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Regulation of yeast fructose-1,6-bisphosphatase in strains containing multicopy plasmids coding for this enzyme

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The structural gene for yeast fructose-1,6-bisphosphatase (FbPase) has been cloned by complementation of a strain carrying the fbp1-1 mutation and lacking FbPase activity. By Northern analysis it could be shown that the fbp1-1 mutation does not interfere with the transcription of the gene although no normal active enzyme is produced. Strains transformed with the cloned gene on a multicopy plasmid overproduce FbPase but are still subject to strong catabolite repression. Catabolite inactivation, however, is slowed down in such strains.

Fructose-1,6-bisphosphatase; Catabolite inactivation; Catabolite repression; Gene expression; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Fructose-1,6-bisphosphatase (FbPase) is subject in *Saccharomyces cerevisiae* to catabolite repression [1] and to catabolite inactivation [2]. Catabolite inactivation occurs in two phases, a very rapid one caused by phosphorylation of the enzyme and a slower one due to proteolytic degradation [3–7]. To study the mechanisms underlying catabolite repression and inactivation a mutant lacking FbPase was isolated in our laboratory [8]. Although the mutation fbp1-1 appeared to affect selectively the FbPase activity, it was not determined whether the mutation was in the structural gene or in a specific regulatory gene. Therefore we used this mutant to clone the corresponding gene by genetic complementation and as a result a DNA fragment was cloned which turned out to be the structural gene for FbPase. In the transformed strains FbPase is repressed by glucose as in a wild-type yeast. Inactivation by glucose, however, is delayed in a strain overproducing FbPase.

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2. MATERIALS AND METHODS

2.1. *Media and growth conditions*

Difco yeast nitrogen base supplemented with 2% glucose or 2% pyruvate was the minimal medium for *S. cerevisiae*. Repressed cells were obtained by growth in minimal medium glucose and harvested during the exponential phase (< 5 mg yeast wet wt/ml). Derepression was achieved by overnight incubation of the cells either in minimal medium pyruvate + 0.5% yeast extract or in YP ethanol (1% yeast extract, 1% peptone, 2% ethanol).

2.2. *Strains and plasmids*

S. cerevisiae CJM 105 (α fbp1-1 ura3) was derived from the FbPase mutant isolated in [8]. *S. cerevisiae* RG1-5d (α fbp1-2 ura3) was derived from the FbPase mutant constructed by Sedivy and Fraenkel [9]. *S. cerevisiae* CJM 88 (α ura3 can^R) was provided by C. Gancedo and *S. cerevisiae* DFY 432 (α leu2 trp1) by D.G. Fraenkel. *Escherichia coli* RYC 7010 contained a Tn5 insertion in the chromosome and was provided by J.C. Perez Diaz. *E. coli* HB101 was used for the plasmid manipulations. For subcloning the shuttle vectors pFL1 [10], pCG542 (donated by R. Serrano) and YEp352 [11] and the integrative vector YIp352 [11] were used.

2.3. *Transformation*

Competent *E. coli* cells were prepared, stored and transformed by standard techniques [12]. For the initial isolation of the gene yeast was transformed by the protoplast method [13]. Subsequent yeast transformations were performed according to the procedure of Ito et al. [14].

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2.4. *Recombinant DNA methods*

DNA manipulations were by standard methods [12]. Subcloning was carried out as follows (see fig.1). pJM2 was obtained by total digestion of pJM1 with *Bam*HI followed by religation of the large resulting fragment. For pRG1, the 3.8 kb fragment *Bgl*II-*Bam*HI from pJM1 was recloned in the *Bam*HI site of pFL1. For pRG2, the 4.4 kb *Hin*dIII-*Hin*dIII fragment from pJM1 was inserted in the *Hin*dIII site of pCG542. For pRG5, pJM1 was digested with *Sma*I which cuts also at the end of the URA3 fragment and the large resulting *Sma*I-*Sma*I fragment was religated. For pRG6, pRG5 was digested with *Xho*I and with *Sal*I which cuts within the Tet^r region of pFL1. The large *Xho*I-*Sal*I fragment was then religated. For pRG7, the 4 kb *Xba*I-*Xba*I fragment from pJM1 was recloned in the *Xba*I site of YEp352. The integrative plasmid pRG11 was prepared by inserting the 4.4 kb *Hin*dIII-*Hin*dIII fragment used to construct pRG2 into the *Hin*dIII site of YIp352. It was linearized with *Bst*EII.

Transposon Tn5 mutagenesis was performed as described by Van Dyk et al. [15].

2.5. *Northern blot hybridization*

Total yeast RNA was extracted as described [16]. 40 μg of the RNA preparation were electrophoresed on a 1.3% agarose gel containing formaldehyde and transferred to a nylon membrane as described [12]. Hybridization and washing was as recommended by the manufacturer.

2.6. *Assay of fructose-1,6-bisphosphatase*

Extracts were prepared as described [6] with 20 mM imidazole, pH 7, and the enzyme was assayed spectrophotometrically [17].

