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Yeast 6-Phosphofructo-2-kinase: Sequence and Mutant[†,‡]

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ABSTRACT: We have reported yeast 6-phosphofructo-2-kinase (EC 2.7.1.105) as having a ca. 96-kDa subunit size, as well as isolation of its structural gene, *PFK26*. Sequencing now shows an open reading frame of 827 amino acids and 93.5 kDa. The deduced amino acid sequence has 42% identity with the 55-kDa subunit of the bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from rat liver with extra material at both ends. Although the yeast sequence is especially similar to the liver one in its bisphosphatase domain, the essential His-258 of the liver enzyme is, in yeast, a serine, which may explain the apparent lack of bisphosphatase activity. Also, the yeast enzyme, known to be activated via protein kinase A, has a putative phosphorylation site near its C-terminus and lacks the N-terminal phosphorylation sequence involved in inhibition of the liver enzyme. In a chromosomal null mutant strain, *pfk26*::*LEU2*, activity was marginal and the protein was not detectable as antigen. The mutant strain grew well on glucose and contained a near-normal level of fructose 2,6-P_2. But in its growth on pyruvate, by contrast with the wild-type strain, no fructose 2,6-P_2 was detectable, and it did not form after glucose addition in the presence of cycloheximide either. Such resting cells, however, metabolized glucose at the normal high rate. Glucose addition to the *pfk26* mutant strain in the absence of cycloheximide, on the other hand, caused a ca. 10% normal rate of fructose 2,6-P_2 accumulation, presumably employing a glucose-inducible second enzyme. Using strains also lacking 6-phosphofructo-1-kinase, affinity chromatography revealed the second enzyme as a minor peak amounting to 6% of 6-phosphofructo-2-kinase activity in a *PFK26* strain and as the sole peak, in similar amount, in a *pfk26* mutant strain.

Fructose 2,6-bisphosphate (fructose 2,6-P_2) has a major role in carbohydrate metabolism in higher cells as activator 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 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) (Pilkis et al., 1987). In liver, muscle, and heart, both activities reside on a bifunctional protein of low specific activity; in liver this enzyme is subject to cAMP-dependent phosphorylation, which causes inhibition of the kinase and activation of the phosphatase activities (Van Schaftingen & Hers, 1982).

Saccharomyces cerevisiae also contains fructose 2,6-P_2, an activator in vitro of its 6-phosphofructo-1-kinase and inhibitor of its fructose 1,6-bisphosphatase (Kessler et al., 1988; Lederer et al., 1981; Clifton & Fraenkel, 1983). 6-Phosphofructo-2-kinase in yeast differs from the mammalian enzymes in apparently not being bifunctional (François et al., 1988; Kretschmer et al., 1987), having a larger subunit size (96 kDa instead of 55 kDa), likely having a much higher catalytic rate, and being present in minute amount ($<10^{-5}$ of cell protein) (Kretschmer et al., 1991). Also, the yeast enzyme is activated by protein kinase A (François et al., 1988), in this respect resembling the enzyme from heart (Sakata et al., 1990). Cloning employed mixed oligonucleotide probes to tryptic peptide sequences, and the gene in multicopy overproduced both enzyme activity and antigen (Kretschmer et al., 1991).

Here we describe (i) the DNA sequence coding for this gene (*PFK26*), (ii) a chromosomal deletion mutant, (iii) a situation of rapid glucose metabolism in the absence of fructose 2,6-P_2, and (iv) evidence for a second, minor, 6-phosphofructo-2-kinase activity apparently induced in growth on glucose.

Materials and Methods

Materials. Fructose 6-P, ATP, and most enzymes were from Boehringer (Mannheim, Germany). Fructose 2,6-P_2,

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[‡]The nucleotide sequence reported in this paper has been submitted to GenBank under Accession Number J05351.

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   1 tcttattgtttggccgggacttatatgactgctttcaaaaggtaactcatacccagtttg
  61 ttaataaaagatgaacaataatacttgaagtagaaaagaaaatagcaacatacaaatttt
 121 gtagaaaagacatggaaattgaagtccttattgaattaggaaaattttgttgaagatact
 181 aacaaaaaaaccggttttctctcgaataaactaaaaaaatatctatacagctcaaacggg
 241 ctgagctaaggctcttttagatcagtaatgttaccaaagctataattgtaattcttctaa
 301 cgttttgatatttccattttgggaagtagtattacccttcttttgatcgaacgtatttgt
 361 catattatacgagtgggatacccgaccgataataataatcacgtttatatcacgtgatca
 421 tctctgaataccaaaatcacaacatacggaaattaggggacataagatttttgaggaga
 481 gatttcttttcctatttatttttaatcgcgccattactgacaatagcaaattaatttata
 541 gtggagtacatggactatttattatagattttgttggcatattctttttttaacaataaa
 601 aggtatacatatatatatatagacaaactcaagtattcagttacgggtaaatcaagtgaa
 661 ataaatttcgccttttagtatctatttgtgtacttttgtgccataagctaaaagagtaaa
 721 aataataacaagaggcgtagaagaagggtttattcaagggaacaaaggcaagaggaataa

 781 aagctcATGTTCAAACCAGTAGACTTCTCTGAAACATCTCCTGTGCCGCCTGATATTGAT
           METPheLysProValAspPheSerGluThrSerProValProProAspIleAsp 18

 841 CTTGCTCCTACACAATCTCCACACCATGTGGCACCTAGTCAAGACTCCAGTTATGATCTT
     LeuAlaProThrGlnSerProHisHisValAlaProSerGlnAspSerSerTyrAspLeu 38

 901 TTATCCCGGAGTTCCGATGATAAAATTGATGCTGAAAAGGGTCCGCATGATGAATTATCT
     LeuSerArgSerSerAspAspLysIleAspAlaGluLysGlyProHisAspGluLeuSer 58

 961 AAGCACTTACCACTTTTTCAGAAAAGACCTTTGAGCGATACTCCTATATCGAGCAATTGG
     LysHisLeuProLeuPheGlnLysArgProLeuSerAspThrProIleSerSerAsnTrp 78

1021 AACTCTCCTGGAATCACTGAAGAAAATACACCTTCTGACTCTCCTGAAAATAGCGCTACT
     AsnSerProGlyIleThrGluGluAsnThrProSerAspSerProGluAsnSerAlaThr 98

1081 AATTTGAAATCGCTACATCGATTGCATATTAACGACGAAACGCAACTAAAAAATGCTAAA
     AsnLeuLysSerLeuHisArgLeuHisIleAsnAspGluThrGlnLeuLysAsnAlaLys 118

1141 ATTCCCACAAACGATACTACTGACTACATGCCTCCTTCAGATGGAGCAAATGAGGTAACT
     IleProThrAsnAspThrThrAspTyrMETProProSerAspGlyAlaAsnGluValThr 138

