The Yeast *FBP26* Gene Codes for a Fructose-2,6-bisphosphatase†

Gerhard Paravicini‡,§ and Matthias Kretschmer*,‖

Division of Biology 147-75, California Institute of Technology, Pasadena, California 91125, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Sequencing of an open reading frame 450 bp downstream from the yeast *VPS35* gene revealed a putative peptide of 452 amino acids and 52.7 kDa. The predicted amino acid sequence has 45% identity with the 55-kDa subunit of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (EC 2.7.1.105/EC 3.1.3.46) from rat liver and 42% identity with 480 amino acids in the center of the recently reported 93.5-kDa subunit of yeast 6-phosphofructo-2-kinase (EC 2.7.1.105). The product of the new yeast gene is similar to the entire sequence of the bifunctional rat liver enzyme and, unlike yeast 6-phosphofructo-2-kinase, has the histidine residue essential for fructose-2,6-bisphosphatase activity. Extracts from a chromosomal null mutant strain, *fbp26*::*HIS3*, incubated in the presence of [2-^{32}P]fructose 2,6-P_2, lacked in autoradiograms the characteristic 56-kDa labeled band observed in wild-type. The same band was intensified 3-fold over wild-type level with the *FBP26* gene introduced on multicopy in the *fbp26*::*HIS3* background. A similar increase was found for fructose-2,6-bisphosphatase activity in the same extracts. The *FBP26* gene did not cause detectable increase in 6-phosphofructo-2-kinase activity when introduced on multicopy in a *pfk26*::*LEU2* mutant, indicating that its gene product is predominantly a fructose-2,6-bisphosphatase. Growth on glucose, fructose, galactose, pyruvate, and glycerol/lactate was not impaired in strains carrying the *fbp26*::*HIS3* allele.

Fructose 2,6-bisphosphate (fructose 2,6-P_2) is a major activator in vitro of 6-phosphofructo-1-kinase (EC 2.7.1.11) and inhibitor of fructose-1,6-bisphosphatase (EC 3.1.3.11) (Hers, 1984). It is formed in higher cells from fructose 6-P and ATP by 6-phosphofructo-2-kinase (EC 2.7.1.105) and hydrolyzed to fructose 6-P and P_i by fructose-2,6-bisphosphatase (EC 3.1.3.46) in a reaction that involves a histidine residue as phosphoacceptor (Pilkis et al., 1987). In mammalian tissues, both activities reside on the same enzyme protein. The liver and the heart enzyme are subject to cAMP-dependent phosphorylation, which in liver causes inactivation of the kinase and activation of the bisphosphatase activity (El-Maghrabi et al., 1981, 1982; Furuya et al., 1982; Van Schaftingen & Hers, 1982), whereas the opposite effect of phosphorylation is found in heart muscle (Kitamura et al., 1987; Sakata et al., 1990).

In *Saccharomyces cerevisiae*, enzymes forming and degrading fructose 2,6-P_2 can be separated (Kretschmer et al., 1987; François et al., 1988). The *PFK26* gene coding for a 6-phosphofructo-2-kinase of 93.5-kDa subunit size was recently isolated (Kretschmer et al., 1991) and its gene product found to account for >90% of the 6-phosphofructo-2-kinase activity in yeast extracts and not to have detectable fructose-2,6-bisphosphatase activity (Kretschmer & Fraenkel, 1991).

Several enzymes have been reported for the degradation of fructose 2,6-P_2 in yeast: two activities of high specificity but different affinity, each producing fructose 6-P and P_i (François et al., 1988), and unspecific phosphatases yielding fructose via fructose 2-P as intermediate product (François et al., 1988; Plankert et al., 1991). By analogy to the fructose-2,6-bisphosphatase activities of the bifunctional mammalian enzymes, the specific, low-K_m fructose-2,6-bisphosphatase in yeast is inhibited by fructose 6-P, is activated by glycerol 3-P (Kretschmer et al., 1987; François et al., 1988), and forms a phosphoprotein intermediate when incubated with [2-^{32}P]fructose 2,6-P_2. The characteristic signal of the labeled peptide, presumably from a phosphohistidine residue, was used to estimate its subunit size as 55 kDa (François et al., 1988).

Here, we (i) describe the DNA sequence (*FBP26*), (ii) describe a chromosomal deletion mutant, and (iii) present evidence that *FBP26* specifies a monofunctional fructose-2,6-bisphosphatase in yeast.

MATERIALS AND METHODS

Materials. Fructose 6-P, ATP, and most enzymes were from Boehringer (Mannheim, Germany). Fructose 2,6-P_2, pyrophosphate:fructose-6-P phosphotransferase, and all other chemicals were from Sigma (St. Louis, MO). Bactotryptone and yeast extract were from Difco (Detroit, MI). SDS–PAGE standards were from BRL (Gaithersburg, MD). [γ-^{32}P]ATP and deoxyadenosine 5′-(α-[^{35}S]thiotriphosphate) were purchased from New England Nuclear (Boston, MA). The plasmid pBluescript KS(−) was from Stratagene (La Jolla, CA), the Miniprep Plus kit was from Pharmacia Inc. (Piscataway, NJ), and the Sequenase version 2.0 DNA sequencing kit was obtained from U.S. Biochemical Corp. (Cleveland, OH).

