor important for regulation of secreted acid phosphatase genes (2, 26). *BAS2* is also required for full GCN4-mediated derepression of the *TRP4* gene in a medium containing large amounts of phosphate (6). *bas1* as well as *bas2* mutants require addition of adenine for normal growth, presumably because both proteins also are involved in the regulation of purine biosynthesis (2). GCN4, previously identified as the general control activator, is involved not only in the general control regulation of the *ARO3* gene but also in maintaining a basal level expression of the same gene (27). Thus, basal level expression generally appears to be controlled by regulatory proteins with global functions.

The *Saccharomyces cerevisiae ILV1* gene encodes the anabolic threonine deaminase, the first enzyme in isoleucine biosynthesis (28). The *ILV1* gene is subject to the general control of amino acid biosynthesis (17). The sequence GAGTCA of the *ILV1* promoter at position −127 binds GCN4 protein in vitro with an affinity only 2.5 times lower than that of the strongest *HIS4* GCN4-binding site (1). Two other potential GCN4-binding sites in the *ILV1* promoter (positions −307 and −21) were found to bind GCN4 in vitro, but with lower affinity (1). Analysis of *ILV1* expression has shown that yeast maintains a relatively high level of expression under noninducing growth conditions (17).

In this article we report the identification of a *cis*-acting element that mediates the control of *ILV1* basal level expression. Within this *cis*-acting element we found a sequence homologous to the REB1 consensus binding sequence CGGGTARNNR. The REB1 consensus binding site is found in the promoter of many unrelated genes. In all cases investigated, DNA fragments with the consensus sequence bind REB1 protein in vitro (9). The *REB1* gene was cloned recently, and the protein shows substantial similarity to the DNA-binding domain of the Myb protein (19). *REB1* is an essential gene, reflecting its important function within the cell (19).

However, the function of this protein in the cell is still unclear. Although a REB1-binding site on its own exerts a modest effect on transcription when tested as an upstream activating sequence (UAS), it can enhance synergistically the effect of another neighboring weak activator (9). Depending upon the circumstances, REB1 can also inhibit transcription (33). The *GAL1-GAL10* REB1-binding site is needed for GCN4 to activate the *HIS3* promoter in a TATA box-independent manner (5). Finally, the binding of REB1 to the *GAL1-GAL10* promoter was shown to influence the chromatin structure by localized exclusion of nucleosomes (13).

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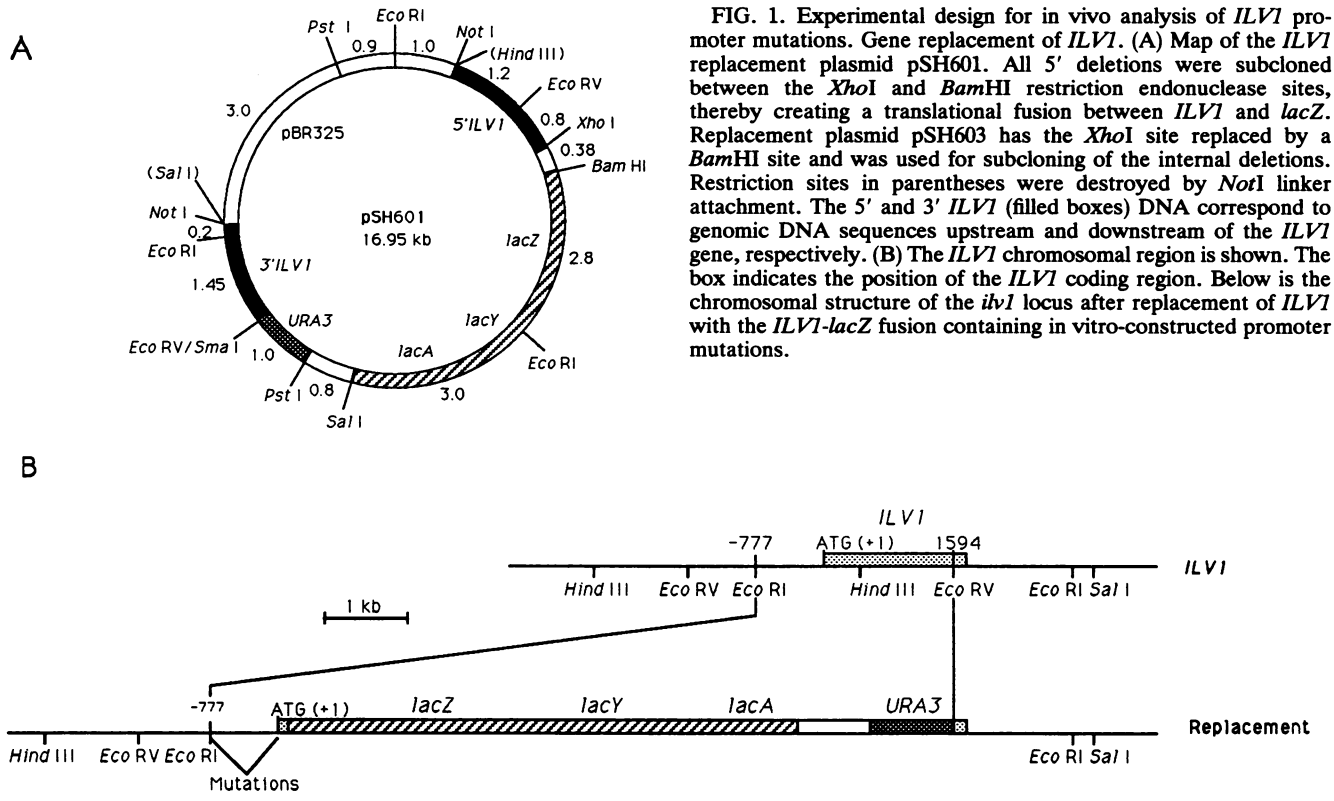


FIG. 1. Experimental design for in vivo analysis of *ILV1* promoter mutations. Gene replacement of *ILV1*. (A) Map of the *ILV1* replacement plasmid pSH601. All 5′ deletions were subcloned between the *Xho*I and *Bam*HI restriction endonuclease sites, thereby creating a translational fusion between *ILV1* and *lacZ*. Replacement plasmid pSH603 has the *Xho*I site replaced by a *Bam*HI site and was used for subcloning of the internal deletions. Restriction sites in parentheses were destroyed by *Not*I linker attachment. The 5′ and 3′ *ILV1* (filled boxes) DNA correspond to genomic DNA sequences upstream and downstream of the *ILV1* gene, respectively. (B) The *ILV1* chromosomal region is shown. The box indicates the position of the *ILV1* coding region. Below is the chromosomal structure of the *ilv1* locus after replacement of *ILV1* with the *ILV1-lacZ* fusion containing in vitro-constructed promoter mutations.

Here we present evidence that REB1 binds specifically to the *ILV1* promoter and is involved in the control of basal level expression. We also suggest that ABF1 can functionally replace REB1 in controlling *ILV1* basal level expression.

A strong activation by a REB1-binding site in its natural context is hereby established for a gene transcribed by RNA polymerase II.

MATERIALS AND METHODS

Strains, chemicals, and media. The following *S. cerevisiae* strains have been used in this study: TD28 (*MAT***a** *ura3-52 ino1 can1*) (kindly provided by G. R. Fink), 9994-6C (*MAT*α *Δgcn4 ura3-52*) (kindly provided by K. Arndt), JHRY 20-2a (*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 Δprc1::HIS3 Δpep4::LEU2*) (kindly provided by J. Rothman). *Escherichia coli* DH5α was used for plasmid propagation. DNA modifying enzymes and restriction enzymes were from New England Biolabs, Beverly, Mass., or Boehringer Mannheim GmbH, Mannheim, Germany. Radiolabelled nucleotides were from New England Nuclear. The *Bam*HI linker (CGGGATC CCG), the *Xho*I linker (CCTCGAGG), and the *Not*I linker (GCGGCCGC) were from New England Biolabs. All chemicals were analytical grade.

