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The promoter of *Saccharomyces cerevisiae* FBA1 gene contains a single positive upstream regulatory element

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The glycolytic enzyme fructose 1,6-bisphosphate aldolase is encoded by the *FBA1* gene of *Saccharomyces cerevisiae*. Transcription of aldolase gene is not regulated by glucose and high levels of expression have been observed also during growth on nonfermentable carbon source. A *FBA1::lacZ* gene fusion was constructed and a deletion analysis demonstrated the presence of a unique *cis*-acting positive upstream element (UAS) required for high levels of *FBA1* expression. This element is located between positions −550 and −440 upstream of the aldolase open reading frame and it contains sequences known to constitute the binding sites for the multifunctional proteins RAP1 and ABF1 and two TTCC motifs.

Fructose 1,6-bisphosphate aldolase; Upstream activation site; Gene regulation; RPG box

1. INTRODUCTION

Aldolase (fructose 1,6-bisphosphate aldolase; EC 4.1.2.13) catalyzes the reversible cleavage of fructose 1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

In *Saccharomyces cerevisiae* aldolase consists of two identical subunits of 40 kDa, each containing one tightly bound zinc atom essential for catalytic function [1].

Like other glycolytic enzymes aldolase is rather abundant in wild-type cells. Unlike phosphoglycerate kinase, enolase and pyruvate kinase, that share the property of being induced by glucose, the aldolase activity is not greatly influenced by the carbon source present in growth media [2].

Cis-acting positive and negative elements, UAS and URS respectively, have been identified in several glycolytic enzyme genes including *ENO1* [3], *ENO2* [4], *PGK* [5], *PYK* [6], *ADH1* [7] and *PDC1* [8].

A conserved sequence is constituted by the RPG box, that has been found initially upstream of many ribosomal protein genes [9] and now has been identified upstream of the silent mating type loci *HML* and *HMR* [10], at telomeres in yeast chromosome [11] and upstream of various glycolytic genes, suggesting a role for the RAP1 protein, the *trans*-acting factor which binds RPG box, in the transcriptional control of glycolytic genes [8,12,13].

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Abbreviations: URS, upstream repression site; UAS, upstream activation site; YEPD, yeast extract-peptone-dextrose; ORF, open reading frame.

In addition the specific activity of many glycolytic enzymes, including aldolase, is reduced 5–50-fold in strains carrying a *gcr1* null mutation. The GCR1 product is known to act as a positive, *trans*-acting transcriptional regulator of glycolytic enzymes [14], although the mechanism by which *GCR1* brings about high level expression of genes encoding glycolytic enzymes remains unknown.

To study the regulation of the aldolase gene expression, we have initiated an analysis of the 5′-noncoding region of the *FBA1* gene in order to identify *cis*-acting regulatory elements required for its expression.

Our results demonstrate that the *FBA1* promoter contains a unique *cis*-acting positive upstream element (UAS) required for high levels of *FBA1* expression. This element contains sequences known to constitute the binding sites for the multifunctional proteins RAP1 and ABF1 and two TTCC motifs.

2. MATERIALS AND METHODS

2.1. *Strains and growth conditions*

Saccharomyces cerevisiae haploid strain W303-1A (MAT *a*, *ade 2-1*, *can 1-100*, *ura 3-1*, *leu 2-3,112*, *trp 1-1*, *his 3-11,15*) [15] and W303 D homozygous diploid were obtained by M.A. Teste (CNRS, Gif-sur-Yvette, France).

Yeast cells were grown in YEPD medium containing 2% peptone, 1% yeast extract or in minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories) supplemented with appropiate nutrients. The carbon source was 2% glucose or 2% glycerol plus 2% ethanol.

E. coli JM101 ((*lac pro*), *thi*, *strA*, *supE*, *endA*, *sbcB*,*hsdR* , *F'traD36*, *proAB*, *lacI*^{q}, *Z M15*) was used for plasmid amplification.

2.2. *Transformation of E.* coli *and S.* cerevisiae

E. coli was transformed by the $CaCl_2$ method [16]. *S. cerevisiae* was transformed by the lithium acetate method described by Ito et al. [17].

2.3. *Cloning of* FBA1 *gene*

An oligonucleotide which was complementary to the sequence from +193 to +213 of the yeast *FBA1* gene was synthesized by a DNA synthesizer (CYCLONE DNA synthesizer, Biosearch Inc.). ^{32}P-labelled and used to screen the yeast DNA genomic library constructed by size fractionation of partial digested DNA in YEp24 [22], kindly provided by G. Lucchini, according to standard techniques [16]). About 15 000 clones were screened.

2.4. *Recombinant DNA techniques*

Standard DNA manipulations were performed according to Sambrook et al. [16].

Plasmid p13A was constructed from YEp13 [23] digested with *Bam*HI and ligated to 5.6 kb *Bam*HI fragment containing the *FBA1* gene.