DNA Sequence Determination and Analysis. A 3 kbp *Bam*HI/*Xba*I fragment containing the 3′ end of the *VSP35* gene (Paravicini et al., 1992) and the complete *FBP26* gene were cloned into the *Escherichia coli* plasmid pBluescript KS(−). Exonuclease III – *mung bean* nuclease deletions were performed from both ends of the insert as described in the Stratagene bluescript manual. Double-stranded DNA from a number of deletion plasmids was isolated from *E. coli* and denatured, and both strands were sequenced by the dideoxynucleotide method (Sanger et al., 1977) using Sequenase version 2.

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‡ California Institute of Technology.

§ Present address: Glaxo Institute for Molecular Biology S.A., 1228 Plan-les-Ouates, Geneva, Switzerland.

‖ Harvard Medical School.

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The predicted protein sequence of *FBP26* was compared with the contents of the NBFR, GenBank, and EMBL databases, using the FASTA and TFASTA programs (Pearson & Lipman, 1988), and amino acid sequence alignment (Needleman & Wunsch, 1970) was done using the program PILEUP of the University of Wisconsin Genetics Computer Group (GCG) sequence package.

Disruption of FBP26. A 1.4 kbp *Bal*I/*Bst*EII fragment containing the *FBP26* open reading frame was replaced by a 1.4 kbp *Xho*I/*Bam*HI fragment containing the *HIS3* gene (Figure 3), and a linear 3 kbp *Bam*HI/*Xba*I fragment was used to transform the haploid wild-type yeast strain SEY6211 [*α*, *leu2*, *his3*, *trp1*, *lys2*, *ura3*, *suc2* (kindly provided by Scott Emr)] by one-step disruption (Rothstein, 1983). His$^+$ transformants, GPY1126, were subjected to Southern blot analysis to confirm the deletion of the *FBP26* gene from the chromosome (data not shown). For studies of the *FBP26* gene product, *fbp26*::*HIS3* was introduced into a strain lacking the *PFK26* gene by cross of GPY1126 (*a*, *fbp26*::*HIS3*, *leu2*, *his3*, *trp1*, *lys2*, *ura3*, *suc2*) with DFY650 [*α*, *pfk26*::*LEU2*, *leu2*, *his3* (Kretschmer & Fraenkel, 1991)]. DFY653 (*pfk26*::*LEU2*, *leu2*, *his3*, *trp1*, *ura3*), DFY655 (*fbp26*::*HIS3*, *leu2*, *his3*, *trp1*, *ura3*), and DFY 657 (*pfk26*::*LEU2*, *fbp26*::*HIS3*, *leu2*, *his3*, *ura3*) were segregants and used in studies of fructose-2,6-bisphosphate activities in extracts and the formation of a phosphoprotein intermediate.

Assay of Enzymes. DFY653 (*pfk26*::*LEU2*), DFY655 (*fbp26*::*HIS3*), DFY 657 (*pfk26*::*LEU2*, *fbp26*::*HIS3*, plasmid YEp352), and DFY 657 [*pfk26*::*LEU2*, *fbp26*::*HIS3*, plasmid pBHY10(FBP26)] were grown in enriched medium A (yeast nitrogen base containing casamino acids; uracil was omitted for growth of plasmid strains), supplemented with 2% glucose to an A_{580} of 6. Cells were harvested, and enzymes were assayed in 11% PEG_{6000} pellets of total cell protein (Kretschmer et al., 1991). Numbers for enzyme activities in Figure 4 are the average of four determinations.

The mixture for 6-phosphofructo-2-kinase contained enzyme, 50 mM Tris-HCl, pH 7.6, 8 mM ATP, 2 mM fructose 6-P, 6.5 mM glucose 6-P, 20 mM magnesium chloride, 2 mM potassium phosphate, and 5 mM mercaptoethanol. Samples were withdrawn at 0, 2.5, 5, and 10 min and assayed for fructose 2,6-P_2 as described (François et al. 1984).

Fructose-2,6-bisphosphatase was assayed by the release of [^{32}P]P_i in a mixture containing enzyme, 30 mM Tris-HCl, pH 7.3, 17 μM fructose 2,6-P_2 (2×10^{16} cpm/mol), 9 mM magnesium chloride, and 5 mM mercaptoethanol. Samples were taken at 0, 15, and 30 min, and [^{32}P]P_i was separated from [2-^{32}P]fructose 2,6-P_2 on Dowex AG-1 as described (Van Schaftingen et al., 1982). One unit of enzyme is defined as 1 μmol of substrate converted/min at 25 °C.

^{32}P in a 56-kDa peptide was determined after incubation for 3 min of PEG_{6000} pelleted total cell protein in the fructose-2,6-bisphosphatase mixture described above, followed immediately by SDS–PAGE (Laemmli, 1970). The 10% acrylamide gel was subsequently dried and exposed to a Molecular Dynamics storage screen. The autoradiogram (Figure 4) was obtained on a Molecular Dynamics Phosphoimager and the amount of ^{32}P labeling in the 56-kDa band quantified using ImageQuant software with a known amount of ^{32}P as standard on the dried gel.