Complex medium for yeast (YPD) contained (per liter) 10 g of yeast extract, 20 g of Bacto Peptone, and 20 g of glucose. Minimal medium for yeast (SD) was 0.67% Bacto Yeast Nitrogen Base without Amino Acids and 2% glucose, buffered with 10 g of succinic acid and 6 g of NaOH per liter (pH 5.8). Required amino acids were supplemented to a final concentration of 50 mg (each) per liter. For growth of strain TD28, an additional 200 mg of inositol per liter was added. SD galactose media contained 3% galactose and 0.01% glucose instead of 2% glucose. For derepression by the general control system, the amino acid analog 5-methyl-DL-tryptophan (MeTrp) was added to the growth medium at a cell density of 1×10^6 to 3×10^6 per ml to a final concentration of 0.5 mM as described previously (17).

Recombinant DNA methodology. Manipulation of nucleic acids was performed by standard procedures (22). Yeast cells were transformed by using the method of Ito et al. (18). Double-stranded DNA was sequenced by the dideoxy primer extension method with the Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio).

Synthetic oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer model 394. The complementary strands of the oligonucleotides were synthesized in such a way that after annealing, each double-stranded oligonucleotide displayed *Xho*I overhangs. The sequences of the upper strand of these oligonucleotides are the following, with the REB1 sites and the ABF1 sites underlined: SIN3REB1, TCGAAGTCCGGGTAATGAT; SIN3REB1muta, TCGAAGTCCaGGTAATGAT; ILV1REB1A, TCGACAAAAAGCGCAGCGGGTAGCAAATTTG; ILV1REB1B, TCGAGAATTCGCAAAAAGAAAAAGCGCAGCGGGTAGCAAATTTGGAATCGCATAAGAGCTC; ARS1ABF1, TCGACTTCTTAGCATTTTTGACGAAATTTGA; and ARS1ABF1mutg, TCGACTTCTTAGCATTTTTGAgGAAATTTGA. The SIN3REB1 oligonucleotide covers the REB1-binding site (box 2) of the *SIN3* gene (32). The SIN3REB1muta oligonucleotide has a G→A mutation described by Chasman et al. (9) for the *RAP1* REB1 site and has a very low affinity for REB1 protein. The ILV1REB1A and ILV1REB1B oligonucleotides cover the *ILV1* promoter region from positions −197 to −172 and from positions −205 to −161, respectively (20). The ARS1ABF1 oligonucleotide contains the ABF1-binding site from *ARS1* (7). The ARS1ABF1mutg oligonucleotide has a C→G muta-

tion which abolishes ABF1 binding (7). In addition, a double-stranded oligonucleotide, rDNAREB1, containing the 35S rRNA promoter REB1-binding site, was kindly provided by J. R. Warner. The upper strand of rDNAREB1 is GATCCA GGTGCCCCGGGTAACCCAGTA (19). The double-stranded rDNAREB1 oligonucleotide has *Bam*HI overhangs and was cloned into the *Xho*I site of plasmid 7X after half of both the *Bam*HI and the *Xho*I overhangs were filled in.

Construction of *ILV1* promoter deletions. To facilitate the construction of deletions in the *ILV1* promoter, the *Eco*RI-*Rsa*I *ILV1* promoter fragment (positions −777 to +33 relative to the ATG) (20) was changed to a *Bam*HI fragment by linker attachment and cloned into the *Bam*HI site of pUC13, giving p41-1. A set of 5′ deletions was generated as follows: *Hin*cII-cut p41-1 DNA was digested with *Bal* 31, blunted with Klenow fragment of DNA polymerase I, ligated to *Xho*I linkers, and cleaved with *Bam*HI and *Xho*I. To obtain a set of 3′ deletions, p41-1 DNA was digested with *Sma*I, treated with *Bal* 31, blunted with Klenow fragment, ligated to *Xho*I linkers, and cleaved with *Bam*HI and *Xho*I. For both sets of deletions, the resulting fragments were inserted into pUC13-1 for sequencing. Plasmid pUC13-1 was constructed as a derivative of pUC13 by inserting an *Xho*I linker in the filled-in *Xba*I site. In order to test the promoter activity of the generated mutations, they were fused to *lacZ* from *E. coli* and subsequently integrated at the *ILV1* locus by one-step gene replacement as follows: the structure of the vector pSH601 is depicted in Fig. 1A. Insertion of the *Xho*I-*Bam*HI 5′ deleted fragments into *Xho*I- and *Bam*HI-cleaved pSH601 results in a translational fusion between *ILV1* and *lacZ*. Before transformation of yeasts, the fusion plasmids were digested with *Not*I and briefly treated with *Bal* 31 to remove the *Not*I linker DNA. Ura$^+$ Ile$^-$ transformants arise by replacing chromosomal *ILV1* DNA from positions −777 to +1594 with the *ILV1* promoter-*lacZ* constructs (Fig. 1B). The 3′ deletions lack the initiation codon and were not assayed as such. Internal deletions were constructed by combining appropriate 5′ and 3′ deletions via the *Xho*I site; hence, they all contain an insertion of CCTCG AGG at the deletion site and were recovered as *Bam*HI fragments. Integration of the internal deletions was carried out as described above but by employing plasmid pSH603 (Fig. 1A).

All integration events were verified by Southern blot hybridization (22).

β-Galactosidase assay. Yeast transformants were grown to a density of 0.5×10^7 to 1×10^7 cells per ml in the media indicated. Assay of β-galactosidase activity was performed after permeabilization of the cells by treatment with Triton X-100 (24). The β-galactosidase activities are expressed in Miller units (23). Each experiment was performed at least twice with independent transformants, and all assays were done in duplicate. Data from experiments done in parallel always varied less than 15%.

Construction of hybrid promoters. To assay for the ability to activate a heterologous (*CYC1*) promoter, the double-stranded ILV1REB1B oligonucleotide was inserted, in both orientations, into the *Xho*I site of pLG670-Z (14).

To assay for the ability to functionally replace a TATA element, the overhangs of the ILV1REB1B oligonucleotide were filled by using Klenow fragment and deoxynucleoside triphosphates, digested with *Sac*I and *Eco*RI, and ligated to the T_R probe plasmid pD-*lacZ* (30) linearized with the same two enzymes. The control plasmid p3801 contained the TFIID-binding sequence CTATAAAGTAATGTG (30). All chimeric promoter constructs were checked by DNA sequencing.