1201 CGGATTGATTTGAAAGACATTAAATCACCTACGAGACACCATAAAAGAAGACCTACCACC
     ArgIleAspLeuLysAspIleLysSerProThrArgHisHisLysArgArgProThrThr 158

1261 ATCGATGTTCCTGGTTTAACAAAGTCTAAAACATCTCCAGATGGTCTCATATCAAAGGAA
     IleAspValProGlyLeuThrLysSerLysThrSerProAspGlyLeuIleSerLysGlu 178

1321 GATAGTGGATCAAAGTTAGTGATTGTCATGGTCGGACTGCCAGCTACGGGAAAGTCATTT
     AspSerGlySerLysLeuValIleValMETValGlyLeuProAlaThrGlyLysSerPhe 198

1381 ATTACAAATAAATTATCCAGATTTTTAAATTATTCTTTATACTATTGTAAAGTGTTTAAT
     IleThrAsnLysLeuSerArgPheLeuAsnTyrSerLeuTyrTyrCysLysValPheAsn 218

1441 GTCGGTAACACTAGAAGGAAGTTTGCTAAGGAGCATGGCCTAAAGGACCAGGATTCAAAG
     ValGlyAsnThrArgArgLysPheAlaLysGluHisGlyLeuLysAspGlnAspSerLys 238
                                   start of NcoI deletion ⟶
1501 TTTTTCGAGCCGAAAAACGCCGACTCTACTAGGTTGAGAGACAAATGGGCCATGGATACT
     PhePheGluProLysAsnAlaAspSerThrArgLeuArgAspLysTrpAlaMETAspThr 258

1561 CTGGATGAATTGCTAGATTATTTATTAGAAGGTTCAGGATCTGTGGGAATTTTTGATGCT
     LeuAspGluLeuLeuAspTyrLeuLeuGluGlySerGlySerValGlyIlePheAspAla 278
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1621 ACAAATACCTCTCGTGAAAGAAGAAAAAACGTTCTGGCTAGAATCAGAAAGAGAAGTCCT
     ThrAsnThrSerArgGluArgArgLysAsnValLeuAlaArgIleArgLysArgSerPro 298

1681 CATTTGAAGGTTTTATTTTTAGAATCTGTTTGTTCGGATCATGCACTGGTACAGAAAAAT
     HisLeuLysValLeuPheLeuGluSerValCysSerAspHisAlaLeuValGlnLysAsn 318

1741 ATTAGACTCAAATTATTTGGTCCAGATTACAAAGGTAAAGATCCTGAAAGCTCTTTAAAA
     IleArgLeuLysLeuPheGlyProAspTyrLysGlyLysAspProGluSerSerLeuLys 338

1801 GATTTTAAAAGTCGCCTGGCAAACTACTTGAAAGCCTATGAACCAATTGAGGATGACGAA
     AspPheLysSerArgLeuAlaAsnTyrLeuLysAlaTyrGluProIleGluAspAspGlu 358

1861 AATTTGCAGTACATCAAAATGATAGATGTGGGAAAGAAAGTCATCGCATACAATATTCAA
     AsnLeuGlnTyrIleLysMETIleAspValGlyLysLysValIleAlaTyrAsnIleGln 378

1921 GGGTTTTTACGTTCGCAGACGGTATATTATTTGTTAAATTTCAATTTGGCTGACAGACAA
     GlyPheLeuArgSerGlnThrValTyrTyrLeuLeuAsnPheAsnLeuAlaAspArgGln 398

1981 ATTTGGATAACGAGAAGTGGCGAGAGCGAAGATAATGTTAGTGGCCGTATAGGCGGAAAT
     IleTrpIleThrArgSerGlyGluSerGluAspAsnValSerGlyArgIleGlyGlyAsn 418

2041 TCCCATTTGACTCCTCGTGGTCTAAGATTTGCTAAAAGTCTACCAAAATTCATTGCCAGA
     SerHisLeuThrProArgGlyLeuArgPheAlaLysSerLeuProLysPheIleAlaArg 438

2101 CAGAGAGAAATATTTTATCAAAATCTCATGCAACAAAAAAAGAATAATGAAAATACAGAT
     GlnArgGluIlePheTyrGlnAsnLeuMETGlnGlnLysLysAsnAsnGluAsnThrAsp 458

2161 GGGAACATTTATAATGACTTTTTCGTTTGGACCAGCATGCGTGCTAGGACTATAGGGACT
     GlyAsnIleTyrAsnAspPhePheValTrpThrSerMETArgAlaArgThrIleGlyThr 478

2221 GCTCAATATTTCAACGAAGATGATTATCCTATCAAACAAATGAAAATGTTAGATGAGTTA
     AlaGlnTyrPheAsnGluAspAspTyrProIleLysGlnMETLysMETLeuAspGluLeu 498

2281 AGTGCAGGTGATTATGATGGTATGACATATCCAGAAATTAAAAACAACTTTCCTGAAGAA
     SerAlaGlyAspTyrAspGlyMETThrTyrProGluIleLysAsnAsnProGluGlu 518

2341 TTCGAAAAAAAGACAGAAAGATAAGTTGAGATACAGATACCCTGGTATTGGCGGTGAATCG
     PheGluLysArgGlnLysAspLysLeuArgTyrArgTyrProGlyIleGlyGlyGluSer 538

2401 TATATGGACGTTATTAATAGACTCAGACCTGTTATCACAGAACTAGAAAGAATCGAGGAT
     TyrMETAspValIleAsnArgLeuArgProValIleThrGluLeuGluArgIleGluAsp 558

2461 AACGTTCTTATTATTACACACCGGGTGGTGGCAAGAGCCTTATTGGGTTATTTTATGAAC
     AsnValLeuIleIleThrHisArgValValAlaArgAlaLeuLeuGlyTyrPheMETAsn 578

2521 TTGAGTATGGGTATTATTGCCAATTTGGATGTCCCATTACATTGTGTATATTGCCTAGAA
     LeuSerMETGlyIleIleAlaAsnLeuAspValProLeuHisCysValTyrCysLeuGlu 598

2581 CCAAAACCATATGGAATCACTTGGTCATTATGGGAGTATGATGAAGCATCGGATTCATTT
     ProLysProTyrGlyIleThrTrpSerLeuTrpGluTyrAspGluAlaSerAspSerPhe 618

2641 TCTAAGGTCCCACAAACGGACTTGAATACCACCAGAGTAAAGGAGGTTGGCCTTGTTTAT

2701 AATGAAAGAAGATATTCTGTTATACCAACAGCTCCGCCAAGTGCAAGAAGCAGCTTTGCA
     AsnGluArgArgTyrSerValIleProThrAlaProProSerAlaArgSerSerPheAla 658
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2761 AGTGACTTTTTGTCAAGAAAAAGATCTAATCCTACTTCTGCATCTTCATCCCAGAGTGAA
     SerAspPheLeuSerArgLysArgSerAsnProThrSerAlaSerSerSerGlnSerGlu 678

