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Isolation and primary structure of the gene encoding fructose-1,6-bisphosphatase from *Saccharomyces cerevisiae*

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The gene encoding *Saccharomyces cerevisiae* fructose-1,6-bisphosphatase *(FBP1)* was isolated. Constructed *fbp1::HIS3* null mutants were unable to grow with ethanol, and growth was restored after transformation with the cloned *fbp* gene. The gene codes for a protein of 347 amino acid residues with an M_r of 38131. Homology with the pig kidney cortex and the sheep liver enzyme is 47.7% and 46.6%, respectively, within a central core of 328 amino acid residues. The cloned promoter size was 318 bp and allowed only low level expression of the gene. This indicates a positive activation site (UAS) upstream of the cloned DNA fragment.

Fructose-1,6-bisphosphatase; Nucleotide sequence; Gluconeogenesis; Null allele; *(Saccharomyces cerevisiae)*

1. INTRODUCTION

Although glycolysis and gluconeogenesis are antithetical, they share most of their reactions. Hence, the regulatory control between both pathways is limited to those reactions catalysed by enzymes which are specific for one or the other. One such key reaction is the phosphorylation of fructose 6-phosphate to fructose-1,6-bisphosphate by phosphofructokinase and another is the inverse reaction by FBPase. In the presence of glucose several control mechanisms reduce FBPase activity: (i) in mammals and in yeast, FBPase is inhibited by AMP and fructose-2,6-bisphosphate [1–3]; (ii) the activity of yeast FBPase is reduced by reversible inactivation immediately after addition of glucose [4,5]; (iii) FBPase is irreversibly degraded, a process that starts about 5 min after addition of glucose to cells growing with non-fermentable carbon sources [6,7]; (iv) in addition to all these regulatory mechanisms mentioned above transcription of *FBP1* is glucose repressible [8]. For FBPase interconversion phosphorylation by a cyclic AMP-dependent protein kinase at a serine residue is probably the mechanism ([9–12], reviewed in [13]). Whereas rapid-reversible inactivation is only observed with FBPase, irreversible inactivation is a common mechanism for most gluconeogenic enzymes, such as cytoplasmic malate dehydrogenase [14] and phospho*enol*-pyruvate carboxykinase [15]. Under conditions that prevent irreversible inactivation generally, the inactivation of FBPase remains reversible; this is indicative of a common mechanism for those enzymes subject to irreversible inactivation [16].

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Abbreviations: FBPase, fructose-1,6,-bisphosphatase (EC 3.1.3.11); *FBP1*, structural gene of FBPase

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number Y00754

2. EXPERIMENTAL

The *FBP1* gene was cloned from strain cat1.S3-14A (*MAT*a *his4 MAL2-8^c MAL3 SUC3*) [17]. Null alleles were constructed in strain WAY.5-4A (*MAT*a *his3-Δ1 ura3-52 MAL2-8^c MAL3 SUC3*) [26]. Recombinant plasmids were amplified in *Escherichia coli* strain RR1 (*F$^-$ hsd520 supE44 ara-14 proA2*

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lacY1 galK2 rpsL20 xyl-5 mtl-1) and BMH71-18 (*Δlac-pro*) *thi supE*; F' ⟨*lacIq lacz ΔM15 proA+B+*⟩). Yeast extract rich (YEP) and chemically defined (synthetic) media (SC) were as in [18]. FBPase activity and protein were estimated as cited in [16] and established protocols were followed for standard techniques of recombinant DNA as given in [18]. DNA was sequenced using the deoxy chain-termination method [19], plasmid sequencing was performed as described in [20] and plasmids were purified in a mini-ultracentrifuge (Beckman, TL100) as described in [21]. Synthetic oligonucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems; Weiterstadt, FRG). DNA/DNA hybridization was performed as described in [22].

