Molecular Cloning of Genomic DNA for Fructose-1,6-bisphosphatase from *Aspergillus oryzae*

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We have cloned and sequenced an *Aspergillus oryzae* genomic DNA fragment that encodes a fructose-1,6-bisphosphatase gene (*fbpA*) with the aim of studying transcriptional regulation mechanisms involved in basic metabolism. Expression of *fbpA* was repressed in the presence of glucose, but not in the presence of pyruvate or sodium acetate in the medium. The CreA and FacB element found in the *fbpA* 5′-flanking region may be important in *fbpA* regulation.

**Key words:** fructose-1,6-bisphosphatase; *Aspergillus oryzae*; genomic DNA; transcriptional regulation

*Aspergillus oryzae*, a filamentous fungus, is regarded as a favorable host for heterologous protein production, but a lack of basic knowledge on its transcription system is impeding more extensive applications. Here, we describe the cloning and characterization of a gene encoding fructose-1,6-bisphosphatase (FBPase) (*EC 3.1.3.1*), which catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate from the filamentous fungus *A. oryzae*. FBPase is a key enzyme in gluconeogenesis, providing a mechanism that permits the reversal of the reaction catalyzed by the glycolytic enzyme 6-phosphofructo-1-kinase (*EC 2.7.1.11*). The enzyme has been extensively studied, particularly the version from a gluconeogenic tissues such as the liver and the kidney cortex and from the yeast *Saccharomyces cerevisiae*. The genes encoding FBPase have been cloned from variety of organisms, including humans, mice, wheat, and yeast; however, no FBPase genes have until now been reported from filamentous fungi.

In *S. cerevisiae*, FBP1 (encoding FBPase) is induced by sodium acetate and repressed by glucose in the medium under the control of Cat8p and Mig1p, respectively. Transcription factors with similar functions to the yeast factors Cat8p and Mig1p have been identified from *A. nidulans* as FacB and CreA, respectively. A high degree of regulation is presumably required for precise control of the enzymes which interplay in glycolysis and gluconeogenesis, making FBPase a very interesting target for molecular and biochemical analyses. This is the first report on the nucleotide sequence of the FBPase of a filamentous fungus. Analysis of the transcription regulation of this gene will be important in gaining an understanding of the *A. oryzae* transcription regulation of genes in relation to the basic metabolic processes of glycolysis and gluconeogenesis.

*A. oryzae* wild-type RIB40 strain was used as both a DNA and an RNA donor. Mixed primers (5′-ACAGGCGATGAYCARAARAAR-3′; Y=T or C and R=A or G) and (5′-CATTCGCTTSCNCNCNCCRTA-3′; S=G or C) were designed by aligning all the FBPase genes registered in the public database, and the degeneration of the mixture of each primer was restricted according to the codon usage of the glucoamylase A gene. An amplified DNA fragment was cloned into the T-vector and partially sequenced. The entire coding region, including the 5′- and 3′-flanking regions of *A. oryzae* FBPase was amplified by PCR-based gene walking from *A. oryzae* genomic DNA, then cloned and sequenced (Fig. 1). Three independent clones were sequenced for the entire region to exclude mutations introduced during PCR. Translation initiation and termination sites were predicted from the alignment of the cloned gene to the other genes encoding FBPase used for the primer design. The corresponding cDNA was cloned by RT-PCR using primers, 5′-ATGTCTGGACACGAGAACCGGT and 5′-TTATTTCTTGACTTGTTATATGT, located at the putative translation in-
Nucleotide Sequence was done using the dyeoxose nucleotide chain termination method using an automated DNA sequencer (Model 4200L, LI-COR, Lincoln, NB, USA). The CAAT sequences in the 5′-flanking region and a polyadenylation signal in the 3′-flanking region are underlined. The regions that show similarity with the A. nidulans CreA- and FacB-binding consensuses are indicated by double underlines and boxes. The nucleotide sequence has been deposited in the DDBJ, GenBank, and EMBL databases under Accession No. AB030248.

Genomic Southern hybridization analysis using cloned fbpA gene as a probe showed a single band on all restriction digests of A. oryzae chromosomal DNA, indicating that the fbpA gene exists as a single copy in the A. oryzae genome (Fig. 3a) and that the fbpA gene we cloned should be functional.

The 5′-upstream sequence of the fbpA gene had putative CAAT boxes but a TATA-box was absent up to about 300 bp upstream of the initiation codon. There are several examples of fungal glycolytic genes which lack a typical TATA sequence, e.g., the A. nidulans pkiA(17) and argB(18) genes.

To investigate the transcription regulation of the A. oryzae fbpA gene, total RNA was extracted from mycelia grown in modified CD medium(15) containing glucose, pyruvate, glucose + pyruvate, or sodium acetate. Approximately 5 μg of total RNA was electrophoresed on a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with the DIG-labeled RNA probe containing the entire fbpA coding region. The results showed that the fbpA gene
Fig. 2. Comparison of Amino Acid Sequences of FBPase Genes.
Amino acid sequences were aligned, allowing gaps to make the best fit. The numbers at the ends of the rows of sequences are the amino acid numbers. Identical amino acids are boxed. When alignment of the sequence suggests an addition or deletion, the gap is represented by a dash.

Fig. 3. Hybridization Analysis of the *A. oryzae* fbpA Gene.

a) Southern hybridization analysis of the *A. oryzae* fbpA gene.
Approximately 10 µg of genomic DNA from *A. oryzae* RIB40 was digested and separated by 1.0% agarose gel electrophoresis. Following transfer to a nylon membrane, the blot was hybridized with a DIG-labeled RNA-probe containing the entire *fbpA* coding region transcribed by T3 RNA polymerase using the DIG RNA-labeling kit (Boehringer-Ingelheim, Germany). The hybridization was done at 42°C in a standard hybridization solution and the washing was done at 42°C in 0.2× SSC containing 0.1% SDS. The hybridization signal was detected by the chemiluminescence reaction using CDP-Star (Tropix, Inc., Bedford, MA, USA) according to the manufacturer's protocol. *A. oryzae* RIB40 genomic DNA was digested with HindIII, (Lane 1); PstI, (Lane 2); SalI, (Lane 3); XhoI (Lane 4).

b) Northern hybridization analysis of the *fbpA* transcript.
RNA was extracted from mycelia grown in modified CD medium containing 2% glucose (Lane 1), 2% pyruvate (Lane 2), 2% glucose + 2% pyruvate (Lane 3), or 2% sodium acetate (Lane 4) for 22 h at 30°C. Hybridization was done in the same way as in the Southern hybridization. The histone H4 transcript was used as an internal standard. Relative intensities of the *fbpA* transcript over those of Histone H4 are indicated under the *fbpA* Northern blot.
was highly expressed when mycelia were grown on pyruvate or sodium acetate but was repressed by glucose in the medium (Fig. 3b). There are three consensus sequences for the binding of A. nidulans CreA (5'-SYGGRG-3'; S=G or C, Y=C or T, R=G or A) and three consensus sequences for the binding of A. nidulans FacB (TCC/GNk-10/C/GGA or GCA/CNk-10/T/GGC) in the 5'-flanking region. These results suggest that some or all of the consensus elements found in the 5'-flanking region are functionally active and the fpbA gene was under the control of A. oryzae factors functionally identical to A. nidulans CreA and FacB. Study of the fpbA gene should be useful in acquiring an understanding of the basic metabolic regulatory mechanism in A. oryzae and to analyze the transcriptional regulation factors, which are involved with the regulation of many industrially important genes.

References