2821 TTATCAGAACAACCCAAGAATAGCGTTAGTGCTCAAACTGGCAGCAATAATACCACTCTC
     LeuSerGluGlnProLysAsnSerValSerAlaGlnThrGlySerAsnAsnThrThrLeu 698

2881 ATTGGGAGCAACTTTAACATCAAGAATGAAAATGGTGATTCGAGAATACCATTATCTGCA
     IleGlySerAsnPheAsnIleLysAsnGluAsnGlyAspSerArgIleProLeuSerAla 718

2941 CCACTTATGGCCACTAATACTTCTAATAACATCTTAGATGGTGGAGGTACCTCAATTTCG
     ProLeuMETAlaThrAsnThrSerAsnAsnIleLeuAspGlyGlyGlyThrSerIleSer 738

3001 ATACATCGTCCCAGGGTTGTTCCAAATCAAAACAACGTGAATCCTCTTTTGGCTAACAAC
     IleHisArgProArgValValProAsnGlnAsnAsnValAsnProLeuLeuAlaAsnAsn 758

3061 AATAAAGCGGCTTCTAATGTACCTAATGTAAAGAAGTCAGCGGCTACACCAAGGCAAATT
     AsnLysAlaAlaSerAsnValProAsnValLysLysSerAlaAlaThrProArgGlnIle 778

3121 TTTGAAATAGATAAAGTGGACGAAAAGTTATCCATGTTGAAAAATAAAAGTTTTCTATTA
     PheGluIleAspLysValAspGluLysLeuSerMETLeuLysAsnLysSerPheLeuLeu 798

3181 CATGGAAAGGATTATCCTAATAATGCTGATAATAATGACAACGAAGATATAAGGGCAAAA
     HisGlyLysAspTyrProAsnAsnAlaAspAsnAsnAspAsnGluAspIleArgAlaLys 818

3241 ACCATGAATCGCAGCCAAAGTCACGTTtaaaataatatgaaaaaaaaaaaaaaaaaaaa
     ThrMETAsnArgSerGlnSerHisVal

3301 aatcagaaatatttTATGaattattcaaaTAGactaatgaagtgataaaaccaaccTTTt
3361 ttttcttctttaaggtatattgttcgacgcaatcatgaagtgttgcggtttttgttta
3421 tatttttctataactgttttccttttttcccaaagaattatgcaaatgaagaatatctacg
3481 tatatatggaaaataatttaatagatatattttgatatgactaagttatgaaatagaaaa
3541 aaataagaaaaaaaaaaccttgactaattactataaagctccgtaaataattccgaaggc
3601 gtcgacccattgaccgactctgccagtcacacctaggatatattggccattaggtggctg
3661 caattcagcgaacccacctccgtctttgttaccaaacatgtcggtcattctcccatgact
3721 cgttataatttggatagcatcaacccaagctccgcacctcagattaaacccaatgattat
3781 ttcacccggttctagtgtagcatccgtaaaattttgtgtttcatgtccaaagagtacact
3841 ttttagattttcttcgtttattgaagcatgatttttgatactatgtgtaatttttgagaa
3901 ataatttcttggtggtacagagggcttggatgcgggacgatctttgttccagttggtttt
                                    <---- end of NcoI deletion
3961 tccttgtaataaaacctcacaccatctaatgcatagCCATGGtatattctcacagcagtg
4021 actaatctaacatcaaaagcgacgacgccgacagcattaccaccgtcgctgcgaccataa
4081 agtggtgatttgataccttgaacatttttagaaaacccatatttcgacatgtctaatggc
4141 tgaacatctaacaaagatggg
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FIGURE 1: Nucleotide sequence of the *PFK26* gene and deduced amino acid sequence. The residues matching the previously reported tryptic peptides are indicated with underlined bold characters. Also indicated are a TATA-like sequence, possible transcription termination signals, and the two *Nco*I sites used for the construction of the null mutant.

pyrophosphate:fructose-6-P phosphotransferase, 4-chloro-1-naphthol, PEG_{6000}, and all other chemicals were from Sigma (St. Louis, MO). Bacto tryptone and yeast extract were from Difco (Detroit, MI), BA85 nitrocellulose (11.5 × 14.5 cm) was from Schleicher & Schuell (Keene, NH), RA 120 filters (4.5-cm diameter, pore size 120 μm) were from Millipore (Bedford, MA), and the biotinylated anti-mouse immunoglobulin G as well as avidin DH-biotinylated horseradish peroxidase H were from Vector Laboratories (Burlingame, CA). Prestained SDS–PAGE standards (nominal molecular masses according to manufacturer) were from BRL (Gaithersburg, MD). The Sequenase version 2.0 DNA sequencing kit was from U.S. Biochemical Corp. (Cleveland, OH), and the T4 DNA ligase was from Takara Biochemicals, Tokyo, Japan. ^{35}S-Deoxyadenosine 5′-α-thiotriphosphate was purchased from New England Nuclear (Boston, MA). Southern blot analysis was performed using the ECL gene detection kit (Amersham Corp., Arlington Heights, IL).

Electrophoresis and Immunoblotting. The procedure for SDS–PAGE with 10% acrylamide gels was according to

Laemmli (1970). Proteins were transferred electrophoretically onto nitrocellulose and 6-phosphofructo-2-kinase was visualized using mouse serum against the 96-kDa subunit protein as described by Kretschmer et al. (1991).

DNA Sequence Determination and Analysis. The *Bam*HI/*Hin*dIII fragment from clone pMK1 (Kretschmer et al., 1991) was subcloned [bacterial host HB101 (Bolivar & Backman, 1979)] as five partially overlapping fragments into the plasmids pT7/T3a-18 and pT7/T3a-19 (Bethesda Research Laboratories, Gaithersburg, MD), and 4.1 kbp was sequenced by the dideoxynucleotide method (Sanger et al., 1977) using Sequenase version 2. The 12 oligonucleotide primers were made on an Applied Biosystems 381A DNA synthesizer. Each subclone was sequenced in one direction only. Two-thirds of the open reading frame was later sequenced again, with no difference observed. Amino acid sequence alignment (Needleman & Wunsch, 1970) was done using the EuGene program (Department of Cell Biology, Baylor College of Medicine, Houston, TX) on a DEC 3100 multiuser system.