3. RESULTS AND DISCUSSION

3.1. *Isolation of the fructose-1,6-bisphosphatase gene*

Four wobbled oligonucleotides from the amino acid sequences of FBPase peptides [23] were derived from peptide 18 (RV1, 5'-GAT(C)AAA(G)-TTT(C)C(T)TT(C)GAT(G)CAT(C)AT-3'; RV2, 5'-GAT(C)AAA(G)TTT(C)C(T)TA(G)GAT(G)C-AT(C)AT-3') and peptide 16 (RV3, 5'-GAT(C)G-AA(G)ATT(C)TTT(C)ATTAAT(C)GC-3'; and RV4, 5'-GAT(C)GAA(G)ATATTT(C)ATC(A)A-AT(C)GC-3'). These oligonucleotides included all possible codons; differences are underlined. They were hybridized against genomic DNA of strain cat1.S3-14A digested with (i) *Eco*RI, (ii) *Hin*dIII, (iii) *Xho*I and (iv) *Hin*dIII/*Xho*I, respectively. Each oligonucleotide gave about 6 signals. Oligonucleotides RV2 and RV3 gave a 4.4 kbp *Hin*dIII signal and a 3.6 kbp *Hin*dIII/*Xho*I signal which were expected to correspond to *FBP1* [8]. The DNA associated with this signal was eluted from the gel by the DEAE paper technique [24] and ligated into the large *Hin*dIII/*Sal*I fragment of pBR322. After transformation of *E. coli* RR1, those clones containing the *FBP1* gene were iden-

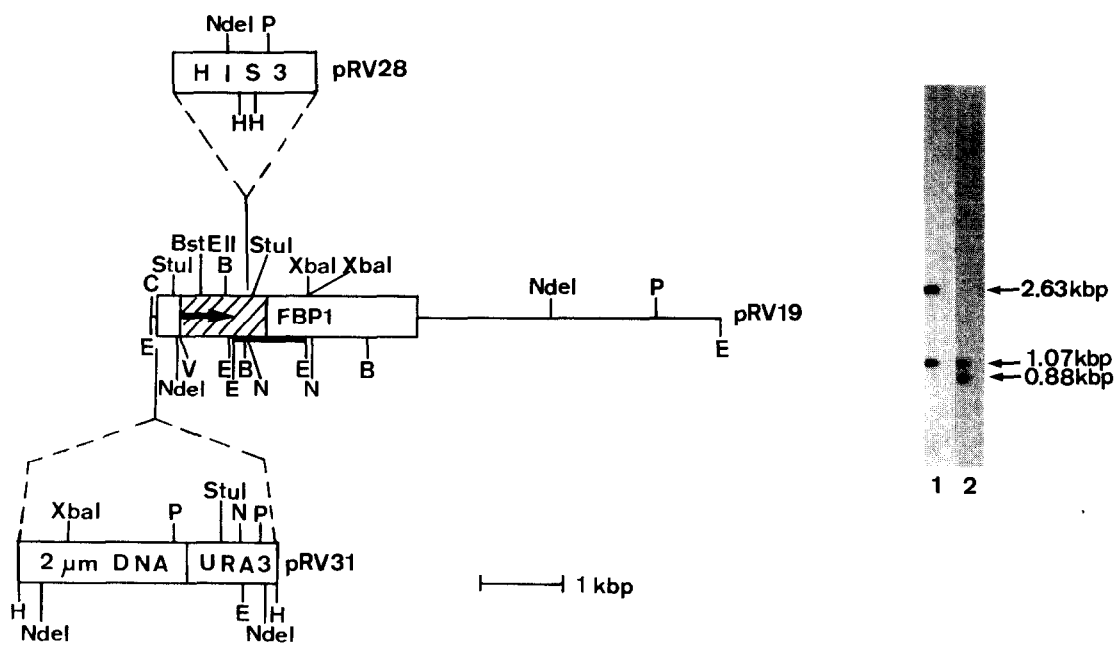

Fig.1. Plasmids (left) and Southern blot analysis of *fbp1::HIS3* null mutant (right). Plasmid pRV19 contains the *Hin*dIII/*Xho*I fragment of *FBP1* ligated into pBR322. Yeast DNA sequences are boxed, *E. coli* DNA sequences correspond to lines. The hatched part inside *FBP1* represents the open reading frame and the arrow indicates the direction of transcription. For construction of pRV28 see section 3.4. To construct plasmid pRV31 the *Stu*I/*Hin*dIII fragment of yeast/*E. coli* shuttle vector YEp24 [31] that contains the 2 μm DNA and the *Sma*I/*Hin*dIII fragment of YEp24 were fused and ligated into the *Hin*dIII site of pRV19. For Southern hybridization DNA from *fbp1::HIS3* null mutant (lane 1) and wild type (lane 2) were restricted with *Eco*RI and hybridized with the 1.9 kbp *Hin*dIII/*Xba*I fragment of *FBP1*. As a result of *HIS3* integration the 0.88 kbp *Eco*RI wild type signal (heavy line in pRV19) is increased to 2.63 kbp in the *fbp::HIS3* null mutant. The 1.07 kbp signal present in wild type and null mutant correspond to a further *Eco*RI restriction site left to the *FBP1* fragment of pRV19. *Abbreviations:* B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; V, *Eco*RV. No restriction sites within the *FBP1* fragment of pRV19 for *Bgl*II, *Cla*I, *Kpn*I, *Pvu*II, *Sac*I and *Sph*I.