[2-^{32}P]fructose 2,6-P_2 was synthesized from fructose 6-P and [γ-^{32}P]ATP using yeast 6-phosphofructo-2-kinase purified from an overproducing strain carrying a 2μ plasmid with the *PFK26* gene under the control of the yeast *ENO1* promoter (Kretschmer and Fraenkel, unpublished results).

Analytical Methods. Fructose 2,6-P_2 was extracted from cells frozen in liquid nitrogen (Clifton & Fraenkel, 1983) and assayed as described (François et al., 1984). Concentrations from at least three experiments were averaged and related to nominal cell water volume (Clifton & Fraenkel, 1983).

Protein was determined with bovine serum albumin as standard as described by Bradford (1976).

RESULTS

Sequence. A 5.8 kbp yeast genomic DNA fragment complementing the *vps35* vacuolar protein missorting phenotype contained not only the *VPS35* gene (Paravicini et al., 1992) but also a second reading frame, starting 450 bp downstream from the *VPS35* gene. This open reading frame comprises 1356 bp and codes for a putative protein of 452 amino acids (Figure 1). A sequence TATATAA, possibly initiating transcription, was found at positions –102 to –96 upstream from the reading frame, and a putative transcription/polyadenylation signal is present as TAG...TATGT...TTTT, starting at position 1449.

When the sequence of the open reading frame was compared to known sequences in the Genbank/NBRF and EMBL databases, it turned out that the predicted protein was highly similar to the mammalian bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. It also showed high similarity to yeast 6-phosphofructo-2-kinase. This enzyme has recently been purified, and the corresponding gene, *PFK26*, has been isolated (Kretschmer & Fraenkel, 1991). Because of the evidence (see below) that the open reading frame described here encodes a protein possessing mainly, if not solely, fructose-2,6-bisphosphatase activity, we refer to the new gene as *FBP26*. Its codon usage is relatively unbiased, the codon bias index (Bennetzen & Hall, 1982) being 0.0226. We have compared the yeast fructose-2,6-bisphosphatase protein with the bifunctional rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the yeast 6-phosphofructo-2-kinase in an alignment shown in Figure 2. The yeast fructose-2,6-bisphosphatase and the liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase sequences show 45% identity. Although the yeast enzyme is presumably a monofunctional bisphosphatase (see below), the similarity between the two enzymes extends over the entire length of the proteins. The nucleotide binding domain (Rossmann et al., 1975) MVGLPARGK (residues 18–26 in the yeast fructose-2,6-bisphosphatase and 46–54 in the rat liver enzyme), thought to be important for kinase activity, is completely conserved. Yeast fructose-2,6-bisphosphatase contains a stretch of residues (231–246) very similar to the active site of the fructose-2,6-bisphosphatase domain of the rat liver enzyme and to yeast and human phosphoglycerate mutases (Bazan et al., 1989). This includes the active site histidine (His-232 in yeast fructose-2,6-bisphosphatase; His-258 in the rat liver enzyme) which acts as phosphoacceptor in the bisphosphatase reaction of the rat liver enzyme. Also, the other important histidine residue (His-367 in yeast fructose-2,6-bisphosphatase; His-392 in rat liver), thought to be a proton donor at the bisphosphatase site (Bazan et al., 1989; Tauler et al., 1990), is present.

No consensus phosphorylation sequence (RRXS) for cAMP-dependent phosphorylation is found in the entire yeast fructose-2,6-bisphosphatase sequence.

Yeast fructose-2,6-bisphosphatase and 6-phosphofructo-2-kinase share 42% identical amino acid residues. With 827 residues, the kinase is considerably longer than the bisphosphatase. Similarity between the two yeast sequences also stretches over the entire length coded for by *FBP26*. However,

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   1 ttcgacgcacttgtgagtacattgaaagtcaaagagaagttgacgatcgtttcaaagtca
  61 tatatgtatagttaaataacgtacttgctcttcgtacatgcccaagataaaaaaaaacta
 121 cacaaataaagctaataaaaatttacctaaagaaggatcatagattaagtggaaggaaac
 181 ttgtacataattcgagtttatttttcaactaagatatcaaggattgtttactataaaaaca
 241 cttaattgggtaatttctatttttttacttttttttctgtgttaaaaaaatgggtcgcgcca
 301 atgcgctcacaaatctattcttatccctaacttacatctacatgtaaagcgccactaaga
                                  start of deletion ⟶
 361 gagctatagcttatcatacatttacatcaggtaaagtatcatttttggccagtggatacga
 421 atttaatatataatctttgctttcccttcgctcttaaattcaccaagcaagtttgttaag
 481 aaataaaggaaaggggctcaaaaaccgaatcaagtaagacaagaaac

 529 ATGGGGTACAGTACTATTTCCAACGATAATGATATCAAAGTATGTGTGATAATGGTTGGC
     METGlyTyrSerThrIleSerAsnAspAsnAspIleLysValCysValIleMETValGly 20