Disruption of PFK26. A 2.7-kbp *Nco*I fragment carrying the *LEU2* gene (Sedivy & Fraenkel, 1985) was introduced into partial *Nco*I digests of *Bam*HI/*Hin*dIII subclones of pMK1 (Kretschmer et al., 1991) and then ligated as *Bam*HI/*Hin*dIII fragments into a vector YEp13 (Broach et al., 1979) from which the *LEU2* gene had been dropped out by *Bgl*II digestion. The resulting plasmids pMK3-6 (Figure 3) were transformed into yeast strain DFY535 [a homozygous diploid, *leu2/leu2 his3/his3*, from strains T8-1C and T8-1D (Whiteway et al., 1987)]. The *Bam*HI/*Hin*dIII fragment of plasmid pMK4, which (see Figure 3) contained *pfk26*::*LEU2*, was used to transform DFY535 by one-step disruption (Rothstein, 1983). Leu$^+$ transformants were purified and segregants obtained. [A medium with pyruvate as carbon source was used in case mutants should prove to not grow on glucose; in fact, they did grow on glucose (see text).] The chromosomal disruptions were confirmed by gel transfer analysis using *Bgl*II/*Sal*I and *Bam*HI/*Xho*I digestion and with *Nco*I fragments of pMK1 as probe (data not shown). For metabolic studies, the pair of strains employed was DFY649 (a *leu2 his3*) and DFY650 (*α leu2 his3 pfk26*::*LEU2*). For enzymological studies (Figure 5), *pfk26*::*LEU2* was introduced into a strain lacking 6-phosphofructo-1-kinase by cross of DFY650 and DFY533 [*pfk1-1 pfk2-2 ade1 leu2* (Kretschmer et al., 1991)] and DFY651 (*pfk1-1 pfk2-2 pfk26*::*LEU2 ade1 leu2*) was a segregant. DFY533 and DFY651 were grown in enriched medium R61 (Fraenkel, 1985) supplemented with 1% pyruvate. At an A_{580} of 6, 1% glucose was added and the cells were harvested 30 min later. Pellets (11% PEG_{6000}) of total cell protein were prepared from 3 g of cells from each culture as described (Kretschmer et al., 1991). The PEG_{6000} pellets (each 60 mg of protein) were dissolved in 5 mL of 0.02 M Tris, pH 7.5, 0.3 M KCl, and 4 mM mercaptoethanol and applied onto 0.5-mL Sephacryl-blue S300 columns equilibrated with the same buffer at a flow rate of 0.048 mL/min. After a 6-mL wash, protein was eluted with the column buffer containing 2 M KCl. Fractions of 0.48 mL for the flowthrough/wash (tubes 1–23) and 0.085 mL during elution (tubes 24–50) were collected and assayed for protein and 6-phosphofructo-2-kinase activity.

Metabolite Concentrations. DFY649 (*leu2*) and DFY650 (*pfk26*::*LEU2*) were grown in enriched medium R61 containing 1% pyruvate to an A_{580} of 6. Portions of each culture received 50 μg/mL cycloheximide at −10 min. Glucose (1%) addition was at 0 min and samples were obtained for analysis (below) as indicated in Figure 6. (The 0-min sample was taken just before adding glucose.)

For glucose utilization in resting cells, the cells were harvested from growth on pyruvate (as above) at an A_{580} of 10, washed in M61 (Fraenkel, 1985), and incubated 1 h with shaking as 500-mL portions, A_{580} of 10, M61 containing 10 μg/mL cycloheximide and 2 μg/mL antimycin D. Then, 2% glucose was added and samples were obtained for assay of glucose in the medium and of metabolites in cell extracts (Table IB).

Analytical Methods. Four hundred twenty A_{580} amounts of cells were collected on Millipore RA 120 filters and frozen in liquid nitrogen (Clifton & Fraenkel, 1983). Fructose 2,6-P_2 was extracted at pH 10 and assayed as described (François et al., 1984). Glucose 6-P and fructose 6-P were assayed in the same samples (Banuelos & Fraenkel, 1982). Fructose 1,6-P_2 was assayed in separate samples using perchloric acid extraction (Banuelos & Fraenkel, 1982). Metabolite concentrations were assayed at least three times and related to nominal cell water volume (Clifton & Fraenkel, 1983), assuming cell water to be 0.6 times wet weight.

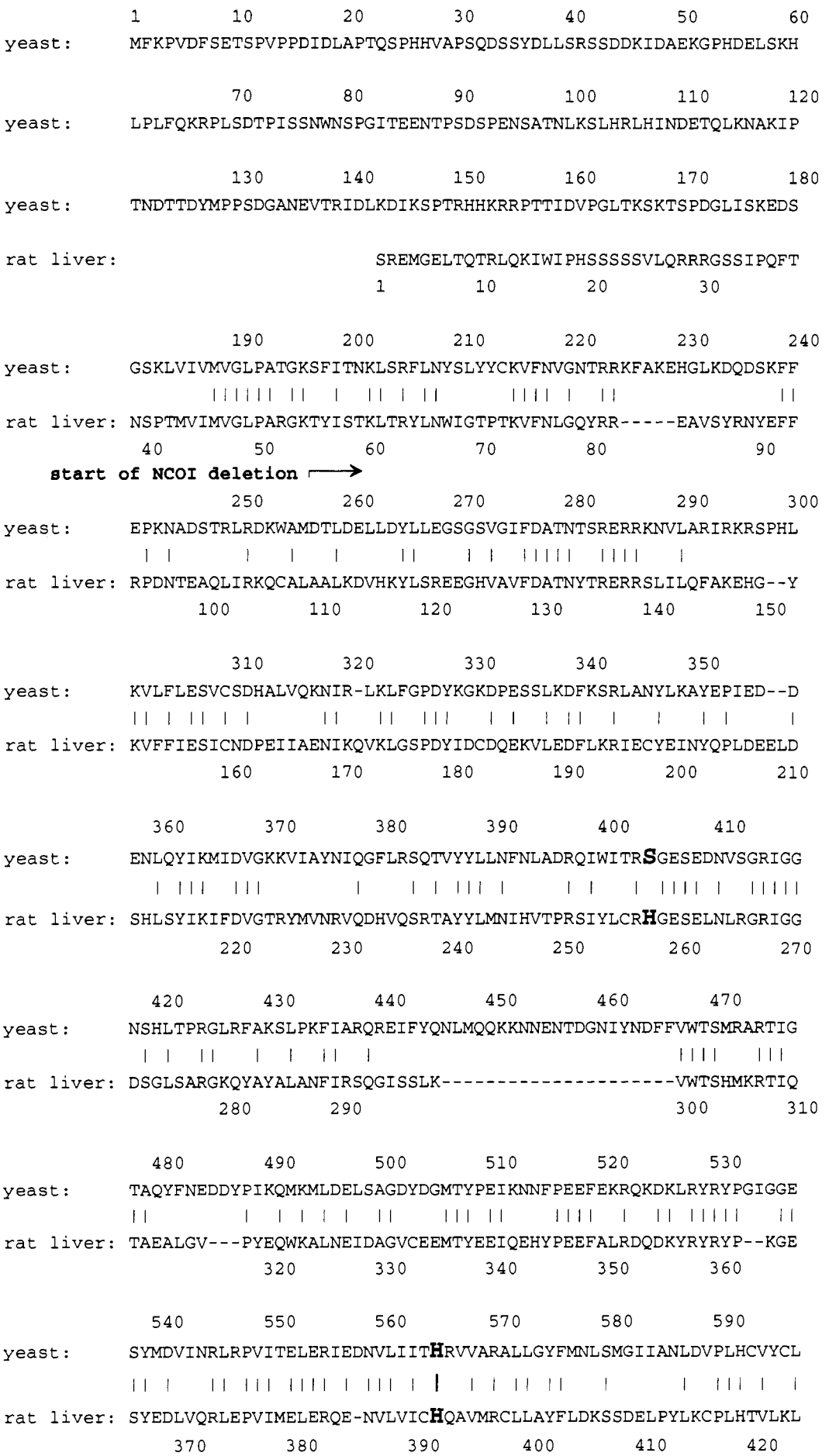
6-Phosphofructo-2-kinase was assayed (Kretschmer et al., 1991) in 11% PEG_{6000} pellets of total cell protein obtained after French press disruption. Samples from an incubation mixture containing enzyme, 50 mM Tris-acetate, pH 7.6, 8 mM ATP, 2 mM fructose 6-P, 6.5 mM glucose 6-P, 20 mM magnesium acetate, 2 mM potassium phosphate, and 5 mM mercaptoethanol were taken at 0, 2.5, and 5 min and assayed for fructose 2,6-P_2 (François et al., 1984). One unit is 1 μmol of substrate converted/min at 25 °C.