2.7. *Fructose-1,6-bisphosphatase inactivation*

Yeast derepressed in YP ethanol was resuspended in 0.1 M potassium phosphate buffer, pH 6, at 20 mg/ml and equilibrated for 15 min at 30°C with shaking. A 20% glucose solution was then added (final concentration 2%). Samples were taken at the indicated intervals, poured on an equal volume of ice-cold water, centrifuged and washed twice with chilled water.

3. RESULTS AND DISCUSSION

S. cerevisiae CJM 105 lacking FbPase is unable to grow on pyruvate as carbon source. To confer to this strain an FBP phenotype a genotheque (gene library) of *S. cerevisiae* in the shuttle vector pFL1 [10] were used. Transformants were isolated by a two-step procedure, selecting first for uracil prototrophy and screening among the selected clones for growth on pyruvate. Total DNA extracts from the transformants were used to transform *E. coli* HB101 selecting for ampicillin resistance. Plasmid DNA from the *E. coli* transformants was analysed and shown to contain the same 8 kb insert. The reisolated plasmid (pJM1) was used to transform again the CJM 105 strain. All transformants regained simultaneously uracil prototrophy and the ability to grow on pyruvate.

To define the DNA region complementing the fbp1-1 mutation two approaches were used: subcloning of the DNA insert and mapping by transposon Tn5 mutagenesis. The results are shown in fig.1. They indicate that the gene complementing the fbp1-1 mutation is included within the 2.1 kb *Xba*I-*Sma*I fragment. To check that the activity hydrolyzing fructose bisphosphate is really due to a specific enzyme, inhibition by AMP and fructose 2,6-bisphosphate was measured. It was found that both compounds inhibited the phosphatase activity at the same concentrations described for native yeast bisphosphatase [18].

While this work was in progress Sedivy and Fraenkel [9] cloned the *S. cerevisiae* FbPase gene by complementation in *E. coli* and used this clone to construct a yeast strain with a disrupted FbPase gene. In a strain derived from this one (RG1-5d) we have found that the lack of FbPase can be corrected by transformation with the pJM1 plasmid and also by the subclones pRG6 and pRG7 showing unequivocally that the cloned gene corresponds to the structural gene for FbPase.

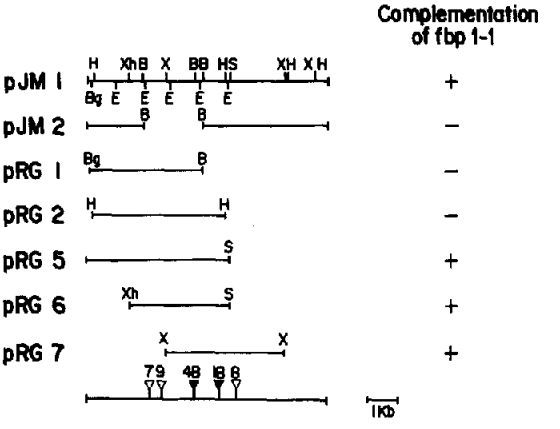

Fig.1. Restriction map of the 8 kb yeast DNA fragment inserted in pJM1, subcloning and location of Tn5 transpositions. The plasmid pJM1 was isolated by its ability to complement the fbp1-1 mutation of *S. cerevisiae* CJM 105 (for details see text). The subclones were constructed as described in section 2. The last line shows the physical location of Tn5 transpositions into the cloned region of plasmid pJM1. Transpositions which inactivated the gene FBP1 are indicated by filled triangles and those not affecting gene expression by open triangles. Only the *S. cerevisiae* DNA segment is shown. Abbreviations: B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; X, *Xba*I; Xh, *Xho*I.

To test whether the fbp1-1 mutation blocked the expression of the gene, RNA from CJM 105 derepressed cells was analyzed by Northern blot hybridization. As seen in fig.2 the mutant produced a 1.3 kb RNA which hybridized with 2 *Eco*RI-*Eco*RI probes internal to the FBP gene. In contrast, when the RNA was extracted from a mutant where the FBP gene has been disrupted [9] no hybridization was observed. Wild-type yeast extracts contain the same 1.3 kb mRNA as the fbp1-1 mutant: this mRNA is present only when the yeast has been grown under derepressed conditions, confirming the results of Sedivy and Fraenkel [9]. From the results of the Northern analysis it is clear that the fbp1-1 mutation is not located in a regulatory region controlling the expression of the FBP structural gene. Most probably the coding region itself is affected, in such a way that no normal active enzyme is produced. We cannot rule out, however, the possibility that the mutation interferes with an efficient translation of the corresponding mRNA.