 589 CTACCAGCTAGAGGAAAGTCTTTTATTTCCCAAAAAATTATCAGGTACTTATCGTGGTTA
     LeuProAlaArgGlyLysSerPheIleSerGlnLysIleIleArgTyrLeuSerTrpLeu 40

 649 TCCATAAAAGCCAAGTGTTTTAATGTGGGAAATTACAGAAGAGACGTGAGTGGAAATGTC
     SerIleLysAlaLysCysPheAsnValGlyAsnTyrArgArgAspValSerGlyAsnVal 60

 709 CCAATGGATGCTGAGTTTTTTAACTTCGAAAATACAGATAATTTTAAACTCAGAGAATTG
     ProMETAspAlaGluPhePheAsnPheGluAsnThrAspAsnPheLysLeuArgGluLeu 80

 769 GCTGCCCAAAATGCCATAAAAGATATTGTTAATTTTTTTACTAAAGAAGACGGATCTGTG
     AlaAlaGlnAsnAlaIleLysAspIleValAsnPhePheThrLysGluAspGlySerVal 100

 829 GCAGTTTTCGATGCTACTAATAGTACACGTAAAAGAAGGAAATGGCTTAAAGATATATGT
     AlaValPheAspAlaThrAsnSerThrArgLysArgArgLysTrpLeuLysAspIleCys 120

 889 GAAAAGAATAATATTCAACCGATGTTTTTAGAGAGCTGGAGTAACGATCATGAACTGATT
     GluLysAsnAsnIleGlnProMETPheLeuGluSerTrpSerAsnAspHisGluLeuIle 140

 949 ATAAATAACGCTAAGGATATCGGTAGCACATCTCCTGATTATGAAAACTCTGAACCTCAT
     IleAsnAsnAlaLysAspIleGlySerThrSerProAspTyrGluAsnSerGluProHis 160

1009 GTGGCGGAAGCTGATTTTTTGGAAAGAATTAGACAATATGAAAGATTTTATGAACCTTTG
     ValAlaGluAlaAspPheLeuGluArgIleArgGlnTyrGluArgPheTyrGluProLeu 180

1069 GACCCCCAAAAAGATAAGGATATGACGTTCATTAAGTTGGTCAATATTATTGAACAAGTA
     AspProGlnLysAspLysAspMETThrPheIleLysLeuValAsnIleIleGluGlnVal 200

1129 GTAATTAATAAGATCAGAACATATTTGGAAAGTAGGATTGTATTTTATGTTATGAATATT
     ValIleAsnLysIleArgThrTyrLeuGluSerArgIleValPheTyrValMETAsnIle 220

1189 CGTCCTAAACCAAAATATATCTGGCTCTCCCGTCACGGCGAATCGATCTATAACGTAGAG
     ArgProLysProLysTyrIleTrpLeuSerArgHisGlyGluSerIleTyrAsnValGlu 240

1249 AAAAAAATTGGCGGGGATTCATCACTGTCTGAAAGAGGCTTTCAGTACGCTAAAAAATTG
     LysLysIleGlyGlyAspSerSerLeuSerGluArgGlyPheGlnTyrAlaLysLysLeu 260

1309 GAGCAGTTAGTGAAAGAGAGCGCAGGAGAAATAAATTTGACCGTGTGGACTTCCACCTTA
     GluGlnLeuValLysGluSerAlaGlyGluIleAsnLeuThrValTrpThrSerThrLeu 280

1369 AAAAGAACACAACAAACGGCAAATTATCTTCCCTATAAGAAACTGCAATGGAAAGCACTT
     LysArgThrGlnGlnThrAlaAsnTyrLeuProTyrLysLysLeuGlnTrpLysAlaLeu 300
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1429 GATGAATTAGACGCTGGCGTTTGTGACGGAATGACGTATGAGGAAATTGAAAAAGAATAT
     AspGluLeuAspAlaGlyValCysAspGlyMETThrTyrGluGluIleGluLysGluTyr 320

1489 CCTGAAGATTTTAAAGCACGTGATAATGACAAATACGAGTACAGATATCGTGGTGGAGAA
     ProGluAspPheLysAlaArgAspAsnAspLysTyrGluTyrArgTyrArgGlyGlyGlu 340

1549 TCATACAGAGATGTAGTGATTCGTTTAGAGCCCGTCATTATGGAATTGGAGCGCCAAGAA
     SerTyrArgAspValValIleArgLeuGluProValIleMETGluLeuGluArgGlnGlu 360

1609 AATGAACTCATTATAACTCATCAAGCCGTACTTCGGTGTATATATGCATATTTTATGAAC
     AsnGluLeuIleIleThrHisGlnAlaValLeuArgCysIleTyrAlaTyrPheMETAsn 380

1669 GTTCCACAGGAGGAATCCCCTTGGATGTCAATCCCACTACACACATTGATCAAGCTGGAG
     ValProGlnGluGluSerProTrpMETSerIleProLeuHisThrLeuIleLysLeuGlu 400
                    ←——┐ end of deletion
1729 CCTAGGGCCTATGGCACAAAGGTCACCAAAATTAAAGCAAACATCCCTGCAGTGAGTACA
     ProArgAlaTyrGlyThrLysValThrLysIleLysAlaAsnIleProAlaValSerThr 420