The *FBA1–lacZ* fusion was constructed from YEp353 [24] digested with *Bam*HI and *Hin*dIII and ligated to *Bam*HI-*Hin*dIII fragment containing sequences from −810 to +383 of the *FBA1* gene, generating plasmid pE1.

5′ deletions were constructed by linearizing pE1 at the unique *Sma*I site in the polylinker. A series of deletions was generated using the exonuclease *Bal*31 [16]. The precise location of each deletion endpoint was determined by dideoxy-chain termination method [25] using alkaline denaturated DNA templates.

Internal deletions were generated by isolating the *Bam*HI-*Mae*I promoter fragment and fusing to the *Sma*I site at the 5′ end of *FBA1–lacZ* fusion in pE1 deletion derivatives.

To perform a gene disruption the 1.2 kb *Hin*dIII fragment containing the yeast *URA3* gene was inserted into the *Hin*dIII site of the *FBA1* gene cloned in a plasmid carrying bacterial ampicillin-resistance gene and origin of replication, obtaining the plasmid pLGAU1. This plasmid was digested with *Bam*HI and *Eco*RI in order to excise the yeast DNA fragment from the vector and the whole mixture used to transform W303 D homozygous diploid. Disruption was tested by Southern blot and by tetrad analysis.

Total yeast DNA was prepared by the method of Nasmyth and Reed [18] and yeast RNA according to Federoff et al. [19].

DNA and RNA transfer from agarose gels to Hybond-N membranes (Amersham) was performed according to the supplier's instruction. ^{32}P-Labelled DNA probes were obtained by nick-translation. Hybridization was performed according to Sambrook et al. [16].

2.5. *β-Galactosidase assay*

β-Galactosidase was extracted from cell pellets with glass beads in Z buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). After centrifugation, the supernatants were used for β-galactosidase and protein assay. The β-galactosidase assay was performed according to the method of Miller [20]. Protein assays were performed according to the microbiuret method [21].

3. RESULTS AND DISCUSSION

3.1. *Cloning and expression of the* FBA1 *gene*

To clone the *FBA1* gene of *S. cerevisiae* an oligonucleotide probe (GGTGGTGCTGCTTACTTCGCT) was synthesized according to the published sequence [26] from +193 to +213 and used to screen a *S. cerevisiae* gene bank in YEp24 vector. Isolation of the *FBA1* gene was confirmed by sequence analysis and by performing a gene disruption in a homozygous diploid strain W303-D. The *URA3* gene was inserted in the *Hin*dIII site of cloned *FBA1* sequence (see Fig. 1) and the construct was used to transform the W303-D to Ura$^+$ phenotype. Several tranformants were checked by Southern blotting (Fig. 1) and by tetrad analysis. Up to 20 tetrads were tested and all of them gave two viable spores on YEPD plates; all the viable spores were Ura$^-$.

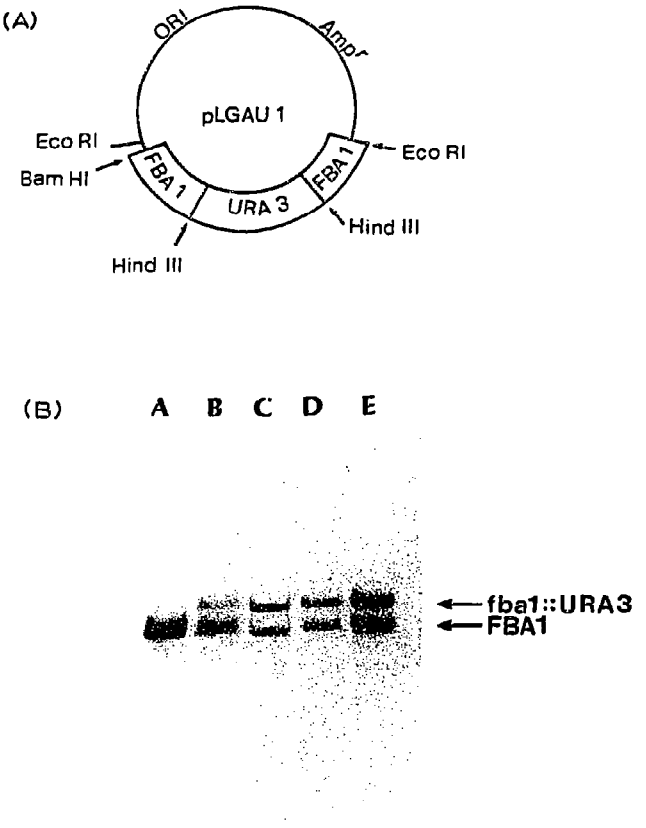

Fig. 1. Disruption of the aldolase gene. (A) A fragment of yeast DNA containing the *URA3* gene was inserted into the *Hin*dIII site of the *FBA1* gene obtaining the plasmid pLGAU1. Homozygous diploid strain W303-D was transformed with *Eco*RI-*Bam*HI digested plasmid, selecting for Ura$^+$ transformants. (B) Southern blot of *Bam*HI-digested total DNA from wild-type cells (lane A) and transformed cells (lanes B,C,D,E) probed with nick-translated *FBA1* gene fragment.