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

Results

Sequence. The *Bam*HI/*Hin*dIII fragment, ca. 4 kbp, of clone pMK2 apparently encodes the whole subunit (Kretschmer et al., 1991). Its sequence is shown in Figure 1. The open reading frame of 2481 bases would code a polypeptide of 93.5 kDa, fitting with the 96-kDa value reported for stained subunit on SDS–PAGE (Kretschmer et al., 1991). The four internal peptides reported (Kretschmer et al., 1991) are all present in the translated sequence, the only difference being (i) a glycine (position 3 of fourth peptide underlined in Figure 1) and a glutamine (penultimate position of third peptide, Figure 1), both instead of glutamates [peptides 93T4 and 96T2, and 93T2, respectively (Kretschmer et al., 1991)]. Two other sequenced peptides are also present (the fifth and sixth peptides, Figure 1). The base sequence also shows a possible TATA element (Dobson et al., 1982) at position -181 from the first ATG codon and a likely downstream polyadenylation/transcription termination signal TATG... TAG...TTT (Zaret & Shermann, 1982) (Figure 1). These results all fit with the cloned gene indeed being expressed and coding for the enzyme previously purified. Since the symbol *PFK2* is already employed in yeast for the gene coding the β subunit of 6-phosphofructo-1-kinase (Clifton & Fraenkel, 1982), we call the new gene *PFK26*. Its codon usage is relatively unbiased, the codon usage index (Bennetzen & Hall, 1982) being 0.098.

Figure 2 compares the derived amino acid sequence of the yeast enzyme with the sequence of the bifunctional rat liver enzyme (Lively et al., 1988; Darville et al., 1987). For best alignment seven gaps in the liver sequence and three in the yeast sequence had to be allowed. The aligned lengths, which are of almost the entire bifunctional liver polypeptide (residues 46–470), and residues 188–643 of yeast, show 42% identity.


1 10 20 30 40 50 60
yeast: MFKPVDFSETSPVPPDIDLAPTQSPHHVAPSQDSSYDLLSRSSDDKIDAEKGPHDELSKH
70 80 90 100 110 120
yeast: LPLFQKRPLSDTPISSNWNSPGITEENTPSDSPENSATNLKSLHRLHINDETQLKNAKIP
130 140 150 160 170 180
yeast: TNDTTDYMPPSDGANEVTRIDLKDIKSPTRHHKRRPTTIDVPGLTKSKTSPDGLISKEDS
rat liver: SREMGELTQTRLQKIWIPHSSSSSVLQRRRGSSIPQFT
1 10 20 30
190 200 210 220 230 240
yeast: GSKLVIVMVGLPATGKSFITNKLSRFLNYSLYYCKVFNVGNTRRKFAKEHGLKDQDSKFF
rat liver: NSPTMVIMVGLPARGKTYISTKLTRYLNWIGTPTKVFNLGQYRR-----EAVSYRNYEFF
40 50 60 70 80 90
start of NCOI deletion
250 260 270 280 290 300
yeast: EPKNADSTRLRDKWAMDTLDELLDYLLEGSGSVGIFDATNTSRERRKNVLARIRKRSPHL
rat liver: RPDNTEAQLIRKQCALAALKDVHKYLSREEGHVAVFDATNYTRERRSLILQFAKEHG--Y
100 110 120 130 140 150
310 320 330 340 350
yeast: KVLFLESVCSDHALVQKNIR-LKLFGPDYKGKDPESSLKDFKSRLANYLKAYEPIED--D
rat liver: KVFFIESICNDPEIIAENIKQVKLGSPDYIDCDQEKVLEDFLKRIECYEINYQPLDEELD
160 170 180 190 200 210
360 370 380 390 400 410
yeast: ENLQYIKMIDVGKKVIAYNIQGFLRSQTVYYLLNFNLADRQIWITRSGESEDNVSGRIGG
rat liver: SHLSYIKIFDVGTRYMVNRVQDHVQSRTAYYLMNIHVTPRSIYLCRHGESELNLRGRIGG
220 230 240 250 260 270
420 430 440 450 460 470
yeast: NSHLTPRGLRFAKSLPKFIARQREIFYQNLMQQKKNNENTDGNIYNDFFVWTSMRARTIG
rat liver: DSGLSARGKQYAYALANFIRSQGISSLK----------VWTSHMKRTIQ
280 290 300 310
480 490 500 510 520 530
yeast: TAQYFNEDDYPIKQMKMLDELSAGDYDGMTYPEIKNNFPEEFEKRQKDKLRYRYPGIGGE
rat liver: TAEALGV---PYEQWKALNEIDAGVCEEMTYEEIQEHYPEEFALRDQDKYRYRYP--KGE
320 330 340 350 360
540 550 560 570 580 590
yeast: SYMDVINRLRPVITELERIEDNVLIITHRVVARALLGYFMNLSMGIIANLDVPLHCVYCL
rat liver: SYEDLVQRLEPVIMELERQE-NVLVICHQAVMRCLLAYFLDKSSDELPYLKCPLHTVLKL
370 380 390 400 410 420

```
                600        610        620        630        640        650
yeast:      EPKPYGI-TWSLWEYDEASDSFSKVPQTDLNTTRVKEVGLVYNERRYSVIPTAPPSARSS
            |  ||    |     ||       |      ||  |  |       |
rat liver: TPVAYGCRVESIYLNVEAVNTHRDKPE-NVDITREAEEALDTVPAHY
                430        440        450        460        470

                660        670        680        690        700        710
yeast:      FASDFLSRKRSNPTSASSSQSELSEQPKNSVSAQTGSNNTTLIGSNFNIKNENGDSRIPL

                720        730        740        750        760        770
yeast:      SAPLMATNTSNNILDGGGTSISIHRPRVVPNQNNVNPLLANNNKAASNVPNVKKSAATPR

                780        790        800        810        820
yeast:      QIFEIDKVDEKLSMLKNKSFLLHGKDYPNNADNNDNEDIRAKTMNRSQSHV
```

FIGURE 2: Alignment of the amino acid sequences of yeast 6-phosphofructo-2-kinase and rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Identical amino acids are indicated by vertical bars between the residues. Dashes indicate gaps introduced to allow maximum similarity. The histidines essential for fructose-2,6-bisphosphatase activity of the rat liver enzyme (His-258 and His-392, see text) are emphasized by enlarged letters.