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                -311       -301       -291       -281       -271       -261       -251       -241       -231       -221
              AAGCTTAA AAAGTTGAGA CAGGGAATTT GAAGGCGAAG ATTGCGGAAC TGGCCAATAC CCACTACTTT TTTTTTTGGT TTGCTTGGTT TCTTCCTGTC

     -211       -201       -191       -181       -171       -161       -151       -141       -131       -121       -111
GCTTGCCAAC TTGTGGCATC TTCCCCACAC TATATTATAA GGATCGTCCT ATGTATAGGC AATATTATCC ATTTCACTCG CTAACAAATG TACGTATATA TATGGAGCAA

      -101        -91        -81        -71        -61        -51        -41        -31        -21        -11         -1
CAAGTAGTGC AATTACAGAC GTGTATTTTG TCTTGATCTT GCTTTTTGTA TGATAGGCCT AAGAATAACA GTGCGAACAT ATAAGAAACA TCCCTCATAC TACCACACAT

                                                                                                                      90
ATG CCA ACT CTA GTA AAT GGA CCA AGA AGA GAC TCT ACC GAA GGG TTT GAT ACC GAT ATC ATC ACT CTT CCT AGA TTC ATA ATC GAG CAC
MET Pro Thr Leu Val Asn Gly Pro Arg Arg Asp Ser Thr Glu Gly Phe Asp Thr Asp Ile Ile Thr Leu Pro Arg Phe Ile Ile Glu His

                                                                                                                     180
CAG AAG CAA TTT AAG AAC GCT ACT GGT GAT TTC ACA TTA GTA CTG AAT GCC TTG CAA TTC GCG TTC AAA TTT GTA TCT CAC ACC ATC AGA
Gln Lys Gln Phe Lys Asn Ala Thr Gly Asp Phe Thr Leu Val Leu Asn Ala Leu Gln Phe Ala Phe Lys Phe Val Ser His Thr Ile Arg

                                                                                                                     270
CGT GCT GAA TTG GTT AAC TTG GTT GGG TTA GCA GGC GCT TCC AAC TTC ACT GGT GAC CAG CAA AAG AAG TTG GAC GTT CTA GGT GAT GAA
Arg Ala Glu Leu Val Asn Leu Val Gly Leu Ala Gly Ala Ser Asn Phe Thr Gly Asp Gln Gln Lys Lys Leu Asp Val Leu Gly Asp Glu

                                                                                                                     360
ATA TTT ATC AAT GCC ATG AGG GCT AGT GGG ATC ATC AAG GTC CTT GTA TCT GAA GAA CAG GAA GAC TTG ATC GTT TTT CCC ACA AAC ACG
Ile Phe Ile Asn Ala MET Arg Ala Ser Gly Ile Ile Lys Val Leu Val Ser Glu Glu Gln Glu Asp Leu Ile Val Phe Pro Thr Asn Thr

                                                                                                                     450
GGC TCA TAC GCA GTG TGT TGT GAT CCT ATT GAT GGC TCC TCA AAT TTG GAC GCC GGT GTC TCC GTT GGA ACT ATC GCG TCT ATA TTC AGA
Gly Ser Tyr Ala Val Cys Cys Asp Pro Ile Asp Gly Ser Ser Asn Leu Asp Ala Gly Val Ser Val Gly Thr Ile Ala Ser Ile Phe Arg

                                                                                                                     540
CTG CTA CCA GAC TCA TCA GGT ACT ATA AAC GAC GTA CTG AGA TGT GGT AAA GAA ATG GTA GCC GCT TGC TAT GCC ATG TAC GGA TCC TCT
Leu Leu Pro Asp Ser Ser Gly Thr Ile Asn Asp Val Leu Arg Cys Gly Lys Glu MET Val Ala Ala Cys Tyr Ala MET Tyr Gly Ser Ser