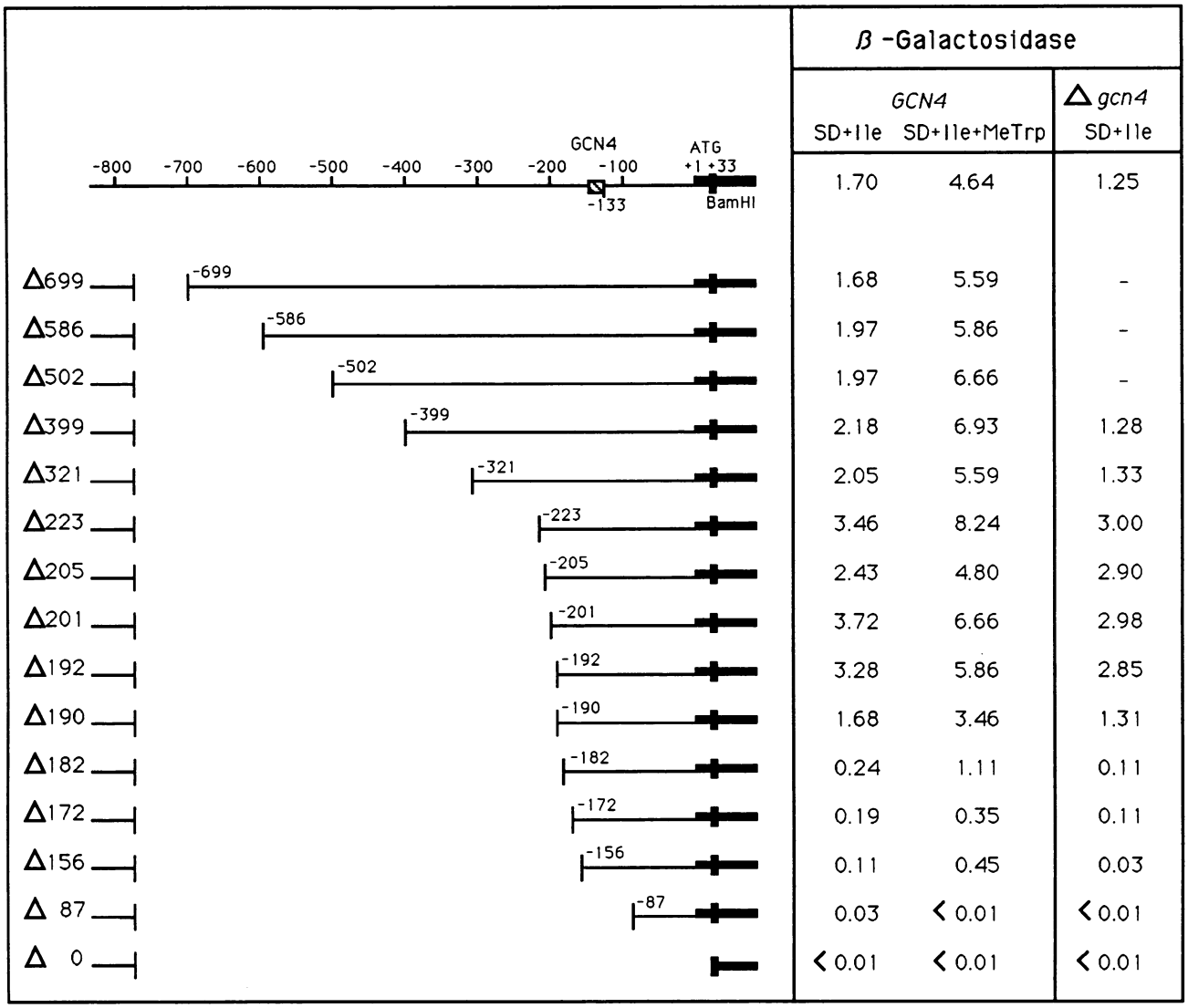
Preparation of the yeast crude nuclear protein extract. The crude nuclear extract (12 mg/ml) was prepared by the method of Machida et al. (21) with strain JHRY20-2a, in which the protease genes *PRC1* and *PEP4* are deleted. Part of the extract (100 mg) was fractionated on a P11 phosphocellulose column by the method of Bram and Kornberg (3). Two fractions were collected: A50 (50 mM KCl) with a protein concentration of 10 mg/ml and A400 (400 mM KCl) with a protein concentration of 0.8 mg/ml.

Gel retardation assays. For use in gel retardation assays, the recessed 3′ ends of the double-stranded oligonucleotide were filled in with [α-^{32}P]deoxynucleoside triphosphates by using the Klenow fragment. A total of 0.5 fmol of the labelled oligonucleotide (5,000 cpm Cerenkov) was added to a 20-μl (final volume) reaction mixture containing 50 mM KCl, 25 mM (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.5), 10% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Poly(dI-dC) (Boehringer), or another DNA as indicated, was used as an unlabelled competitor. Finally, the protein extract was added, and the binding reaction was allowed to proceed for 15 min at 25°C. Samples were loaded immediately (under voltage) onto a 6 or 8% polyacrylamide gel (acrylamide-bisacrylamide weight ratio of 19:1) made in 1× Tris-borate-EDTA (TBE). Electrophoresis was performed at 200 V for 2 to 3 h at room temperature. Afterwards, the gel was fixed, dried, and autoradiographed.

RESULTS

Deletion analysis of the *ILV1* 5′-flanking region. We initiated the analysis of the *ILV1* upstream region by generating a set of nested 5′ deletions fused to the *lacZ* gene of *E. coli* as described in Materials and Methods. DNA containing the various *ILV1-lacZ* fusions was introduced into the yeast strains TD28 (*GCN4*) and 9994-6C (Δ*gcn4*) in such a way that it replaced the original chromosomal wild-type *ILV1* gene in single copy at the *ilv1* locus (Fig. 1B). The pattern of β-galactosidase production supported by the various *ILV1-lacZ* deletion constructs is shown in Fig. 2.

In the *GCN4* strain TD28 grown in SD plus isoleucine (SD+Ile) medium, the wild-type *ILV1* promoter supported 1.7 U of β-galactosidase activity (Fig. 2). Derepression by general control of amino acid biosynthesis induced the expression from the wild-type promoter 2.6-fold. This is in agreement with published data both for enzyme activities and for mRNA levels (17). In SD+Ile we observed a slightly reduced expression from the wild-type promoter in the Δ*gcn4* strain 9994-6C compared with that in the *GCN4* strain. This difference could be interpreted either as the result of a pleiotropic effect associated with the deletion of *GCN4* or as a slight derepression by general control when strain TD28 is grown in SD+Ile medium (17). Unless otherwise indicated, we define the basal level as the expression observed in the Δ*gcn4* strain. Deletion of sequences down to position −321 relative to the ATG (+1) (Δ699, Δ586, Δ502, Δ399, and Δ321) retained the wild-type *ILV1* basal level of expression in both strains, as well as the depressed level (starvation conditions) in the *GCN4*$^+$ strain (Fig. 2). Some further deletions (Δ223, Δ205, Δ201, and Δ192) displayed a twofold increase of the *ILV1* expression both in the wild-type strain and the Δ*gcn4* strain. This increase of expression is also observed under derepressing conditions and might indicate the presence of a weak upstream repressing se-



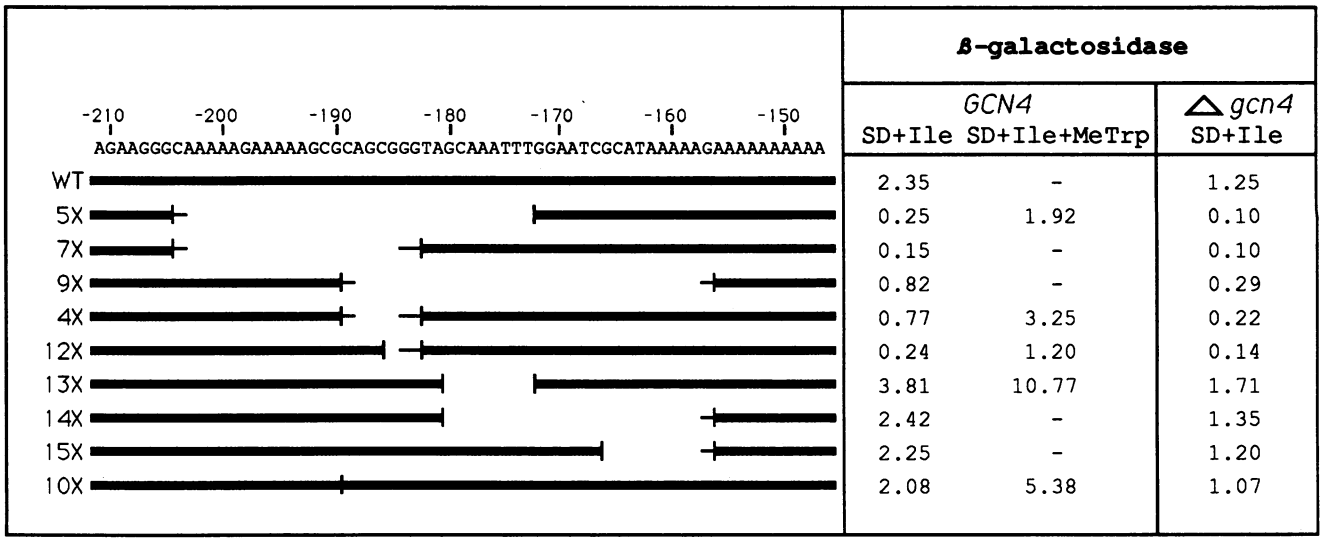
β-Galactosidase		
GCN4		Δ *gcn4*
SD+Ile	SD+Ile+MeTrp	SD+Ile
1.70	4.64	1.25
1.68	5.59	-
1.97	5.86	-
1.97	6.66	-
2.18	6.93	1.28
2.05	5.59	1.33
3.46	8.24	3.00
2.43	4.80	2.90
3.72	6.66	2.98
3.28	5.86	2.85
1.68	3.46	1.31
0.24	1.11	0.11
0.19	0.35	0.11
0.11	0.45	0.03
0.03	< 0.01	< 0.01
< 0.01	< 0.01	< 0.01