1789 TATAAAGAGAAGGGTACAAGCCAAGTAGGTGAGCTTTCTCAAAGCTCAACTAAACTTCAT
     TyrLysGluLysGlyThrSerGlnValGlyGluLeuSerGlnSerSerThrLysLeuHis 440

1849 CAACTGCTCAATGACTCTCCTTTCCAAGACAAATTTtaa
     GlnLeuLeuAsnAspSerProPheGlnAspLysPhe***

1888 attttacttttttatttttgtgtatcttaagtccaagcttacttgtggctgatacgcacaa
1948 tcttttatgtacgaaaagatcaggaaacgtagcattgatggttttttatgtacgtctgca
2008 gcattgtatgtaattattacccaaactaattttaagtgaaattgtagaacgccacggcgg
2068 aagtatttttgagtaaaaaaaaaaaagtaagattcgaaaatgaaattatacagtacagaa
2128 aacgtaaatatacaataaaggttgaataaacatactagatatcctcctttcttttgcat
2188 gtacatagtactggtgtaaactcgatataccgatggtcagatttttggtttaaacaaga
2248 aaaagaacgaagaaaaggaaaatacagacttgcctgcagacaatgaacaaaacgcagcag
2308 aaacgtcgtctagcaacgtatctggaaatgaagaaagaatagacccaaacagtcatgata
2368 cgaaccctgaaaatgcaaacaatgatgatgcgtctacgacttttggttcgtccataccat
2428 cgtcatcctatattctaga
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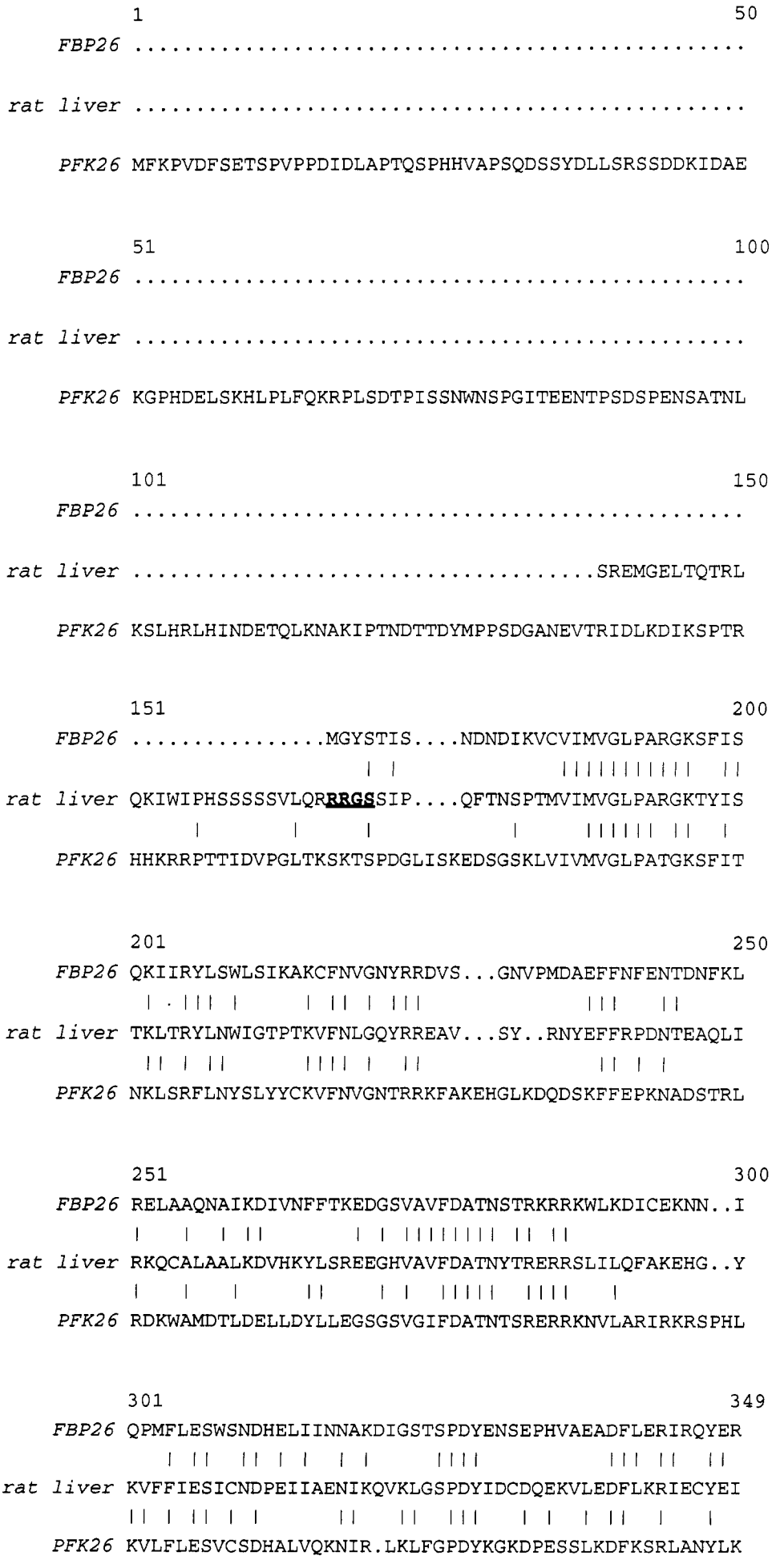
FIGURE 1: Nucleotide sequence of the FBP26 gene and deduced amino acid sequence. Indicated are a TATA-like sequence, possible transcription termination signals, and the *Bal*I and *Bst*EII sites used for the construction of the null mutant.

yeast 6-phosphofructo-2-kinase is missing the key histidine (His-258 in rat liver), which represents the phosphoacceptor in the bisphosphatase reaction, and it does possess a consensus sequence RRYS for cAMP-dependent phosphorylation.