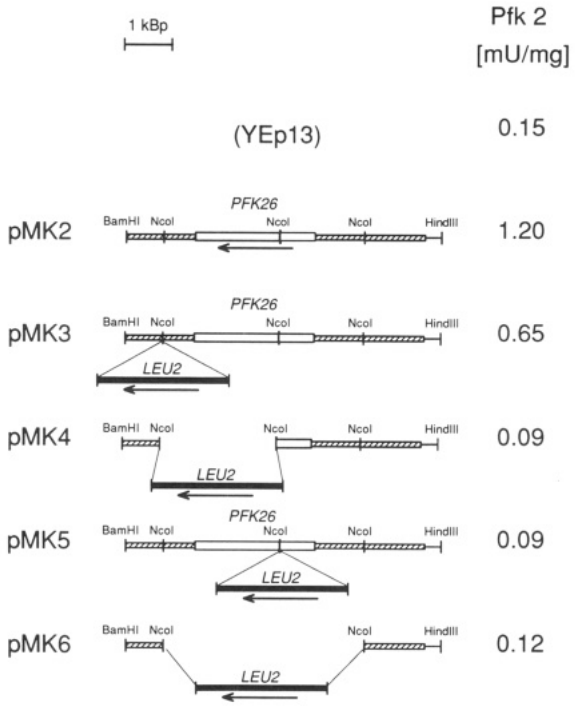


FIGURE 3: Plasmids and 6-phosphofructo-2-kinase activity. 6-Phosphofructo-2-kinase activity, milliunits per milligram of protein ("Pfk 2, mU/mg"), was assayed in extracts of strain DFY535 carrying the indicated plasmids (YEp13 is the vector). White and black bars indicate *PFK26* and *LEU2*, respectively; arrows denote the reading direction.

Although presumably a monofunctional kinase (see also below), the yeast enzyme does contain a stretch (residues 403–417) very similar to the active sites of fructose-2,6-bisphosphatase and phosphoglycerate mutases (Bazan et al., 1989). However, this peptide lacks the histidine residue (His-258, rat liver) which is phosphoacceptor in the bisphosphatase reaction (Tauler et al., 1990), instead having a serine (Ser-404, yeast). The other important histidine residue (His-392, rat liver), which is thought to act as proton donor at the bisphosphatase site (Bazan et al., 1989; Tauler et al., 1990), is present (His-565, yeast).

Also conserved between the yeast and rat liver enzymes is the nucleotide binding domain (Rossmann et al., 1975) MVGLPATGK (residues 188–196 in yeast and 46–54 in liver). Less conserved are residues 251–266, 343–348, and 522–530 of the yeast enzyme, corresponding to sites likely involved in fructose 6-P and fructose 2,6-P_2 binding of the liver enzyme (Kitamura et al., 1988; Kitajima et al., 1985).

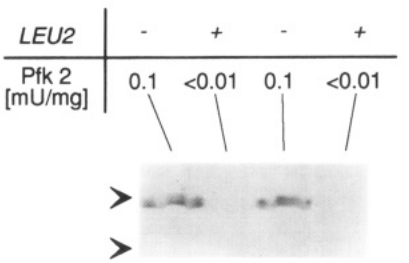


FIGURE 4: Immunoblot and 6-phosphofructo-2-kinase activity in extracts from a tetrad. Protein (600 μg) was loaded in each lane. Positions of prestained molecular mass markers, phosphorylase B (top, nominal mass 97.4 kDa) and bovine serum albumin (68.0 kDa), are indicated by arrows. Strains DFY649 (a *leu2 his3*) and DFY650 (α *leu2 his3 pfk26::LEU2*), used later, are the two left-hand entries, respectively.

If the cAMP-dependent activation of the yeast enzyme (François et al., 1988) employs a consensus phosphorylation sequence (Taylor, 1987), the only such sequence is the RRYS (Ser-644) near the C-terminus. The one involved in inhibition of the rat liver enzyme (its Ser-32) is not present.

A Null Mutant. pMK2, a multicopy plasmid carrying *PKF26*, causes a substantial increase in 6-phosphofructo-2-kinase activity and antigen in wild-type yeast (Kretschmer et al., 1991). Employing its three *Nco*I sites, several *LEU2* insertion or substitution mutations were also tested in multicopy. According to the sequence, plasmids pMK4, -5, and -6 were expected to be mutants but pMK3 was not, and this was the result obtained by assay (Figure 3). A Western blot (data not shown) revealed high levels of antigen for pMK3 as well as pMK2, but only the weak wild-type signal for pMK4, -5, and -6. The mutant gene from pMK4, therefore, was introduced into the chromosome of diploid wild-type strain DFY535 by transplacement, employing the entire *Bam*HI–*Hin*dIII fragment, giving a putative heterozygous diploid strain. Haploid segregants were obtained after sporulation.

Figure 4 shows that the antibody detected the usual faint signal for 6-phosphofructo-2-kinase polypeptide in the two Leu^- spores and no signal in the two Leu^+ spores. Enzyme assay gave the normal low 6-phosphofructo-2-kinase activity in the