FIG. 2. Activity of 5′ deletions made in an *ILV1-lacZ* fusion. Deletions with the indicated end points were integrated at the *ilv1* locus in yeast strains TD28 (*GCN4*) and 9994-6C (Δ*gcn4*). β-Galactosidase activity was measured after growth in SD+Ile. For derepression by the general control system, 5-methyl-DL-tryptophan (MeTrp) was added when the culture was at 1×10^6 to 3×10^6 cells per ml, at a final concentration of 0.5 mM. The 5′ end of the *ILV1* mRNA is heterogeneous, displaying 20 different termini from positions −39 to −116 with the major initiation sites at −40 and −39 (16). The hatched box indicates the position of the strongest binding site for GCN4 protein (1).

quence (URS) between positions −321 and −222. Deletion of 10 bp downstream of −192 generated the greatest loss of activity: deletion Δ182, having an endpoint at −182, displayed a very low *ILV1* basal level of expression both in strain TD28 and in strain 9994-6C, corresponding to 14- and 25-fold drops in expression, respectively. It should be noted that deletion of the 10 bp causes a significant decrease of expression even under derepressing conditions (fivefold). However, the remaining *lacZ* expression was highly derepressible by GCN4, indicating that DNA downstream of −182 still contains sufficient information to support derepression. This is in agreement with the observation that purified GCN4 protein binds to its target sequence located at position −127 in the *ILV1* promoter (1). In summary, an element important for the *ILV1* basal level expression is present between positions −192 and −182. We call this decamer sequence $ILV1_{BAS}$. The element controls *ILV1* expression in a Δ*gcn4* strain and is thus able to work independently of GCN4. However, in the *GCN4* strain the level of derepression observed in deletion Δ182 is only 20% of the derepressed level of the wild-type promoter, indicating that the combination of $ILV1_{BAS}$ and activation by GCN4 is needed for maximal expression of the *ILV1* gene under conditions of amino acid starvation.

The two next deletion constructs, Δ172 and Δ156, having endpoints at −172 and −156, respectively, exhibited phenotypes similar to that of Δ182. Finally, deletion Δ87, ending at −87, had a very low basal level expression. Derepression by GCN4 was undetectable in the deletion Δ87, consistent with the absence of the GCN4-binding site.

Internal deletion analysis. To further characterize $ILV1_{BAS}$, we constructed a series of internal deletion/linker mutations as described in Materials and Methods. These mutations were integrated at the *ilv1* locus, both in the



	β-galactosidase		
	GCN4		△ *gcn4*
	SD+Ile	SD+Ile+MeTrp	SD+Ile
WT	2.35	-	1.25
5X	0.25	1.92	0.10
7X	0.15	-	0.10
9X	0.82	-	0.29
4X	0.77	3.25	0.22
12X	0.24	1.20	0.14
13X	3.81	10.77	1.71
14X	2.42	-	1.35
15X	2.25	-	1.20
10X	2.08	5.38	1.07

FIG. 3. Activity of internal deletions. Internal deletions with the indicated end points were integrated at the *ilv1* locus in strains TD28 (*GCN4*) and 9994-6C (Δ*gcn4*). Thin lines denote wild-type sequences which are part of the linker sequence CCTCGAGG. Derepression by the general control system was accomplished with MeTrp (c.f. Fig. 2).

GCN4 strain TD28 and in the Δ*gcn4* strain 9994-6C, in the same way as described for the 5′ deletion-*lacZ* fusions (Fig. 1). Figure 3 shows the internal deletions and their β-galactosidase activities measured from cells grown in SD+Ile medium.

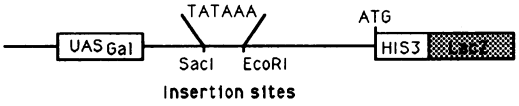
Deletions 4X, 5X, 7X, 9X, and 12X, which remove all or part of the region comprising positions −189 to −182, displayed a 3- to 13-fold reduction of the *ILV1* basal level expression in both strains. This result operationally defines the element to this region and shows that no sequence upstream of the element can substitute for its function. The deletions downstream of the element had no effect on *ILV1* basal level expression when the basal element was present (13X, 14X, and 15X) and no additional effect when it was absent (9X). However, in the absence of the sequence from −188 to −184, further deletion up to −203 (7X) still had an effect (two- to fivefold). Deletion 5X gave results similar to those with deletion 7X. Therefore, the region between −203 to −192 contributes to the activity of $ILV1_{BAS}$. A 10-fold decrease of basal level expression is seen in both the *GCN4* strain and the Δ*gcn4* strain from the 12X construct, in which the guanine at position −184 has been replaced by CCTCGA.

When $ILV1_{BAS}$ is affected (4X, 5X, and 12X), derepression by the general control system can still take place. Thus, in agreement with the results obtained with the 5′ deletions described above, $ILV1_{BAS}$ is not required for GCN4-dependent transcriptional activation by amino acid starvation.

In a reconstituted in vitro transcription system, the sequence CATAAA was shown to behave as a TATA element. However, the activity of this element was 70% lower than the activity of the canonical TATAAA element (15). In the 15X construct, we deleted this CATAAA sequence without any effect on basal level *ILV1* expression, indicating that this potential binding site for TFIID is not important for basal level expression of the *ILV1* promoter.

The $ILV1_{BAS}$ element does not act as a TATA element. The absence of the motifs TATAAA, TATATA, and TATTTA downstream of $ILV1_{BAS}$ suggested to us that the sequence might have a function related to the TATA element. Singer et al. (30) have constructed a centromere plasmid, pD-*lacZ*, containing a *GAL-HIS3* promoter deleted for the TATA element, with four GAL4-binding sites from the *GAL1-GAL10* locus, the wild-type *HIS3* initiator, and the amino-terminal part of *HIS3* fused to *lacZ* (Fig. 4). By inserting randomly synthesized DNA between *Sac*I and *Eco*RI, Singer et al. (30) showed that the consensus TATAAA sequence can be functionally substituted in vivo by GC-rich elements.

When a 2-kb *Drosophila* *Sac*I-*Eco*RI fragment was present in the pD-*lacZ* plasmid, no effect of replacing



SacI-EcoRI DNA fragments	β-galactosidase	
	Galactose	Glucose
Drosophila DNA	1.21	1.17
Canonical TATAAA	26.17	1.39
ILV1REB1B	1.13	1.27

FIG. 4. $ILV1_{BAS}$ shows no effect in a TATA-probe plasmid. The ILV1REB1B oligonucleotide was inserted between the *Sac*I and *Eco*RI sites of the plasmid pD-*lacZ* as described in Materials and Methods. Subsequently, the resulting plasmid and the positive (containing the consensus TATAAA sequence) (30) and negative (containing a 2-kb *Sac*I-*Eco*RI *Drosophila* DNA insert) (30) control plasmids were introduced into the Δ*gcn4* strain 9994-6C. β-Galactosidase activity was measured from cells grown either on glucose- or on galactose-containing medium.