Localization and Gene Disruption of FBP26. The yeast *FBP26* gene is located 450 bp downstream of *VPS35*, and the *VPS35* gene is situated 350 bp downstream of *INO1*, which has previously been mapped to the left arm of chromosome X. This assigns *FBP26* also to chromosome X of *S. cerevisiae*.

To examine the phenotype of a *fbp26* null mutant, we deleted all but the C-terminal 134 bp of the *FBP26* gene from the chromosome. The 1.4 kbp *Bal*I/*Bst*EII *FBP26* fragment was replaced by a 1.4 kbp *Xho*I/*Bam*HI *HIS3* fragment (Figure 3), and a linear 3 kb *Bam*HI/*Xba*I fragment containing the deletion was used to transform the haploid wild-type strain SEY6211. His^+ transformants were subjected to Southern blot analysis to confirm the deletion of the *FBP26* gene from the chromosome (data not shown). The viability of the haploid transformant indicated that *FBP26* is not an essential gene in yeast. The *fbp26* null mutant was tested for growth under different conditions. *fbp26*::*HIS3* cells were restreaked on rich medium R61 (Fraenkel, 1986) containing glucose, fructose, galactose, pyruvate, and glycerol/lactate, respectively, and incubated at 30 and 37 °C. Under all of the conditions examined, growth of the *fbp26*::*HIS3* cells was indistinguishable from that of the wild-type cells. The same result was found for cells carrying both, *fbp26*::*HIS3* and *pfk26*::*LEU2*, mutations.

Eznyme Activities. The presence of His-232 in the *FBP26* gene product suggested that the putative polypeptide might act in analogy to the bifunctional mammalian enzyme as a fructose-2,6-bisphosphatase via a phosphohistidine intermediate. Indeed, extracts from wild-type strains carrying the *FBP26* gene showed a characteristic band of a 56-kDa ^{32}P-labeled polypeptide after incubation with [2-^{32}P]fructose 2,6-P_2 (Figure 4, second lane). The size of 56 kDa on SDS–PAGE fits well with the predicted molecular mass of 53.5 kDa for the *FBP26* gene product and the previously reported size for a peptide labeled under the same conditions in yeast extracts which copurified with a low-K_m fructose-2,6-bisphosphatase (François et al., 1988). In a chromosomal mutant carrying the *fbp26*::*HIS3* allele, this band was not detectable, with fructose 2,6-P_2 hydrolysis reduced to 10–25% of wild-


1 50
FBP26 .....
rat liver .....
PFK26 MFKPVDFSETSPVPPDIDLAPTQSPHHVAPSQDSSYDLLSRSSDDKIDAE
51 100
FBP26 .....
rat liver .....
PFK26 KGPHDELSKHLPLFQKRPLSDTPISSNWNSPGITEENTPSDSPENSATNL
101 150
FBP26 .....
rat liver .....SREMGELTQTRL
PFK26 KSLHRLHINDETQLKNAKIPTNDTTDYMPPSDGANEVTRIDLKDIKSPTR
151 200
FBP26 .....MGYSTIS....NDNDIKVCVIMVGLPARGKSFIS
rat liver QKIWIPHSSSSSVLQRRRGSSIP....QFTNSPTMVIMVGLPARGKTYIS
PFK26 HHKRRPTTIDVPGLTKSKTSPDGLISKEDSGSKLVIVMVGLPATGKSFIT
201 250
FBP26 QKIIRYLSWLSIKAKCFNVGNYRRDVS...GNVPMDAEFFNFENTDNFKL
rat liver TKLTRYLNWIGTPTKVFNLGQYRREAV...SY..RNYEFFRPDNTEAQLI
PFK26 NKLSRFLNYSLYYCKVFNVGNTRRKFAKEHGLKDQDSKFFEPKNADSTRL
251 300
FBP26 RELAAQNAIKDIVNFFTKEDGSVAVFDATNSTRKRRKWLKDICEKNN..I
rat liver RKQCALAALKDVHKYLSREEGHVAVFDATNYTRERRSLILQFAKEHG..Y
PFK26 RDKWAMDTLDELLDYLLEGSGSVGIFDATNTSRERRKNVLARIRKRSPHL
301 349
FBP26 QPMFLESWSNDHELIINNAKDIGSTSPDYENSEPHVAEADFLERIRQYER
rat liver KVFFIESICNDPEIIAENIKQVKLGSPDYIDCDQEKVLEDFLKRIECYEI
PFK26 KVLFLESVCSDHALVQKNIR.LKLFGPDYKGKDPESSLKDFKSRLANYLK