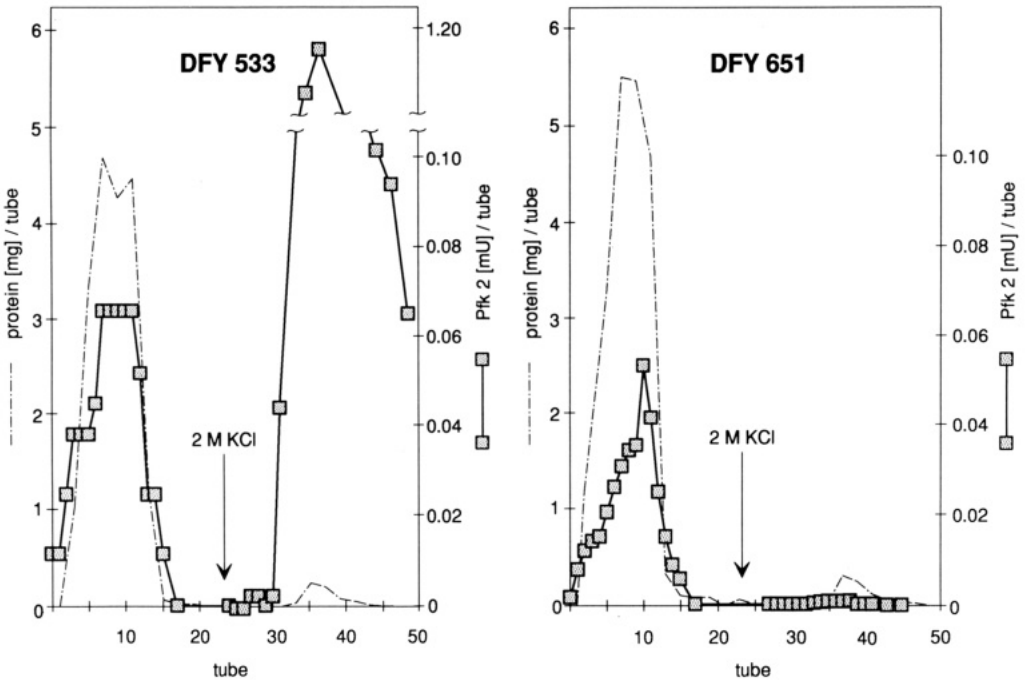


FIGURE 5: Chromatography of extracts from *PFK26* (DFY533) and *pfk26* (DFY651) strains. Both strains lack 6-phosphofructo-1-kinase. The same amounts of protein (60 mg each) were applied on Sephacryl-blue S300 columns and eluted with 2M KCl (see Materials and Methods).

Table I: Levels of Glucose (in Medium) and Metabolites (in Cells)

	DFY649 (*PFK26*)				DFY650 (*pfk26*::*LEU2*)			
	glucose (mM)	fructose 2,6-P_2 (μM)	fructose 1,6-P_2 (mM)	fructose 6-P (mM)	glucose (mM)	fructose 2,6-P_2 (μM)	fructose 1,6-P_2 (mM)	fructose 6-P (mM)
				(A) Growth on Glucose				
	ca. 90	2.0	7.4	0.9	ca. 90	1.6	5.6	0.6
				(B) Resting Cells[a]				
0 min	103	0.20	0.10	0.20	104	<0.02	0.10	0.20
30 min	99	1.1	3.9	1.1	100	<0.02	3.6	1.6
120 min	89	1.0	3.5	1.2	89	<0.02	2.5	1.9
240 min	74	0.9	3.0	1.3	78	<0.02	2.3	1.8

[a] Cells were incubated in buffer with cycloheximide and antimycin A, and glucose was added at 0 min; see Materials and Methods.

former two spores and a marginal activity, 10% or less, in the two Leu^+ spores. Since the normal level of this enzyme is so low, it is difficult to discriminate 10% from zero in a strain containing the normal (10^4-fold higher) level of 6-phosphofructo-1-kinase activity, which uses the same substrates. Therefore, to assay residual activities, strains also lacking 6-phosphofructo-1-kinase were employed. Figure 5 shows affinity chromatography on Sephacryl-blue S300 of extracts from such strains. A minor peak amounting to 6% of total 6-phosphofructo-2-kinase activity in the *PFK26* strain did not bind to the dye matrix, and a similar quantity of activity, in the same position, was present as the sole peak in the *pfk26* mutant strain.

Physiology. The null mutant in the wild-type 6-phosphofructo-1-kinase background grew well on glucose. Concentrations of key metabolites are shown in Table IA. The striking result was the presence of fructose 2,6-P_2 at almost wild-type concentration. The amounts of fructose 1,6-P_2, fructose 6-P, and glucose 6-P were also a little below that of wild type.

It is known that in growth on respiratory carbon sources fructose 2,6-P_2 is present in much lower amounts than in growth on glucose. Addition of glucose causes an increase of fructose 6-P, activation of preformed 6-phosphofructo-2-kinase by its cAMP-dependent phosphorylation (François et al., 1987), and a rapid increase in fructose 2,6-P_2 concentration. Figure 6 shows the metabolite patterns after glucose addition to cultures growing on pyruvate and the effect of the protein synthesis inhibitor cycloheximide. In the wild-type strain the usual increase in fructose 2,6-P_2 (panel A) was observed, fitting with the known mechanism of activation of the kinase. The results were quite different in the mutant. Fructose 2,6-P_2 was neither detectable in its growth on pyruvate nor after glucose addition in the presence of cycloheximide. Glucose addition in the absence of cycloheximide caused slow appearance of fructose 2,6-P_2, reaching a steady-state level near that in long-term growth on glucose (Table I) by ca. 30 min, as compared with 2 min in the wild-type strain.

By contrast to the data for fructose 2,6-P_2, the time course of concentration changes in fructose 6-P (panel B) and fructose 1,6-P_2 (panel C) after glucose addition were fairly similar in wild-type and mutant strains, with or without cycloheximide.

As tested in a slightly different condition (washed cells at higher density in a buffer containing both cycloheximide and antimycin D [S. Benevolensky and D. G. Fraenkel, to be reported]), the rate of glucose metabolism in the mutant strain obtained from growth on pyruvate was similar to that of wild type: plots of data from Table IB giving values of 11 and 12 nmol min^{-1} A_{580}^{-1}, respectively, in spite of the mutant containing no fructose 2,6-P_2. Glucose-grown cells in the same conditions also had similar rates of glucose metabolism, 16 and 15 nmol min^{-1} A_{580}^{-1} for mutant and wild-type strains

