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Isolation and characterization of the gene encoding phosphoenolpyruvate carboxykinase from *Saccharomyces cerevisiae*

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The yeast PCK1 gene coding for phosphoenolpyruvate carboxykinase (PEPCK) was isolated by functional complementation of pck1 strains from *S cerevisiae* Only one copy of the gene was found per haploid yeast genome An RNA of about 2 kb which hybridized with a DNA probe internal to the PCK1 gene was found only in cells growing in non-fermentable carbon sources Yeast strains carrying multiple copies of the PCK1 gene showed normal catabolite repression of PEPCK except those carrying the shortest insertion complementing the mutation (2 2 kb) that presented an altered kinetics of derepression Catabolite inactivation was decreased in strains transformed with multicopy plasmids carrying the PCK1 gene

Phosphoenolpyruvate carboxykinase, Gluconeogenesis, Catabolite repression, Catabolite inactivation

1. INTRODUCTION

Growth of yeasts on non-carbohydrate carbon sources requires gluconeogenesis. All steps of gluconeogenesis implicate enzymes that are shared by the glycolytic pathway except those catalyzed by fructose-1,6-bisphosphatase (FbPase) and phosphoenolpyruvate carboxykinase (PEPCK). Both enzymes are regulated by catabolite repression [1,2] and catabolite inactivation [3-5]. In order to obtain more knowledge about the molecular processes underlying these phenomena in the case of PEPCK we undertook the isolation and characterization of the gene encoding this enzyme.

2. MATERIALS AND METHODS

2 1. *Strains*

S. cerevisiae strains PUK-3B, MAT α pck1 ura3 ade1 was derived from strain JPM2 [6] and used to clone the PCK1 gene. Strains X2180 (wild-type); CJM46, MAT a his1 ade1 can1; CJM88, MAT α ura3, CJM-D2, MATa ura3 and PUK-2B, MAT a pck ura3 were also utilized *Escherichia coli* HB-101 [7] was used for transformation and amplification of plasmids A yeast genomic library constructed in pFL1 [8] was used to isolate plasmids containing PCK1 sequences Vectors YEp352 and YIp352 [9] were used for subcloning

2 2 *Media and growth conditions*

Rich medium was 1% (w/v) yeast extract, 1% (w/v) peptone Minimal medium was 0.7% (w/v) Difco yeast nitrogen base with the required supplements As carbon sources 2% (w/v) glucose or pyruvate were added

2 3. *Transformation of* E coli *and yeast*

Competent *E coli* cells were prepared, stored and transformed by standard techniques [10] For the initial isolation of the gene, yeast was transformed using the protoplast method [11] Subsequent yeast transformations were performed according to [12]

2 4 *DNA/RNA manipulations*

DNA manipulations were by standard methods [7] Probes were labelled as described in [13]

2.5. *Enzymatic assays*

Yeast extracts were prepared as in [14] PEPCK was assayed as in [15] as modified in [6] FbPase was assayed as in [14] and malate dehydrogenase as in [16]. Protein was assayed as in [17]

2.6 *Isolation of the PCK1 gene*

The PCK1 gene was isolated by functional complementation of *S cerevisiae* PUK-3B unable to grow on pyruvate. First the yeast was transformed to uracil prototrophy All the transformants were screened for growth on pyruvate Among 30000 colonies only one colony was able to grow on pyruvate. A plasmid was isolated from this transformant (pMV1), amplified in *E coli* and used to retransform strain PUK-3B All the transformants were prototrophic for uracil and grew on pyruvate When grown on rich medium glucose for ten generations 50% of the population lost both the pyruvate positive phenotype and the uracil prototrophy.

3. RESULTS AND DISCUSSION

Plasmid pMV1 was analyzed by restriction endonuclease digestion to generate the map shown in fig.1. Several subclones were tested for their ability to complement the pck mutation (fig.1). The smallest fragment complementing the pck mutation was located in a 2.2-kb fragment in plasmid pMV7. Genomic DNA probed with the fragment *Hin*dIII-*Sal*I internal to the gene showed hybridization of the cloned DNA to a single locus (results not shown). To eliminate the possibility that a suppressor of the mutation had been cloned two approaches were used: integration of the complementing DNA into the genome and disruption of the