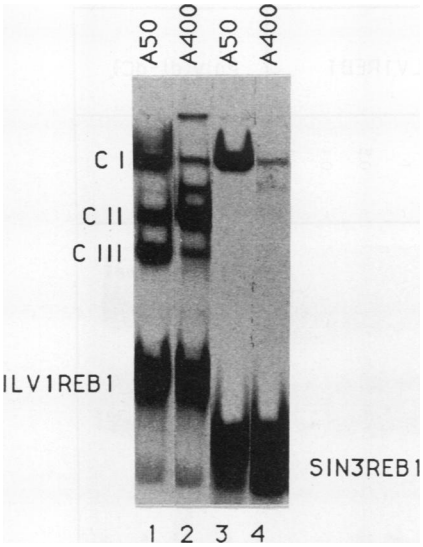


FIG. 5. Gel retardation analysis. Complexes (CI, CII, and CIII) were formed with two yeast nuclear protein fractions from a P11 column (A50 and A400, respectively) and two double-stranded oligonucleotides containing either $ILV1_{BAS}$ (ILV1REB1A, lanes 1 and 2) or the REB1 site from *SIN3* (SIN3REB1, lanes 3 and 4) as probes. The binding reactions were done without addition of poly(dI-dC) as a nonspecific competitor.

glucose by galactose was seen (Fig. 4) (30). On the other hand, when a consensus TATA box was tested, a 20-fold increase in β-galactosidase activity was observed, indicating that this sequence restores promoter activity. To investigate a putative TATA box function of $ILV1_{BAS}$, an oligonucleotide covering this sequence (oligonucleotide ILV1REB1B; see Materials and Methods) was inserted into plasmid pD-*lacZ*. In this construct, no increase in expression was observed, either on galactose- or on glucose-containing medium (Fig. 4). Therefore, in contrast to some other GC-rich sequences $ILV1_{BAS}$ cannot mediate the activity of UAS_{GAL}.

REB1 protein binds specifically to $ILV1_{BAS}$. A comparison of $ILV1_{BAS}$ and UASs identified in yeasts revealed a sequence within the *ILV1* element that matches perfectly the consensus binding sequence CGGGTARNNR of the REB1 protein (9).

To investigate whether REB1 specifically binds to $ILV1_{BAS}$, gel retardation assays were performed with three different double-stranded oligonucleotide probes. The oligonucleotide ILV1REB1A covers the *ILV1* promoter region from −197 to −172. The oligonucleotide SIN3REB1 contains the REB1-binding site from the promoter (box 2) of the *SIN3* gene (32). The oligonucleotide rDNAREB1 contains the REB1-binding site from the 35S rRNA promoter (19). The SIN3REB1 and rDNAREB1 oligonucleotides are known to specifically bind purified REB1 (19, 32). As an initial source of REB1 protein, we prepared a yeast crude nuclear protein extract as described in Materials and Methods. The extract was separated into two fractions on a phosphocellulose column: A50 (eluted with 50 mM KCl) and A400 (400 mM KCl). Using the SIN3REB1 probe, we found that the REB1 protein eluted mainly with the A50 fraction (Fig. 5, lane 3). A weak binding activity is also visible with the A400 fraction. Using the ILV1REB1A probe together with the A50 fraction, we obtained three different DNA-protein complexes, CI, CII, and CIII (Fig. 5, lane 1). It is also seen that complex I migrates at the same position in the gel as the DNA-REB1 protein complex identified with the SIN3REB1 probe. Moreover, as for the SIN3REB1 complex, the binding activity resulting in complex I is primarily present in the A50 fraction. A weak complex I is also visible when oligonucleotide ILV1REB1A was incubated with the A400 fraction. Together with the nucleotide sequence, this result suggests that the binding activity identified in complex I is REB1, as the difference in length (13 bp) between the SIN3REB1 and ILV1REB1A oligonucleotides is expected to be too small to influence migration of the DNA-protein complexes.

In Fig. 6 the results of various competition experiments are presented. The ILV1REB1A oligonucleotide was labelled with ^{32}P and incubated with the A50 protein fraction described above. Without competitor DNA, a very strong binding activity is seen, corresponding to complex I identified above. Complexes II and III are also present as in Fig. 5. The competition experiments shown in Fig. 6 gave the following results. One nanogram of the ILV1REB1A or SIN3REB1 oligonucleotide as competitor was sufficient to compete almost totally the binding activity in complex I, whereas complexes II and III were unaffected. Moreover, a 500-fold surplus of poly(dI-dC) compared with that in the oligonucleotides was required to compete complex I, and in this case complexes II and III were affected to the same degree as complex I.

To confirm that REB1 binds to $ILV1_{BAS}$, REB1 purified from yeast cells (25) or partially purified from *E. coli* cells expressing *REB1* (both REB1 purified fractions were a gift from B. Morrow) were used in gel retardation. The result is shown in Fig. 7. When the ILV1REB1A and rDNAREB1 oligonucleotide probes were incubated with the protein extract prepared from bacterial cells expressing *REB1*, a strong binding activity was seen (lanes 2 and 6), whereas no binding activity was detectable when the extract was prepared from cells containing the expression vector peT11a without the *REB1* insert (lanes 1 and 5). When REB1 protein purified from yeast cells (25) was used, a strong binding activity was observed with both probes (lanes 3 and 7). Moreover, the complex identified with ILV1REB1A migrated at the same position in the gel as the complex corresponding to binding of REB1 to the rDNAREB1 site. Our conclusion from these experiments is that REB1 binds specifically to $ILV1_{BAS}$.

Independent proof for the specific binding of REB1 to the *ILV1* sequence is shown in Fig. 8. When the A50 protein fraction is incubated with ILV1REB1A (Fig. 8, lane 2), complexes I, II, and III are seen as before. However, if the A50 protein fraction is incubated with antibody directed against REB1 protein (a gift from B. Morrow) prior to mixing with the probe, only complex I is further retarded by the presence of REB1 antibody, whereas complexes II and III remain unaffected (Fig. 8, lane 1). Thus, complex I corresponds to the specific binding of REB1 to the *ILV1* sequence.

Finally, we performed a competition experiment using the ILV1REB1A oligonucleotide as the labelled probe, the purified REB1 protein from yeasts, and as nonradioactive competitor DNA the rDNAREB1 and ILV1REB1A oligonucleotides. From this experiment (Fig. 9) we can see that 10 ng of ILV1REB1 nonradioactive oligonucleotide is required to almost compete the REB1 binding, whereas 1 ng of rDNAREB1 oligonucleotide is sufficient to reach the same level of competition. A similar 10-fold difference was ob-

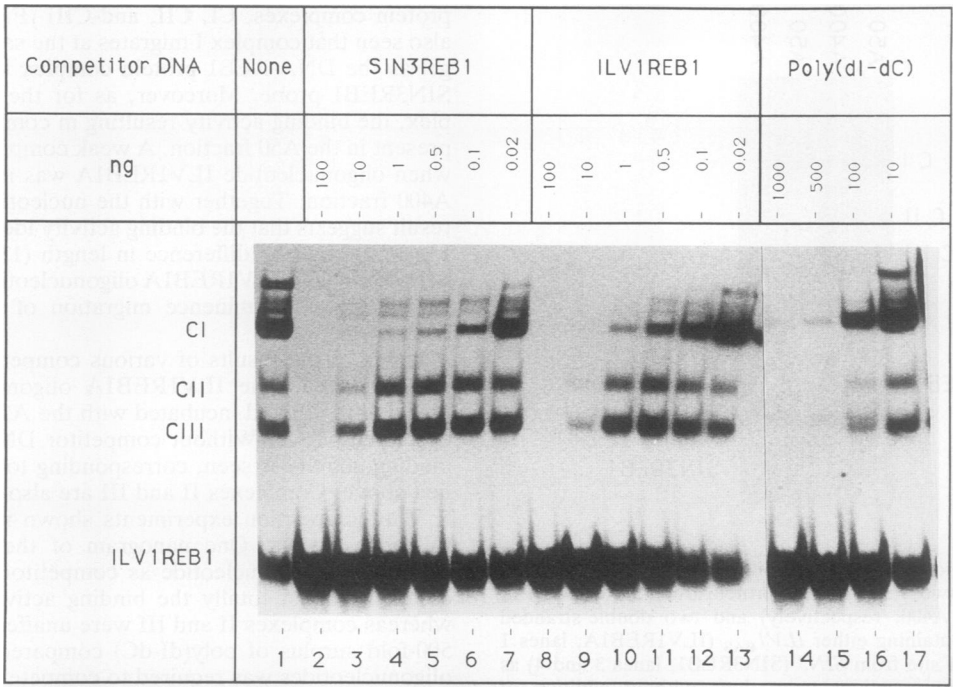


FIG. 6. The CI complex with the ILV1REB1A probe is specifically competed by SIN3REB1 and ILV1REB1A. A total of 10 pg of ^{32}P-labelled ILV1REB1A oligonucleotide was used as a probe with 10 μg of the A50 protein fraction.

served when the rDNAREB1 oligonucleotide was the labelled probe (data not shown). This shows that the affinity of REB1 for the *ILV1* sequence is about 10-fold lower than for the rDNA REB1 site. This rDNA REB1-binding site was found by Chasman et al. (9) to have the highest affinity for REB1 among 15 REB1 sites tested.