                                                                                                                     630
ACG CAT CTA GTA TTG ACA TTG GGT GAT GGA GTT GAT GGG TTT ACC TTA GAC ACA AAC TTG GGC GAA TTC ATC TTG ACT CAT CCT AAC TTA
Thr His Leu Val Leu Thr Leu Gly Asp Gly Val Asp Gly Phe Thr Leu Asp Thr Asn Leu Gly Glu Phe Ile Leu Thr His Pro Asn Leu

                                                                                                                     720
AGA ATT CCG CCT CAA AAG GCC ATC TAC TCA ATT AAT GAA GGT AAC ACC CTC TAC TGG AAC GAG ACT ATA AGA ACA TTT ATT GAG AAA GTC
Arg Ile Pro Pro Gln Lys Ala Ile Tyr Ser Ile Asn Glu Gly Asn Thr Leu Tyr Trp Asn Glu Thr Ile Arg Thr Phe Ile Glu Lys Val

                                                                                                                     810
AAA CAA CCC CAA GCA GAC AAC AAC AAC AAG CCT TTC TCG GCT AGG TAT GTT GGA TCC ATG GTT GCT GAT GTT CAC AGG ACG TTT CTT TAC
Lys Gln Pro Gln Ala Asp Asn Asn Asn Lys Pro Phe Ser Ala Arg Tyr Val Gly Ser MET Val Ala Asp Val His Arg Thr Phe Leu Tyr

                                                                                                                     900
GGT GGC CTT TTC GCA TAC CCT TGC GAC AAG AAG AGC CCC AAC GGA AAA CTG AGG TTG CTT TAT GAG GCC TTC CCA ATG GCT TTC TTA ATG
Gly Gly Leu Phe Ala Tyr Pro Cys Asp Lys Lys Ser Pro Asn Gly Lys Leu Arg Leu Leu Tyr Glu Ala Phe Pro MET Ala Phe Leu MET

                                                                                                                     990
GAA CAA GCA GGG GGA AAA GCG GTC AAC GAT CGC GGA GAG AGA ATC TTG GAT TTG GTG CCA AGT CAT ATC CAT GAC AAA TCT TCT ATT TGG
Glu Gln Ala Gly Gly Lys Ala Val Asn Asp Arg Gly Glu Arg Ile Leu Asp Leu Val Pro Ser His Ile His Asp Lys Ser Ser Ile Trp

                                                                                                1050       1060       1070       1080
TTG GGT TCT TCA GGT GAA ATT GAC AAA TTT TTA GAC CAT ATT GGC AAG TCA CAG TAG                      TTC AATGATCGCC TTCTTTTCTT ATTTTCTTTG
Leu Gly Ser Ser Gly Glu Ile Asp Lys Phe Leu Asp His Ile Gly Lys Ser Gln

      1090       1100       1110       1120       1130       1140       1150       1160       1170       1180       1190
TTCTGTACTT TAGTACGCGA AAAAAAAAAT CTGTATATGT CCTTATATAT ATATATATTT ATATATATAT ATGTGTATGT ATGTGTACCG TAAGCATTAC TCCTTCTAAT

      1200       1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
AATGAAAATT CTTAGGAAAA GAGAAAGGAA GTAGCGAATG GAATGGGATG GAAGTTTTAA AGAACATTAG AATTTATCCT TTGTCAAACT TCATCACATC AACCAAGAAC

      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400       1410
TATATAAACC TACCAAATGA ATTAAGAAAC CTAATTAGTG AAGAGCAGGA GAGTAAACTA GGGTTCTTGC ACATCATTGA AAGTGATTTT AAACCTTCGG TAGCGCTGCA

      1420       1430       1440       1450       1460       1470       1480       1490       1500       1510       1520
AAAGTTGGTG AATTGTACTA CGGGGGACGA AAAGATCCTA ATCATAGATA TAGTATCAAT ATGGTCCCAA CAAAAGCAAA GGCAGCATGG CGCGATCTAC ATGAATTCGC