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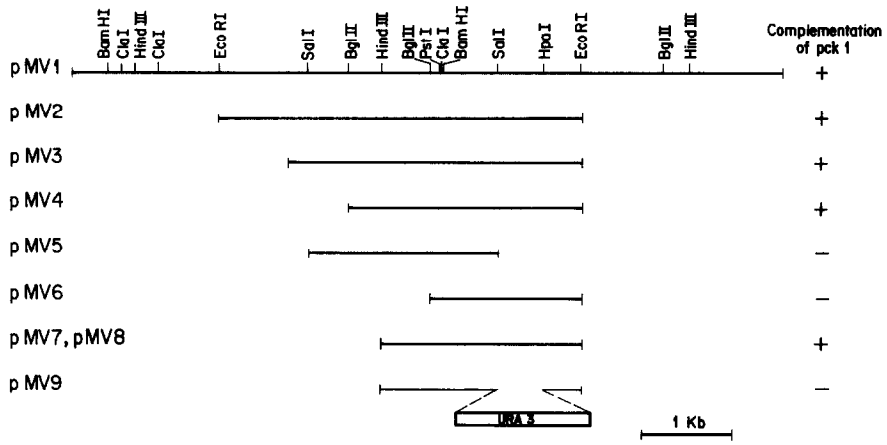

Fig.1. Restriction maps of plasmids used to characterize PCK1. pMV1 was isolated from a yeast genomic library in pFL1. To construct pMV2 through pMV7, fragments from pMV1 were ligated into YEP352. pMV8 had the same insert as pMV7 but ligated into YIP352. To obtain pMV9 the fragment *Sal*I-*Hpa*I was eliminated from pMV8, and substituted by the URA3 gene.

gene. Plasmid pMV8 (fig.1) was used to transform PUK-3B to uracil prototrophy and clones growing on pyruvate were selected. Genomic DNA from one transformant and from wild-type were digested with *Eco*RI or with *Hin*dIII and probed with the *Hin*dIII-*Eco*RI fragment. The bands obtained were in accordance with the values expected if the plasmid had been inserted at a site homologous to the DNA fragment that complements pck1 (fig.2). The transformant was crossed with CJM46 (PCK1, URA3) and with PUK-2B (pck1, ura3). In 9 complete tetrads of the first cross all spores grew on pyruvate ($4^+:0^-$). Segregation of Ura^+ phenotype was $4^+:0^-$ in 3 tetrads and $3^+:1^-$ in 6 of them. In 21 complete tetrads from the second cross growth on pyruvate segregated $2^+:2^-$ and the Ura^+ phenotype cosegregated with the positive growth on pyruvate. These results demonstrate that pMV8 had integrated into the genome at a site tightly linked to PCK1 locus. The fragment *Bgl*II-*Eco*RI of pVM9 containing the disrupted PCK1 gene was introduced into CJM88 (PCK1, ura3) to replace the resident copy of PCK. Uracil prototrophs were isolated and those unable to grow on pyruvate selected. PEPCK activity was not detectable in these clones. Genomic DNA from wild-type and a pck1::URA3 were digested with *Hin*dIII or *Eco*RI and probed with the fragment *Hin*dIII-*Eco*RI (fig.2). The results are in agreement with the expected fragment size of the wild-type and disrupted gene. The transformant with the disrupted gene was crossed with CJM-D2 (PCK1, ura3) and sporulated. In 9 complete tetrads, growth on pyruvate and uracil prototrophy cosegregated $2^+:2^-$. These results allow us to conclude that the PCK1 gene and not a suppressor has been cloned. Moreover, the sequence of the smallest insert [18] codes for a protein with an estimated molecular weight in accordance with the one reported for the PEPCK

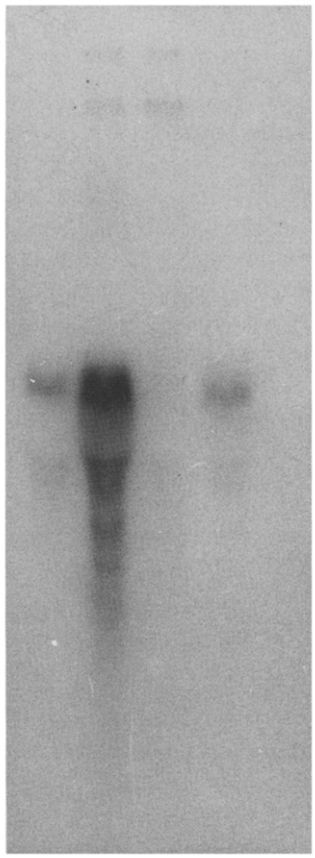

Fig.2. Southern-blot analysis of strains carrying a PCK1 integrating plasmid or a disruption of the chromosomal PCK1 gene. DNA was digested with *Eco*RI (lanes 1, 3, 5) or *Hin*dIII (lanes 2, 4, 6). The probe used was the 2.2 kb *Hin*dIII-*Eco*RI fragment (see fig.1). (Lanes 1 and 2) DNA from X2180 (wild-type); (lanes 3 and 4) DNA from a yeast carrying pMV8; (lanes 5 and 6) DNA from a yeast carrying fragment *Bgl*II-*Eco*RI from pMV9.