A 5.6 kb *Bam*HI fragment, obtained from the original YEp24 clone, was inserted into the *Bam*HI site of the vector YEp13 and the recombinant plasmid p13A was used to transform the haploid strain W303–1A. Aldolase, that is highly expressed produced in wild-type cells growing on glucose, representing about 0.5% of total cell protein, was over-expressed about 5-fold in *S. cerevisiae* W303–1A[p13A] transformants (1.8 U/mg of protein).

The aldolase activity was not greatly influenced by the carbon source present in the media, in fact we found about the same enzyme level in cell extracts from glucose (0.38 U/μg) and glycerol/ethanol (0.3 U/μg) cultures. Northern blot analysis (Fig. 2), performed on total RNA from haploid W303–1A cells showed that comparable levels of aldolase mRNA were also present in cells growing on glucose and glycerol plus ethanol media, indicating that the aldolase gene is constitutively expressed.

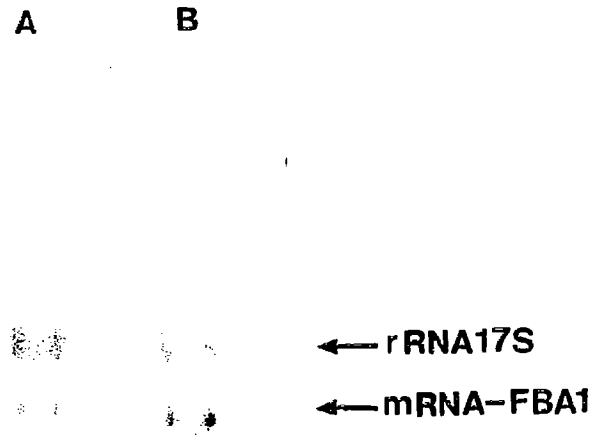

Fig. 2. Northern blot analysis showing carbon source regulation of the *FBA1* mRNA levels. Total RNA (10 μg) was isolated from W303-1A cells grown in glucose medium (lane A) and in glycerol plus ethanol (lane B). The filter was probed with a nick-translated *FBA1* gene fragment and a 17 S ribosomal-specific probe to measure rRNA as a loading control (both nick-translated to a specific activity of 10^8 cpm/μg for *FBA1* and 10^6 cpm/μg for 17 S probe).

3.2. *Mapping of the* cis*-acting regulatory sequences within the 5'-flanking region of* FBA1 *gene*

To study transcriptional regulation and promoter elements of *FBA1* gene we constructed a *FBA1–lacZ* gene fusion. The YEp353 plasmid was digested with *Bam*HI and *Hin*dIII and ligated to *Bam*HI-*Hin*dIII fragment containing sequences from −810 to +383 of the *FBA1* gene, generating plasmid pE1 (Fig. 3). β-Galactosidase expression was monitored in W303–1A cells transformed with plasmid pE1 during the exponential phase of growth (5×10^6 cells/ml) in media containing glucose or glycerol plus ethanol. A comparable level of β-galactosidase activity was observed on both media (Fig. 3), in agreement with the levels of aldolase activity and of mRNA measured previously.

Plasmids carrying deletions within the 5'-flanking region of the *FBA1* gene were constructed by cutting pE1 plasmid at the unique *Sma*I site in the polylinker and by a series of digestions with the exonuclease *Bal*31. The exact location of each deletion endpoint was determined by plasmid sequencing using appropriate oligonucleotides.

Strain W303–1A was transformed with the plasmids carrying the different deletions. About 10 copies/cell of plasmid were found in the different transformants (data not shown) and the expression of the *FBA1–lacZ* fusion carrying promoter deletions was measured in cells grown on media containing glucose or glycerol plus ethanol (Fig. 3).

As shown in Fig. 3 deletions extending from position −880 to −550 do not influence the expression level on both media. Deleting a further 88 bp downstream (−462) reduces expression of 2.5-fold on both media; a further deletion extending till position −364 dramatically reduces expression.