Taken together, the binding experiments described above show that the REB1 protein binds specifically to $ILV1_{BAS}$. Furthermore, since the deletion analysis of the *ILV1* promoter demonstrated that the integrity of the REB1-binding site at position −177 is required for efficient promoter activity, we suggest that REB1 plays an important role in controlling the basal level expression of the *ILV1* gene.

The *SIN3* and rDNA REB1-binding sites can functionally substitute for $ILV1_{BAS}$. The internal deletion 7X (Fig. 3), which replaces the region between positions −204 and −182 with an 8-bp *Xho*I linker, supported a very low basal level expression, and the 7X construct could thus be used as a probe for sequences that can mediate basal level expression in the *ILV1* context. In order to test whether the low basal level expression of 7X was mainly due to the absence of the REB1-binding site, we tested whether the REB1 sites from *SIN3* (box 2) and the rDNA, respectively, could functionally substitute $ILV1_{BAS}$, i.e., we inserted into the *Xho*I site of the 7X deletion the SIN3REB1 and rDNAREB1 oligonucleotides. The β-galactosidase activities supported by these constructs integrated at the *ilv1* locus are listed in Fig. 10. It is seen that insertion of both the SIN3REB1 and rDNAREB1 oligonucleotides restored the *ILV1* basal level expression and did so in an orientation-independent manner. Increasing the copy number of the SIN3REB1 oligonucleotide gave rise to an increased expression. Furthermore, insertion of the oligonucleotide SIN3REB1muta, which contains a point mutation in the REB1-binding site, does not restore basal level expression (Fig. 10). Chasman et al. (9) showed that the corresponding point mutation gave rise to a low affinity for REB1 binding. Using a protein extract prepared from *E. coli* expressing *REB1*, we also showed that this SIN3REB1muta oligonucleotide failed to bind REB1 (Fig. 11, lane 4). We conclude that binding of REB1 to its target sequence in the *ILV1* promoter is important for *ILV1* basal level expression.

ABF1 can functionally substitute for REB1 in controlling *ILV1* basal level expression. REB1 is a member of a family of DNA binding proteins that are remarkable for their abundance in yeast protein extracts, their many binding sites in the yeast genome, and their involvement in diverse chromosomal functions. Another member of this family is ABF1, which binds to UASs, *ARS* elements, and the mating type silencer (7, 29). We tested if the *ARS1* ABF1-binding site was able to restore the *ILV1* basal level expression in the 7X probe construct. Insertion of the ARS1ABF1 oligonucleotide into the *Xho*I site of 7X gave rise to an even higher basal level expression than the *ILV1* wild-type promoter (Fig. 10). The oligonucleotide ARS1ABF1mutg contains a point mutation in the ABF1-binding site. Buchman and Kornberg (7) showed that the corresponding mutation abolishes ABF1 binding. By using the yeast A50 protein fraction, ABF1 binds to ARS1ABF1 (Fig. 12). One nanogram of nonradioactive ARS1ABF1 oligonucleotide is sufficient to compete the ABF1 binding (Fig. 12, lane 3), whereas more than 100 ng of ARS1ABF1mutg (Fig. 12, lane 5) and more than 250 ng of poly(dI-dC) (Fig. 12, lane 9) are required to compete out ABF1 binding. This competition experiment confirmed the very low affinity of ARS1ABF1mutg for ABF1. Insertion of ARS1ABF1mutg in deletion 7X does not restore *ILV1* basal level expression (Fig. 10). Also, neither ARS1ABF1mutg nor ARS1ABF1 binds REB1 produced in *E. coli* (Fig. 11, lanes 6 and 8, respectively). We therefore suggest that ABF1 can functionally replace REB1 in controlling *ILV1* basal level expression.

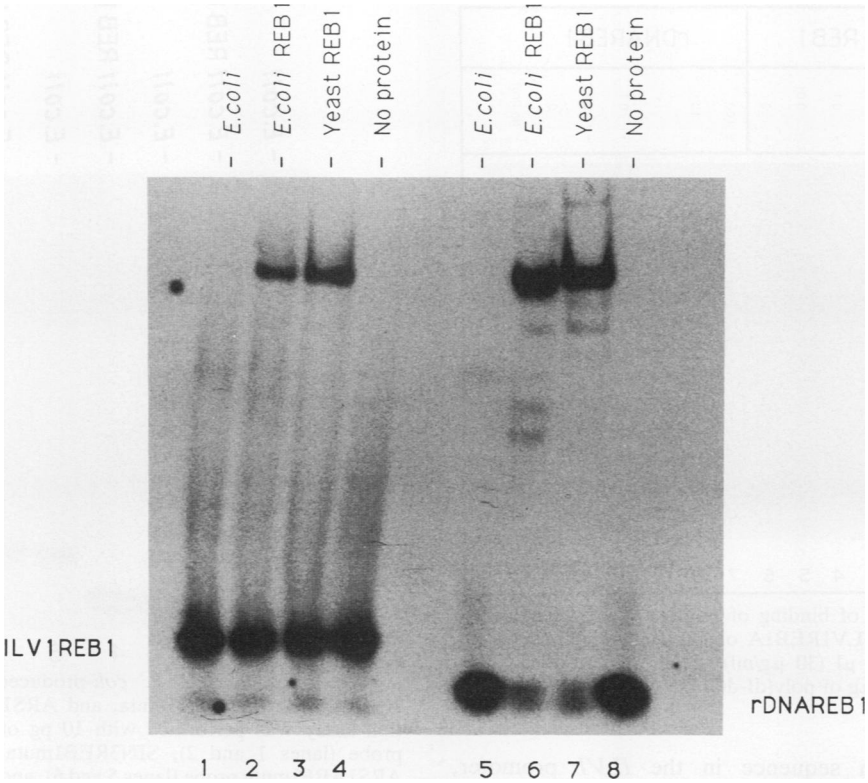


FIG. 7. Binding of purified yeast REB1 and *E. coli*-produced REB1 to the REB1 sites of *ILV1* and 35S rRNA, respectively. Gel retardation assay was performed with 10 pg of ^{32}P-labelled ILV1REB1A probe (lanes 1 to 4) and 10 pg of ^{32}P-labelled rDNAREB1 probe (lanes 5 to 8). Lanes 1 and 5, probes were incubated with 4 μl (1 mg/ml) of protein extract prepared from *E. coli* containing the expression vector peT11a as a control; lanes 2 and 6, incubation with 2 μl (1 mg/ml) of a protein extract prepared from *E. coli* expressing *REB1* from the plasmid peT11a containing the *REB1* insert; lanes 3 and 7, 10 μl of purified yeast REB1 (25) (30 μg/ml); lanes 4 and 8, no protein was added. In all lanes, 1 μg of poly(dI-dC) was included.