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          350                                                 397
FBP26     FYEPLDPQKDKDMTFIKLVNIIEQVVINKIRTYLESRIVFYVMNIRPKPK
rat liver NYQPLDEELDSHLSYIKIFDVGTRYMVNRVQDHVQSRTAYYLMNIHVTPR
PFK26     AYEPIED..DENLQYIKMIDVGKKVIAYNIQGFLRSQTVYYLLNFNLADR

          398                                                 447
FBP26     YIWLSRHGESIYNVEKKIGGDSSLSERGFQYAKKLEQLVKESAGEI....
rat liver SIYLCRHGESELNLRGRIGGDSGLSARGKQYAYALANFIR.SQGIS....
PFK26     QIWITRSGESEDNVSGRIGGNSHLTPRGLRFAKSLPKFIARQREIFYQNL

          448                                                 497
FBP26     ..............NLTVWTSTLKRTQQTANYL...PYKKLQWKALDE
rat liver ..............SLKVWTSHMKRTIQTAEAL...GVPYEQWKALNE
PFK26     MQQKKNNENTDGNIYNDFFVWTSMRARTIGTAQYFNEDDYPIKQMKMLDE

          498                                                 547
FBP26     LDAGVCDGMTYEEIEKEYPEDFKARDNDKYEYRYR..GGESYRDVVIRLE
rat liver IDAGVCEEMTYEEIQEHYPEEFALRDQDKYRYRYP..KGESYEDLVQRLE
PFK26     LSAGDYDGMTYPEIKNNFPEEFEKRQKDKLRYRYPGIGGESYMDVINRLR

          548                                                 597
FBP26     PVIMELER.QENELIITHQAVLRCIYAYFMNVPQEESPWMSIPLHTLIKL
rat liver PVIMELER.QENVLVICHQAVMRCLLAYFLDKSSDELPYLKCPLHTVLKL
PFK26     PVITELERIEDNVLIITHRVVARALLGYFMNLSMGIIANLDVPLHCVYCL

          598                                                 646
FBP26     EPRAYGTKVTKIKANIPAVSTYKEKGTSQVGELSQSSTKL.........
rat liver TPVAYGCRVESIYLNVEAVNTHRDK.PENVDITREAEEAL.........
PFK26     EPKPYGI.TWSLWEYDEASDSFSKVPQTDLNTTRVKEVGLVYNERRYSVI
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                647                                                 696
    FBP26 ..HQLLNDSPFQDKF.....................................

rat liver ..DTVPAHY.....................................

    PFK26 PTAPPSARSSFASDFLSRKRSNPTSASSSQSELSEQPKNSVSAQTGSNNT


                697                                                 746
    FBP26 .....................................................

rat liver .....................................................

    PFK26 TLIGSNFNIKNENGDSRIPLSAPLMATNTSNNILDGGGTSISIHRPRVVP


                747                                                 796
    FBP26 .....................................................

rat liver .....................................................

    PFK26 NQNNVNPLLANNNKAASNVPNVKKSAATPRQIFEIDKVDEKLSMLKNKSF


                797                         827
    FBP26 ...........................

rat liver ...........................