      1530       1540       1550
TATCTTGCAT AAACATCACG GGATTAATCG TA
```

Fig.2. Nucleotide and deduced amino acid sequence of *FBP1*. A possible TATATA sequence for transcription initiation is underlined.

tified by colony hybridization with the above mentioned oligonucleotides. The restriction maps of plasmids that were constructed are given in fig.1. Restriction sites of *FBP1* corresponded closely with that reported by Sedivy and Fraenkel [8].

3.2. *Nucleotide sequence of the* FBP1 *gene*

Since restriction sites were suitable for subclone construction, most sequencing was done with pUC19 subclones; otherwise *FBP1* specific oligonucleotides were synthesized and used as sequencing primer. The sequence is shown in fig.2. An open reading frame of 348 codons was identified, which excluding the starting methionine, corresponded to an M_r of 38131. A codon bias index (cbi; defined in [25]) of 0.25 was calculated from these sequencing data. The cbi has a value of 1 if only 22 codons (identified by their occurrence in highly expressed glycolytic genes) out of 61 possible codons are used, while a value of 0 represents a random codon distribution. Hence, FBPase has a relatively weak codon usage, which is only slightly above that of a number of regulatory proteins and enzymes (see also [26]).

3.3. *Sequence comparison between mammalian and yeast FBPase*

The comparison of sequence homologies between such strongly diverged organisms like *Saccharomyces cerevisiae* on one hand and pig and sheep on the other, are useful for identifying those regions containing essential functions. There is a central core with 47.7% (pig kidney cortex) and 46.6% (sheep liver) homology, spanning 328

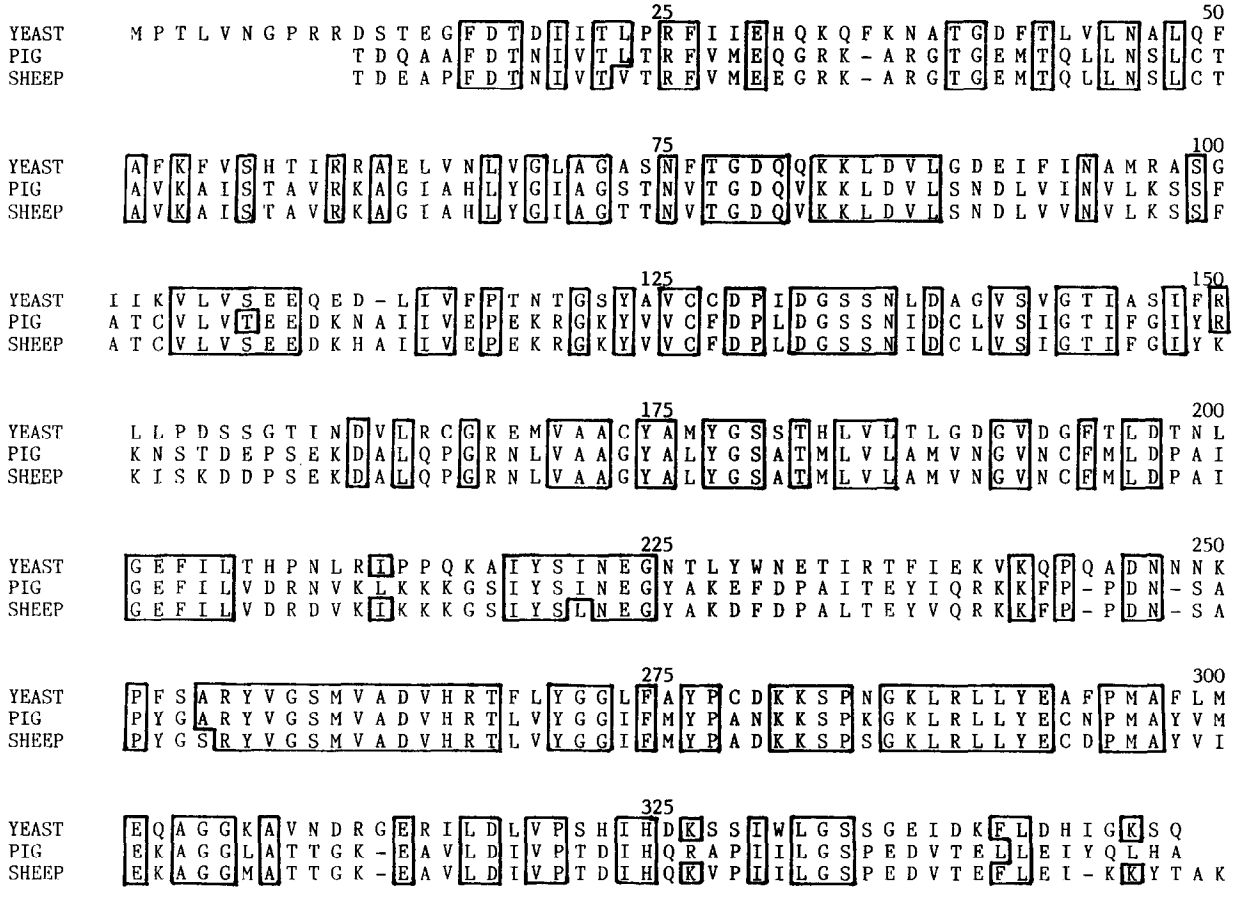

Fig.3. Amino acid sequence homologies between yeast, sheep liver [28] and pig kidney cortex [27] FBPase. Those amino acid residues present in yeast and mammalian FBPases are boxed.

amino acid residues between yeast, pig kidney cortex [27] and sheep liver [28] FBPase (fig.3). In addition, many changes seem to be of minor importance, as similar amino acids were replaced. There is no homology whatever with respect to the N-terminus. The N-terminus of the yeast enzyme extends further than that of the mammalian enzyme by 9 amino acid residues. This was also observed for cytoplasmic malate dehydrogenase in comparison to the mitochondrial isoenzyme [29], an enzyme which is also inactivated after glucose addition and may indicate a role of the N-terminal sequence for irreversible glucose inactivation. Notably, the N-terminal amino residue of FBPase is proline. This is also found for cytoplasmic malate dehydrogenase [29] and may become a common characteristic of those gluconeogenic enzymes that are subject to irreversible