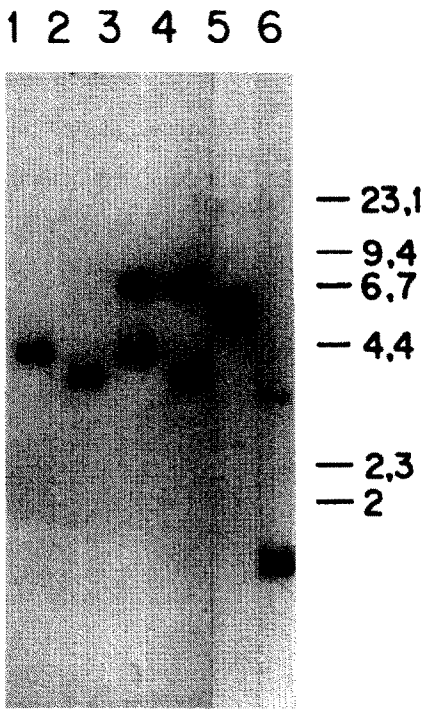

Fig.3. Expression of PCK1 mRNA. Total RNA was extracted as in [24] from glucose repressed or derepressed cultures, treated as in [23] and probed with the *Bam*HI-*Sal*I fragment internal to the PCK1 gene (see fig.1). Size in kb indicated by the arrow was determined by comparison with *S. cerevisiae* ribosomal RNA. (Lane 1) PUK-3B derepressed; (lane 2) PUK-3B/pMV4 derepressed; (lane 3) Id repressed; (lane 4) X2180 derepressed; (lane 5) Id repressed.

subunit [19] and the overall amino acid composition is in accordance with the one published for the yeast enzyme [20] thus indicating that PCK1 is the structural gene for PEPCK.

Extracts from wild-type and mutant cells grown in derepressed conditions contained a 2.1 kb RNA that hybridized with a *Bam*HI-*Sal*I probe internal to the gene (fig.3). In conditions of glucose repression this RNA was not detected (fig.3). It is therefore likely that the PCK1 gene is regulated by differential transcription, although a different stability of the mRNA depending on the carbon source of the medium cannot be excluded. Repression by glucose was as marked in the transformants as in the wild-type. However, in strains transformed with pMV7 activity began to appear when glucose in the medium was still as high as 90 mM.

PEPCK is subject to catabolite inactivation [4,5,19] as well as FbPase [3,14] and malate dehydrogenase [21, 22]. In strains carrying a multicopy plasmid with the PCK1 gene catabolite inactivation of PEPCK and of FbPase was delayed while that of malate dehydrogenase was unaffected (fig.4). This result is parallel to that reported by De la Guerra et al. [23] with strains overproducing FbPase. Taken together these results suggest that PEPCK and FbPase are the substrates of the same inactivating system while a different one would be operative with malate dehydrogenase.

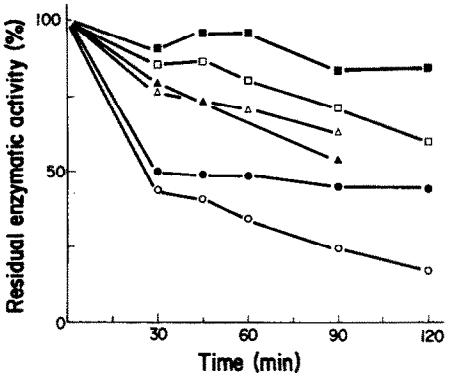

Fig.4. Catabolite inactivation of PEPCK, FbPase and malate dehydrogenase in yeast strains carrying multicopy or integrative plasmids with the PCK gene. Inactivation was performed as described [23]. Squares, PEPCK; circles, FbPase; triangles, malate dehydrogenase. Open symbols, integrative plasmid pMV8; filled symbols, multicopy plasmid pMV1. During the time of the experiment the content of total protein in the cells did not vary. Initial specific activities (mμ/mg) for strains carrying the plasmid pMV8 were: PEPCK, 500; FbPase, 60; malate dehydrogenase, 2000; and for strains carrying pMV1: PEPCK, 3000; FbPase, 100; malate dehydrogenase, 4000.

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