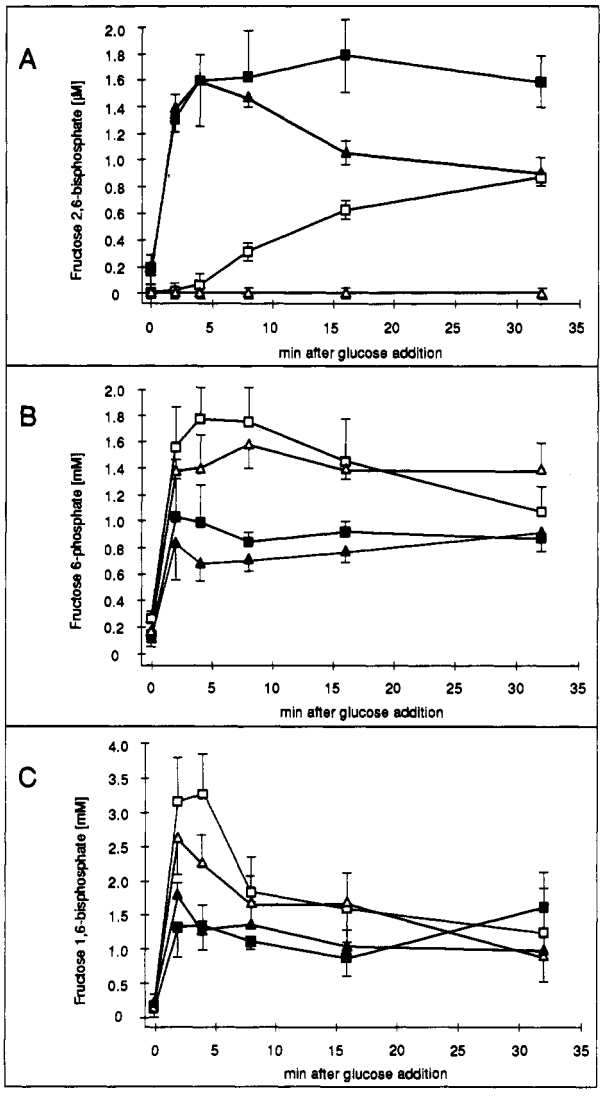


FIGURE 6: Metabolites after addition of glucose to strains grown on pyruvate. Filled and open symbols indicate wild-type strain DFY649 and mutant strain DFY650, respectively. Triangles specify cultures also containing cycloheximide (added at −10 min, see text and Materials and Methods). Error bars represent standard deviations obtained from three independent cultures with each metabolite concentration measured twice. To avoid crowding, error bars are sometimes shown only for one direction.

respectively (data not shown).

Discussion

The present work establishes that the cloned protein kinase A activated 6-phosphofructo-2-kinase of yeast is a protein with extensive sequence similarity to the well-known enzyme from liver. But there are at least three major differences between the enzymes. First, the yeast polypeptide is much larger than the liver one. Since the 96-kDa size of the stained protein (Kretschmer et al., 1991) and the 103-kDa estimate of major phosphorylatable peptide in a partially purified preparation (François et al., 1988), both according to SDS–PAGE, are similar to the 93.5-kDa value for the open reading frame in the clone, it is likely that the entire open reading frame is employed. However, there is no independent size estimate yet, and N-terminal sequencing was not possible (Kretschmer et al., 1991). Nonetheless, it appears that the yeast enzyme has extensive stretches of peptide at both ends which will not necessarily be involved in catalysis. Sequence comparisons to date have not revealed similarity of these regions to other genes.

Second, the yeast polypeptide has especially strong similarity to the bisphosphatase region of the bifunctional liver polypeptide but with serine in place of a key histidine. Several lines of evidence point to the fructose-2,6-bisphosphatase activities of yeast being different proteins from 6-phosphofructo-2-kinase (François et al., 1988; Kretschmer et al., 1987; Plankert et al., 1991). But in view of the sequences we have assayed bisphosphatase activity in a new 10^4-fold purified preparation of the kinase from an overproducing strain (to be reported). Kinase and bisphosphatase activities (the latter assayed with 1 μM fructose 2,6-P_2) were, respectively, 750 and less than 0.3 milliunits/mg of protein, confirming earlier findings. Whether the present enzyme lacks the bisphosphatase activity in other conditions, and the role of its serine-404, merit study.

The third difference is the putative cAMP-dependent phosphorylation site near the C-terminus, rather than the N-terminal position in the liver enzyme. A C-terminal site is likewise found in the heart enzyme (Sakata et al., 1990), which is also activated rather than inhibited by such phosphorylation.

The role of fructose 2,6-P_2 is not well understood in yeast, although the fact of its presence, a regulated 6-phosphofructo-2-kinase activity, and a sensitivity of 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase to this effector fit with it having the expected functions. We have shown previously that its presence helps explain rapid 6-phosphofructo-1-kinase activity in spite of low concentrations of fructose 6-P, particularly in mutants with altered 6-phosphofructo-1-kinase (Clifton & Fraenkel, 1983). In part, the present work was undertaken to obtain 6-phosphofructo-2-kinase mutants to explore the same question. In this regard there are two findings. One of them, of substantial fructose 2,6-P_2 in the null mutant and a 6-phosphofructo-2-kinase not binding to Sephacryl-blue S300, suggests there may be a second 6-phosphofructo-2-kinase in yeast. The other is that glucose metabolism can be rapid in the absence of fructose 2,6-P_2.

The implication of a second activity for making fructose 2,6-P_2 rests on the following grounds: The chromosomal mutant is almost certainly a null on the basis of its structure. It lacks (Figure 2) the distal 71% of the open reading frame. Of the remaining 256 residues, 186 are unrelated to the 45 N-terminal residues of the liver enzyme and 70 show similarity to a portion of the 6-phosphofructo-2-kinase domain of the liver enzyme. It is unlikely, though conceivable, that the fragment, if expressed, retains some kinase activity. However, no such activity above the negligible 10% or less value was found in the chromosomal mutant strain even when the mutant gene was present in multicopy (data not shown). Also, the requirement for protein synthesis to obtain fructose 2,6-P_2 in the mutant incubated with glucose best fits with synthesis of the second activity, unlike the known enzyme, being induced by glucose.

Interestingly, the low assayed amount of the second activity would suffice for the rate of accumulation of fructose 2,6-P_2 in the mutant (Figure 6A), as well as for its steady-state level, assuming no turnover. It might be related to a second activity peak observed in chromatography of crude extract (Aragón et al., 1987), although that report was of a relatively higher activity. One speculation about the second kinase is that it might be employed in steady-state growth on glucose, the normal function of the protein kinase A activated enzyme being in metabolic transitions. However, in the mutant, there was no indication of a delayed transition to more rapid growth after

glucose addition to a culture growing on pyruvate (data not shown).

Because of inducibility of the putative second kinase, when grown without glucose the mutant apparently lacks ability to synthesize fructose 2,6-P_2. This fact allows one to show that relatively rapid glucose metabolism in resting cells does not require detectable fructose 2,6-P_2. The rate of glucose metabolism in this situation (prevention of both protein synthesis and respiration) is, for glucose-grown cells, more than 50% of the flux during growth (to be reported). It is almost certain that glucose is employing the glycolytic pathway and 6-phosphofructo-1-kinase: no other pathway for rapid glucose metabolism in yeast is known, and a strain blocked in both 6-phosphofructo-1-kinase and 6-phosphofructo-2-kinase (DFY651, *pfk1 pfk2 pfk26*) was, like mutants lacking just 6-phosphofructo-1-kinase (Clifton & Fraenkel, 1982), unable to grow on glucose (data not shown). Also, fructose 6-P was present in near-normal levels during the rapid glucose metabolism of the *pfk26* mutant (Table IB) and not low, as might have been expected for a different metabolic pathway. Interestingly, these levels are not particularly high either, as if other effectors may be adequate.

The question of whether growth of yeast on glucose or other carbohydrates can also occur in the absence of fructose 2,6-P_2 remains to be answered. A mutant in the second enzyme would be useful. But if attainment of adequate glucose flux is the only necessity, the answer is likely to be that fructose 2,6-P_2 is not required.

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