glucose inactivation. Notably, the N-terminal amino residue of FBPase is proline which is also found for cytoplasmic malate dehydrogenase [29]. The serine residue at which yeast FBPase is phosphorylated is also absent from the mammalian enzymes. Comparing the overall homologies, the largest region of homology is found between amino acid residues 251 and 298. Possibly, this region may correspond to the substrate binding site. Three further regions of strong homology are present between amino acid residues 16 and 150, and amino acid residues 161 and 205. These strong homologies may correspond to binding sites of the allosteric effectors common to the two enzymes (see also section 1).

3.4. *Construction of fructose-1,6-bisphosphatase null alleles*

For the construction of null alleles, a *Bam*HI fragment encoding the yeast *HIS3* gene was introduced into the open reading frame of *FBP1*. The *Cla*I/*Xba*I fragment of the resulting plasmid pRV28 (fig.1) was introduced into the yeast genome replacing the native *FBP1* gene (fig.1). The resulting null mutant (*fbp1::HIS3*) was unable to grow on complete rich media (YEP) with 3% ethanol as the carbon source and had no FBPase activity (table 1).

3.5. *Expression of the* FBP1 *gene*

A 3.2 kbp cassette, containing a yeast 2 μm ARS sequence and a *URA3* gene, was ligated into pRV19 and the resulting multi-copy plasmid pRV31 (fig.1) was used for transformation of the FBPase null mutant. The resulting transformants had their capacity to grow with ethanol as carbon source restored, although the FBPase activity was low (table 1). Hence, the cloned fragment did not contain all information for proper expression of the gene. The isolated promoter has a size of 318 bases and contains the structural characteristics for transcription initiation. Therefore, we conclude that the isolated gene lacks an upstream activation sequence. This is consistent with the assumption that derepression of gluconeogenic enzymes requires positively acting transcriptional factors (reviewed in [30]).

Table 1

FBPase activities of wild type, *fbp1::HIS3* null mutant and *FBP1* transformant (plasmid pRV19)

Strain	Genotype	Plasmid	Specific activity of FBPase (nmol/min per mg)	
			Glucose	Ethanol
WAY.5-4A	*FBP1*	none	2	50
WAY.5-4A/1	*fbp1::HIS3*	none	not detectable	–[a]
WAY.5-4A/1	*fbp1::HIS3*	pRV19	1.8	9

[a] No growth

The FBPase sequence reported here [during submission of the manuscript similar data were reported by Rogers et al. (1988) J. Biol. Chem. 263, 6051–6057] will provide the basis for future investigation of those enzyme structures that are responsible for the multiple regulatory control mechanisms regulating FBPase activity in mammals and yeast.

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