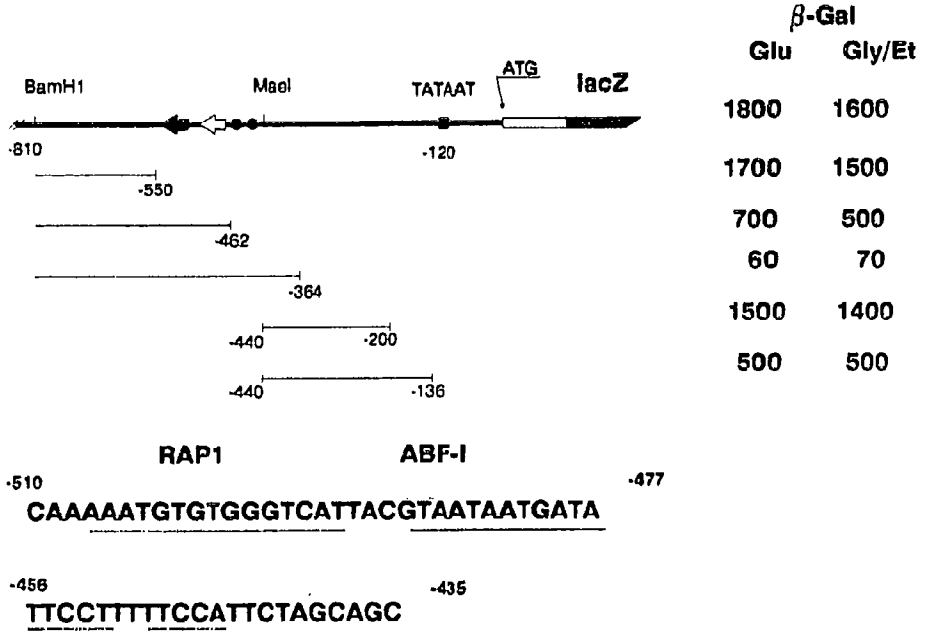

Fig. 3. Deletion analysis of *FBA1* promoter: effect of 5' and internal deletions on *FBA1–lacZ* expression. All deletion endpoints are numbered with respect to the ATG of the aldolase ORF and were determined by sequencing. Random transformants were used as soon as they were large enough to serve as inocula and cultures assayed immediately. β-Galactosidase assay was performed on whole-cell extracts isolated from log-phase cells grown in minimal medium containing glucose or glycerol plus ethanol as the carbon source. Enzyme activities, mesured in units [20] per milligram of total protein, are the average of two experiments. Filled arrow indicates RAP1-binding site direction, open arrow ABF1-binding site direction and filled circles TTCC box, as shown at the bottom.

These data mapped the apparent boundary of a positive regulatory region (UAS_{FBA}) between position −550 and −364. Internal deletions within the *FBA1* upstream sequence were then constructed by fusing the *Bam*HI–*Mae*I fragment to the *Sma*I site at the 5′ end of pE1 deletion derivatives.

Restoring sequences upstream from −440 as in internal deletion −440/−200, results in a normal level of expression while a further 64 bp deletion (till −136) reduces the expression 3-fold.

Probably there are not repressor sequences involved in the modulation of the basal levels of gene expression, like in the *ENO1* promoter, since the −440/−200 deletion yields a wild-type level of expression. In the −440/−136 deletion the UAS_{FBA} is located very close to the TATA box (13 bp) and this could explain the reduced expression observed, as recently reported by Nishizawa et al. [28] for the UAS_{PYK}, in which shorter distance between UAS and TATA box causes a similar reduced expression. In addition, in all the deletions tested no significant difference of expression was found in glucose and in glycerol/ethanol media, suggesting that there are no sequences subjected to carbon source regulation.

Taken together these data indicate the presence of a unique *cis*-acting positive upstream element required for high levels of *FBA1* expression. This element is located between position −550 and −440.

When we looked for homology of the *FBA1* UAS region with other known yeast regulatory sequences [7,8,12,13], two interesting sequences were found: between −506 and −493 there is a motif homologous (11 to 13 nucleotide) to an RPG box and a nucleotide sequence between −490 and −478 is identical to ABFI consensus sequence (Fig. 3), that has been found also in the UAS of *ENO2* gene [12].

Another sequence was found between −456 and −444 which contains two TTCC blocks; this sequence seems able to activate the transcription even alone (Fig. 3, deletion −462). RAP1-binding sites in the promoter of glycolytic genes are often found adjacent to one or more sequence elements with the motif CTTCC and the presence of this latter element has been correlated with efficient transcriptional activation [8,13,27].

It is possible that proteins binding to these sequences may interact with the RPG-binding protein to produce maximal expression.

According to our results the *FBA1* promoter appears to be quite simple: it contains a unique UAS region composed of sequences known to constitute binding sites for the multi-functional proteins RAP1 and ABFI and two TTCC motifs. The RPG and ABFI boxes are necessary , but not sufficient, for maximal level of expression of *FBA1*, since deletion of these boxes reduces the expression only 2.5-fold.

No negative regulatory elements were found and this may be related to the observation that aldolase expression is not modulated by carbon sources and is not subjected to glucose induction or repression. A similar situation has been recently described for the yeast *TPI* gene [29].

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