$ILV1_{BAS}$ is a poor activator of the *CYC1* downstream promoter. To address whether $ILV1_{BAS}$ alone could support reporter gene expression, the ILV1REB1B oligonucleotide was inserted into the *Xho*I site of pLG670-Z (Fig. 13), a heterologous expression vector lacking a UAS element (14). In the Δ*gcn4* strain 9994-6C, pLG670-Z supported a β-galactosidase activity of 0.88 U. Insertion of the ILV1REB1B oligonucleotide, in the same orientation as in the *ILV1* promoter, raised the *lacZ* expression 2.5-fold. Interestingly, when inserted in the opposite orientation, the *lacZ* expression was increased sevenfold. One explanation for this effect might be that, when the ILV1REB1B oligonucleotide is reversed, the A-rich element (10 A's in 11 nucleotides) normally situated upstream of the *ILV1* REB1 site now becomes a T-rich element downstream of the REB1 site. In fact, Chasman et al. (9) showed that the *RAP1* REB1-binding site functions as a powerful auxiliary transcription activator when placed in the same *CYC1* context upstream of the T-rich element of the *DED1* gene.

In conclusion, $ILV1_{BAS}$ confers low UAS activity in this heterologous context. This is in agreement with results obtained with the *GAL1,10* REB1 site and the *SIN3* REB1 site in the same *CYC1* context (7, 32).

DISCUSSION

A REB1-binding site controls *ILV1* basal level expression but is not required for activation of *ILV1* by GCN4. We have

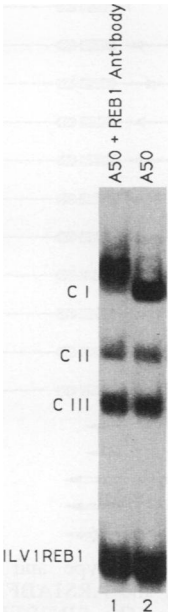


FIG. 8. REB1 antibody specifically binds to complex CI. Gel retardation assay was performed with ILV1REB1A as the probe and with the A50 protein fraction. Lane 1, 10 μg of the A50 protein fraction was mixed with antibody directed against REB1 before complex formation; lane 2, 10 μg of the A50 protein fraction.

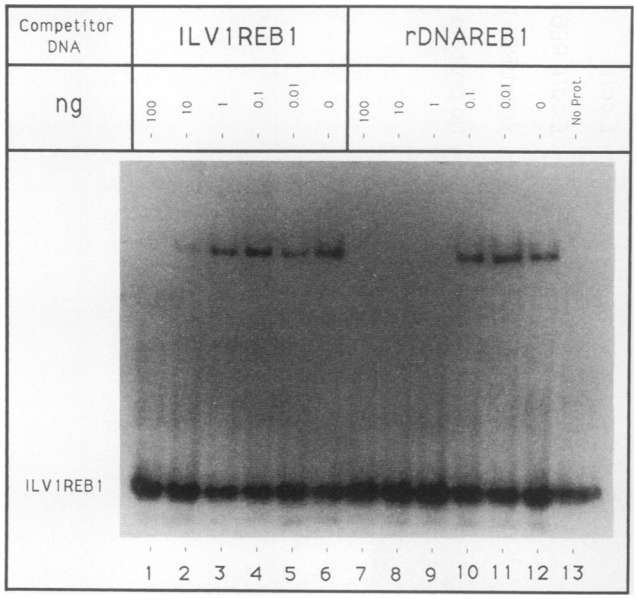


FIG. 9. Competition of binding of purified yeast REB1 to the *ILV1* REB1 site. The ILV1REB1A oligonucleotide probe (10 pg) was incubated with 10 μl (30 μg/ml) of partially purified REB1 protein. In all lanes, 1 μg of poly(dI-dC) DNA was included.

identified a GC-rich sequence in the *ILV1* promoter, $ILV1_{BAS}$, required for basal level expression. In a test plasmid an oligonucleotide including this sequence did not exhibit TATA-like activity. Analysis of this *cis*-acting element revealed an overlapping sequence homologous to the REB1 consensus binding sequence CGGGTARNNR (9). Gel retardation assays, performed with REB1 protein purified from yeast or partially purified from a bacterial strain expressing the *REB1* gene, showed that REB1 binds specifically to the *ILV1* sequence. Moreover, interruption of the *ILV1* REB1-binding sequence by replacing the first guanine with the sequence CCTCGA (Fig. 3, construct 12X) resulted in a 10-fold reduction of expression in nonstarvation conditions, reinforcing the idea that the REB1 element is crucial for basal level expression of the *ILV1* gene.

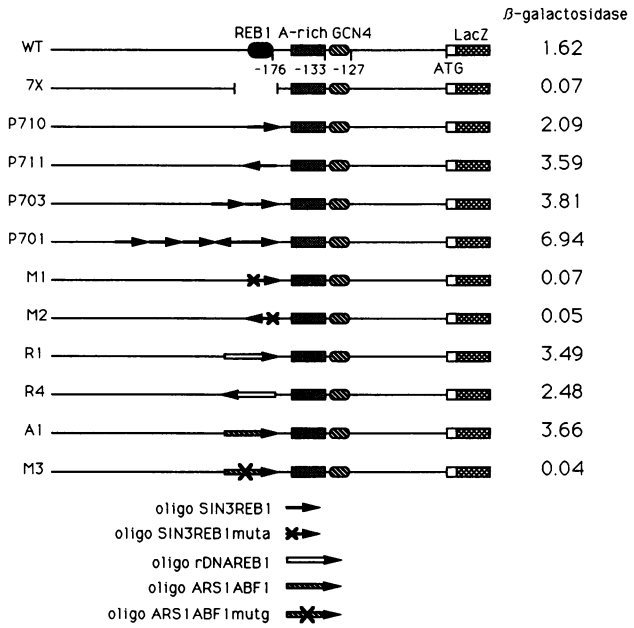


FIG. 10. Activities of wild-type and mutated forms of the SIN3REB1, rDNAREB1, and ARS1ABF1 oligonucleotides when used instead of $ILV1_{BAS}$. The SIN3REB1, rDNAREB1, ARS1 ABF1, SIN3REB1muta, and ARS1ABF1mutg oligonucleotides were inserted in one or more copies (as indicated) into the *Xho*I site of the 7X internal deletion. All constructs were integrated at the *ilv1* locus in strain 9994-6C (Δ*gcn4*). β-Galactosidase activity was measured after growth in SD+Ile.

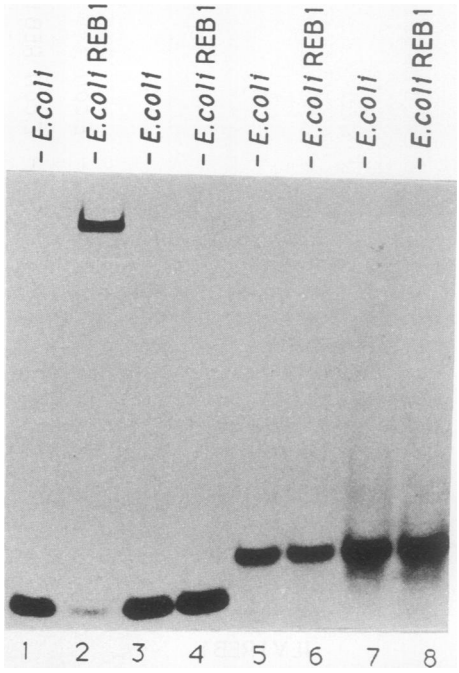


FIG. 11. Binding of *E. coli*-produced REB1 to SIN3REB1, ARS1ABF1, SIN3REB1muta, and ARS1ABF1mutg. Gel retardation assay was performed with 10 pg of ^{32}P-labelled SIN3REB1 probe (lanes 1 and 2), SIN3REB1muta probe (lanes 3 and 4), ARS1ABF1mutg probe (lanes 5 and 6), and ARS1ABF1 probe (lanes 7 and 8). Lanes 1, 3, 5, and 7, the probes were incubated with 4 μl (1 mg/ml) of a protein extract prepared from an *E. coli* strain containing the expression vector peT11a as a control; lanes 2, 4, 6 and 8, incubation was with 2 μl (1 mg/ml) of a protein extract from *E. coli* expressing *REB1* from peT11a containing *REB1*. In all lanes, 1 μg of poly(dI-dC) was included.