    PFK26 LLHGKDYPNNADNNDNEDIRAKTMNRSQSHV
```

FIGURE 2: Alignment of the amino acid sequences yeast fructose-2,6-bisphosphatase, rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, and yeast 6-phosphofructo-2-kinase. Identical amino acids are indicated by vertical bars between the residues. Dashes indicate gaps introduced to allow maximum similarity. Histidines known to be essential for fructose-2,6-bisphosphatase activity of the rat liver enzyme and consensus sites for cAMP-dependent phosphorylation are emphasized by enlarged and bold letters, respectively. Numbers correspond to amino acids in the largest polypeptide, yeast 6-phosphofructo-2-kinase (827 amino acids).

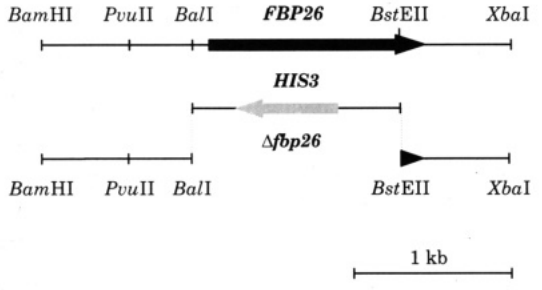

FIGURE 3: Construction of a chromosomal null mutant. Replacement of a 1.4 kbp *Bal*I/*Bst*EII fragment containing *FBP26* by a 1.4 kbp *Xho*I/*Bam*HI fragment containing the *HIS3* gene. Black and hatched arrows indicate *FBP26* and *HIS3*, respectively, and their direction.

type level (Figure 4, first and third lanes vs second lane), but reemerged ca. 2.5-fold stronger than wild-type when the *FBP26* gene was introduced on multicopy (Figure 4, lane 4). The ratio of phosphoprotein formation in these extracts compared well with that of fructose-2,6-bisphosphatase assayed as release of phosphate from [2-^{32}P]fructose 2,6-P_2.

What about 6-phosphofructo-2-kinase activity—a particularly interesting question considering that the *pfk26* null mutant turned out to contain substantial fructose 2,6-P_2 in its growth on glucose and still contains a separable 2-kinase in 5% amount. As shown for the null mutant background, *FBP26* on a multicopy plasmid gave no detectable increase in the 2-kinase (Figure 4, compare activities for lane 4 with those of lanes 2 and 3), and no increase was seen in a *PFK26* strain either (data not shown). Furthermore, a double mutant, *fbp26*::*HIS3*, *pfk26*::*LEU2*, harvested from growth in medium R61 (Fraenkel, 1986) with 2% glucose at A_{580} of 4, contained fructose 2,6-P_2 at 3 μM, about the same concentration reported for the single *pfk26* mutant. And like the latter strain, the double mutant still contains the marginal 5% 2-kinase activity.

These results show that the new gene does not contribute detectable 6-phosphofructo-2-kinase activity and specifies fructose-2,6-bisphosphatase. It is for this reason we have named it *FBP26*.

DISCUSSION

The present work identifies the *FBP26* gene as coding for the only protein in yeast which is labeled upon incubation of cell extracts with [2-^{32}P]fructose 2,6-P_2. This distinctive

Strain:	DFY655 *PFK26* *fbp26*	DFY653 *pfk26* *FBP26*	DFY657 *pfk26* *fbp26*	DFY657 *pfk26* *fbp26*
Plasmid:	-	-	YEp352	pBHY10(*FBP26*)
Pfk-2 [μU/mg]	70 ± 7	<5	<5	<5
Fbp-2 [μU/mg]	0.2 ± 0.1	1.6 ± 0.3	0.4 ± 0.2	3.0 ± 0.4
Phosphate in 56 kDa band [pmol/mg]	0	0.064 ± 0.016	0	0.160 ± 0.016

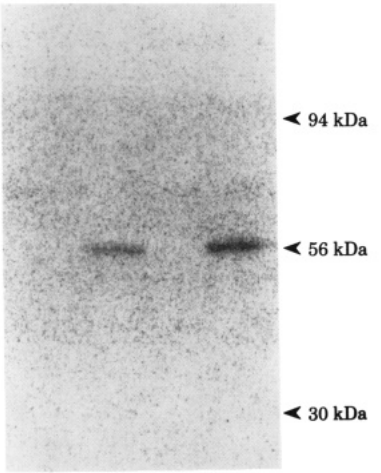

FIGURE 4: Enzyme activities and protein labeling with [2-^{32}P]fructose 2,6-P_2 in extracts. 6-Phosphofructo-2-kinase activity, microunits per milligram of protein ("Pfk-2, μU/mg"), fructose-2,6-bisphosphatase ("Fbp-2, μU/mg"), and labeling ("Phosphate in 56 kDa band, pmol/mg") were assayed as described under Materials and Methods and related to extract protein. The top and bottom arrows on the autoradiogram denote the positions of molecular mass markers on the stained SDS–PAGE gel: phosphorylase (94 kDa) and carboanhydrase (30 kDa).

attribute matches the previously reported high-specificity fructose-2,6-bisphosphatase, characterized by high substrate specificity and a low K_m value for its substrate [0.1–0.3 μM (Kretschmer et al., 1987; François et al., 1988)]. It hydrolyzes fructose 2,6-P_2 to fructose 6-P and P_i and can be separated from the 96-kDa phosphofructo-2-kinase of high specific activity, which is coded for by the *PFK26* gene (Kretschmer & Fraenkel, 1991). The subunit size of yeast fructose-2,6-bisphosphatase (56 kDa in SDS–PAGE; 53.5 kDa as predicted from the open reading frame) corresponds to the size of the mammalian bifunctional enzymes (Lively et al., 1988; Darville et al., 1987; Sakata & Uyeda, 1990). The amino acid sequence has no obvious consensus sequence for cAMP-dependent phosphorylation and shows 45% similarity with the bifunctional rat liver enzyme and 42% similarity with 480 amino acids in the central region of the recently reported 93.5-kDa subunit of yeast 6-phosphofructo-2-kinase. However, the 56-kDa yeast enzyme has no significant 6-phosphofructo-2-kinase activity: it can be separated from 6-phosphofructo-2-kinase on Sephacryl-blue (Kretschmer et al., 1987; François et al., 1988), and its overproduction from a 2μ plasmid, while increasing fructose-2,6-bisphosphatase activity 15-fold over the *fbp26* mutant level, has no influence on 6-phosphofructo-2-kinase activity in yeast extracts, nor does its loss.

Thus, just as *PFK26* specifies the major 6-phosphofructo-2-kinase of yeast—an enzyme which apparently does not also have fructose-2,6-bisphosphatase activity—the new gene, *FBP26*, specifies a fructose-2,6-bisphosphatase which analogously does not have 2-kinase activity. Interestingly, considering the respective single and double mutant strains, the two gene products cannot be the only ones for synthesis and degradation of fructose 2,6-P_2 in vivo in *S. cerevisiae*.

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