The regulation of FbPase expression was investigated in the transformed strains. As shown in table 1, both in the case of an integrative plasmid

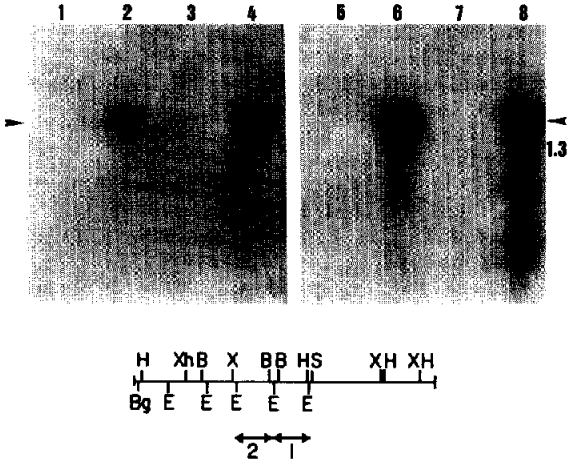

Fig.2. Expression of FBP1 mRNA. Northern blot hybridization analysis of total yeast RNA was done as described in section 2. Cells were harvested from repressed (R) or derepressed (D) cultures. Lanes: 1 and 5, RG1-5d (D); 2 and 6, CJM 105 (D); 3 and 7, DFY 432 (R); 4 and 8, DFY 432 (D). The probes were ^{32}P nick-translated *Eco*RI-*Eco*RI DNA fragments isolated from pJM1 as shown in the restriction map. Lanes 1–4 were hybridized with probe 1 and lanes 5–8 with probe 2. Size (in kilobases) indicated by the arrow was determined by comparison with *S. cerevisiae* ribosomal RNA.

Table 1

Fructose-1,6-bisphosphatase activity of strain CJM 105 transformed with various plasmids

	Fructose-1,6-bisphosphatase activity[a]	
	Repressed[b]	Derepressed[b]
Multicopy plasmid		
none	<1	<1
pJM1	<1	200
pRG2	<1	5
pRG5	2	280
pRG6	2	250
pRG7	<1	200
Integrative plasmid		
pRG11	<1	55

[a] Fructose bisphosphatase activity is expressed as nmol/min per mg protein. Results are the mean of at least 3 separate cultures

[b] Conditions of repression and derepression are given in section 2

and of a multicopy plasmid, repression by glucose was as marked as in a wild-type yeast. The amount of FbPase in the transformants containing multicopy plasmids is 4–5-fold higher than in the yeast transformed with an integrative plasmid. It is striking that the level of FbPase decreases about 50-fold in the yeast transformed with pRG2 which lacks the upstream region *Sma*I-*Hin*dIII. This result is consistent with data recently published [19] but contrasts with earlier work [9]. We have transformed the RG1-5d strain with the plasmid PRG2 and observed that in this case the specific activity of FbPase was much higher (80 mU/mg protein). From these results we conclude that the *Sma*I-*Hin*dIII region contains an upstream activating sequence which is required for a high level of transcription of FBP in some genetic backgrounds but which is dispensable in strains from other genetic stocks.

Since yeast transformed with a multicopy plasmid overproduces FbPase it could happen that in this transformed strain the system which inactivates FbPase upon glucose addition becomes rate-limiting and the inactivation by glucose takes place with delayed kinetics. This possibility was tested and the results are shown in fig.3. The first phase of inactivation occurs as rapidly in the strain with high FbPase as in the control strain transformed with pFL1. The second phase, however, is delayed in the strain overproducing FbPase. These

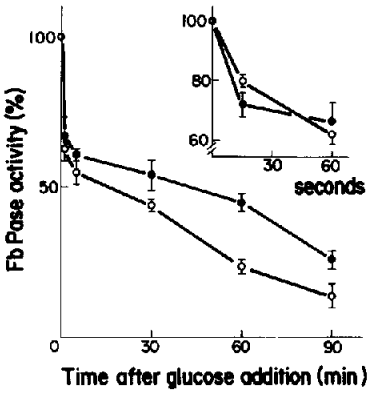

Fig.3. Inactivation of fructose bisphosphatase by glucose in yeast strains with different levels of fructose bisphosphatase. Inactivation was carried out as described in section 2. Inset: course of inactivation during the first minute. Each point represents the mean of 4 experiments. (○) CJM 88/pFL1 (initial activity 37 ± 6 mU/mg protein); (●) CJM 88/pRG5 (initial activity 200 ± 50 mU/mg protein).

results indicate that the system which phosphorylates FbPase works at excess capacity and can handle an increased supply of its substrate. The proteolytic system, on the other hand, appears to be overtaxed by an excess FbPase and the second phase of inactivation proceeds at a slower pace. This conclusion would be supported by the observation that in a yeast transformed with the FBP gene under the control of the PHO5 promoter, FbPase accumulates even in the presence of glucose, suggesting that an increase in the level of FbPase had saturated the inactivating system [20]. Catabolite inactivation accompanied by a specific loss of antigenic material has been shown to occur with other enzymes: phospho*enol*pyruvate carboxykinase (PEPCK) [21-23] and malic dehydrogenase (MDH) [24,25]. To test whether these enzymes are degraded by the same system as FbPase, their inactivation was measured in the same strains. It was observed that inactivation of PEPCK is slower in the strain with high levels of FbPase (30% and 49% inactivation at 60 and 90 min vs 41% and 62% for the control strain). On the other hand, MDH is inactivated with similar kinetics in both strains (about 30% after 60 min with no further inactivation at 90 min). These results would suggest that the gluconeogenic enzymes FbPase and PEPCK share a common inactivating system, while MDH is degraded by a different one.

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