The REB1-binding sites from the *SIN3* gene (box 2) and from the 35S rRNA promoter have been shown to bind the REB1 protein specifically (7, 32). Interestingly, these two REB1 sites were able to functionally replace $ILV1_{BAS}$. Furthermore, a mutated SIN3REB1 site that does not bind REB1 cannot substitute for $ILV1_{BAS}$ function. These results support the notion of an important in vivo role of REB1 binding in controlling the *ILV1* basal level expression. We also observed an increasing activation of the *ILV1* basal level expression when multiple copies of the SIN3REB1 oligonucleotide replaced the *ILV1* REB1-binding site. It is interesting to note that the rDNA REB1-binding site, which influences transcription by RNA polymerase I, can also control the basal level expression of a gene transcribed by RNA polymerase II.

Most of the REB1-binding sites analyzed conferred only low UAS activity when combined with the *CYC1* down-

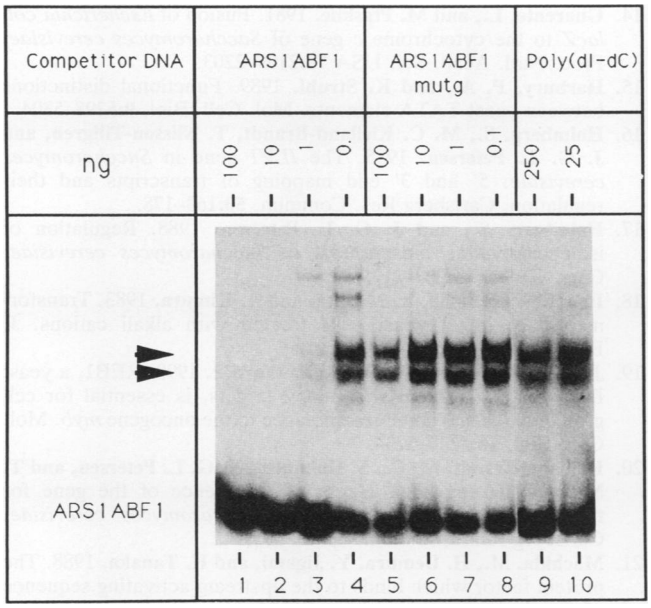


FIG. 12. Binding of ABF1 to ARS1ABF1 is specifically competed by ARS1ABF1 but not by ARS1ABF1mutg. A total of 10 pg of ^{32}P-labelled ARS1ABF1 was used as probe with 10 μg of the yeast A50 protein fraction.

stream promoter elements (9). For example, the *SIN3* REB1-binding site (box 2) activated *CYC1* transcription only 3.2-fold (33). Therefore, it was not surprising to find that the *ILV1* REB1 site stimulated *CYC1* transcription only about twofold. Although the *SIN3* REB1 site gives low UAS activity in the *CYC1* promoter context, it confers a 30-fold increase in transcription when inserted in the deletion 7X (deletion of $ILV1_{BAS}$). Likewise, the 35S rDNA REB1 site, in the same context, increased transcription 50-fold. Thus, these REB1 sites function as strong activating regions in the *ILV1* promoter. Chasman et al. (9) showed that REB1 protein is a powerful auxiliary activator in combination with other weak activators. We may reason that the *ILV1* REB1 site has its strong function in the *ILV1* promoter because binding of REB1 to the site has a synergistic effect together with a nearby weak transcription activator. The effect of the presence of a REB1-binding site in the *ILV1* promoter was observed not only in the *GCN4* strain but also in the Δ*gcn4* strain, suggesting that REB1 may enhance the effect on transcription of an element other than the GCN4 UAS. Recent studies (28a) have revealed a synergistic activation between the REB1 site and the downstream A-rich element (−134 and −164).

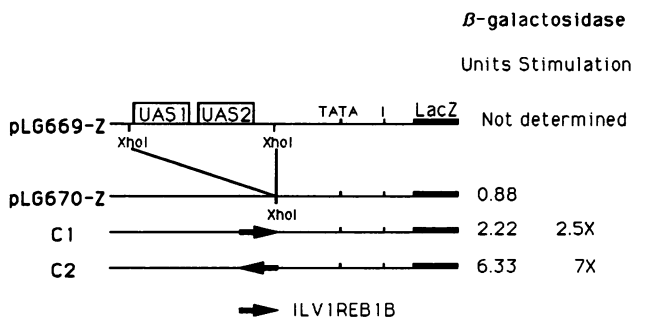


FIG. 13. UAS activity of $ILV1_{BAS}$. The ILV1REB1B oligonucleotide was inserted into the *Xho*I site of 2 μm-based plasmid pLG670-Z (14) in both orientations. The three plasmids pLG670-Z, C1, and C2 were introduced into the Δ*gcn4* strain 9994-6C. β-Galactosidase activity was measured after growth in SD+Ile.

Chen and Struhl (10) replaced the TATA element (T_R element) of the *GAL-HIS3* promoter with a binding site for GCN4 protein. Surprisingly, GCN4 was able to activate transcription of this hybrid promoter. Subsequently, Brandl and Struhl (5) demonstrated that the REB1 site present in the *GAL* fragment was required for this TATA-independent GCN4-mediated transcriptional activation. In addition, Fedor et al. (13) have shown that this *GAL1-GAL10* REB1 site influences the chromatin structure of the DNA, leading to a localized exclusion of nucleosomes. The mechanism remains to be established. Similarly, a recent study has revealed that binding of RAP1 to the *HIS4* promoter is required for BAS1/BAS2- and GCN4-dependent transcription of the *HIS4* gene (11). Micrococcal nuclease experiments revealed that the presence of the RAP1 site makes the region containing the GCN4- and BAS1/BAS2-binding sites more accessible to the nuclease, suggesting that bound RAP1 protein keeps the *HIS4* promoter region free of nucleosomes (11).

In the case of the *ILV1* promoter deleted for the REB1-binding site, GCN4 is still able to activate transcription. We suggest that binding of REB1 to the *ILV1* basal element influences chromatin structure within the *ILV1* promoter and that the resulting chromatin structure is important for the *ILV1* basal level expression but not for the GCN4 activation. One aspect of such changes in chromatin structure could be nucleosome exclusion, while the effect of multiple copies of SIN3REB1 suggests additional effects. Although we have not demonstrated the occurrence of nucleosome-free regions in the constructs studied here, such regions might be expected to extend about 100 bp from the REB1 site and downstream in the promoter (13). Thus, both the A-rich element and the GCN4 site are within the region expected to be free from nucleosomes. It should be noticed that Chasman et al. (9) found no synergism between REB1 and another acidic activator protein, GAL4. In addition, Workman et al. (33) suggested that the function of the acidic activation domains might be to overcome nucleosome-mediated repression activity. In agreement with this hypothesis, GCN4 still activates an *ILV1* promoter deleted for its REB1 site four- to fivefold.

ABF1 and REB1 can act in a similar manner to control the *ILV1* basal level expression. Although there is no evident homology between ABF1 and REB1, they share several characteristics. Both proteins, as well as RAP1 and CBF1, belong to a class of regulatory proteins characterized by their abundance (10^2 to 10^3 molecules per haploid cell), their many binding sites in the genome, and their involvement in diverse chromosomal functions (4, 8, 29). Also, *ABF1*, *RAP1*, and *REB1* are essential genes (12, 19, 29). Homology between *RAP1* and *ABF1* has been reported (12). ABF1 and REB1 act as weak transcription activators on their own but as powerful auxiliary transcription activators when associated with other weak regulatory elements such as the *DED1* T-rich sequence (6, 7).

These similarities suggest that these proteins have related functions within the cell. When bound to DNA, these proteins might influence the higher-order chromatin structure to facilitate transcription, replication, centromere function, or telomere elongation.

If these proteins have a similar functional role in the cell they should be able to substitute for each other. Indeed,

when we replaced the REB1 site in the *ILV1* promoter with the *ARS1* ABF1 site, the basal level expression from this new promoter was largely unaffected, whereas a mutated *ARS1* ABF1 site that does not bind ABF1 did not support *ILV1* basal level expression. This result argues in favor of a related function of these two proteins.

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