Schizosaccharomyces pombe possesses an unusual and a conventional hexokinase: biochemical and molecular characterization of both hexokinases

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Abstract Two hexokinases were characterized in *Schizosaccharomyces pombe*: hexokinase 1, with a low phosphorylation coefficient on glucose (K_m 8.5 mM) and hexokinase 2, a kinetically conventional hexokinase. Genes *hxk1*$^+$ and *hxk2*$^+$ encoding these enzymes were cloned and sequenced. Disruption of *hxk1*$^+$ had no effect on growth but disruption of *hxk2*$^+$ doubled the generation time in glucose. Spores carrying the double disruption *hxk1*$^+$ *hxk2*$^+$ did not grow on glucose or fructose after one week. Expression of *hxk1*$^+$ increased strongly during growth in fructose or glycerol. Expression of *hxk2*$^+$ was highest during growth in glycerol. A NADP-dependent glucose dehydrogenase was detected, but not a glucokinase.

Key words: Hexokinase; Sugar phosphorylation; Fructose; Glucose; *Schizosaccharomyces pombe*

1. Introduction

Hexokinases initiate the metabolism of sugars in a variety of organisms and have also important regulatory functions. In mammals glucokinase acts as a glucose sensor in the pancreas and participates in the regulation of insulin secretion [1]. In *Saccharomyces cerevisiae*, hexokinases interact with sugar transporters and influence their kinetic characteristics [2] and hexokinase II participates in the transcriptional control of some genes repressed by glucose [3,4]. The inhibition of hexokinases by trehalose-6-P [5] appears to play a role in the control of the glycolytic flux in *S. cerevisiae*. Since trehalose-6-P does not affect glucose phosphorylating activity in extracts of *Schizosaccharomyces pombe* [6] we have initiated a study of the characteristics of the hexokinase(s) of this yeast. Surprisingly, although *S. pombe* has been widely used in molecular and cell biology studies, there is scarce information on its glycolytic enzymes and their regulation [7]. We present here our results on the isolation of the genes encoding two hexokinases from this yeast, their expression and the kinetic characteristics of the encoded enzymes.

2. Materials and methods

2.1. Yeast strains and culture conditions

The *S. pombe* strain PB003, *h*$^+$ *ade6-M216 leu1-32 ura4-D18* and its isogenic *h*$^-$ strain PB004 were used in this study. The following *S. cerevisiae* strains were used: W303-1A MATa *ade2 leu2,3-112, ura3-52 trp1 his3* [8]. Strain WF8-2C, *MAT***a** *leu2-1 ura3-52 hxk1::LEU2 hxk2::LEU2*, carrying disruptions in the *HXK1* and *HXK2* genes was obtained from a cross between DFY568 [9] and W303-1A. Strain THG1, *MAT***a** *leu2-1 ura3-52 hxk1::LEU2 hxk2::LEU2 glk1::LEU2 lys2*, carrying disruptions in genes *HXK1*, *HXK2* and *GLK1* was obtained from a cross between WF8-2C and DFY582 [9].

S. pombe was grown in a complete medium or in a synthetic medium [10] with 2% glucose, fructose or 3% glycerol as carbon sources and the adequate auxotrophic requirements. *S. cerevisiae* was grown on minimal medium with 2% fructose, glucose or glycerol as carbon sources and the adequate auxotrophic requirements. Crosses, sporulation and tetrad dissection were carried out by standard procedures.

2.2. Enzyme fractionation

Cell free extracts were obtained with glass beads as described in ref. [5] using 10 mM potassium phosphate pH 7 with 1 mM PMSF. To the extract protamine sulfate dissolved in the same buffer was added to a final concentration of 0.1%. After 30 min centrifugation in the cold at 18,000 × *g*, the supernatant was dialyzed for 3 h against extraction buffer and loaded into an hydroxylapatite (BioRad HT) column. Elution was carried out with a gradient of phosphate pH 7 between 10 and 100 mM. When necessary, the fractions containing activity were concentrated using an Amicon Centriplus TM (model 50) membrane. Gel filtration was carried out in a Sephadex G-200 column equilibrated with 50 mM NaCl in extraction buffer.

2.3. Enzyme determinations

Hexose phosphorylating activity was determined spectrophotometrically using a coupled system with phosphoglucose isomerase and glucose-6-P dehydrogenase [11] or with 2.5 mM PEP, 0.25 mM NADH and 1 unit each pyruvate kinase and lactate dehydrogenase. Kinetic constants were determined graphically using the Eadie-Hofstee transformation. Protein was assayed as in [12] with a commercial reagent (Pierce).

2.4. Bacterial strains, DNA and RNA manipulations

Escherichia coli TG1 was used for plasmid multiplication. Transformation of *S. pombe* was done as in [10] and transformation of *S. cerevisiae* as in [13]. Genomic DNA from *S. pombe* was obtained as in [14]. Other manipulations were carried out as described in [15]. DNA sequencing was performed by the dideoxy chain termination method of Sanger [16]. All DNA fragments were sequenced in both strands. Total RNA was extracted from 100 mg (wet weight) samples with the Gibco TRIzol reagent [17].

2.5. Cloning and disruption of the hxk1$^+$ and hxk2$^+$ genes from S. pombe

S. cerevisiae strain WF8-2C was transformed with a cDNA *S. pombe* library under the control of the *S. cerevisiae ADH1* promoter [18] and 25000 transformants were selected on glucose plates without uracil. From these, 36 grew on fructose plates without uracil. Two different plasmids, pTP3 and pTP5, were recovered from the transformants. The insert of each plasmid was cloned into the *Not*I site of plasmid pBluescript (Stratagene) to yield plasmids pTP16 and pTP18 (Fig. 2). Disruption was carried out as follows: the *Hind*III site from pBluescript was eliminated and the insert *Not*I–*Not*I from pTP16 and pTP18 introduced to yield pTP22 and pTP23, respectively. The 1.8 kb *Hind*III–*Hind*III fragment from pREP4 containing the *ura4*$^+$ gene [19] was inserted into the *Hind*III site of pTP22 or pTP23 to yield pTP32 and

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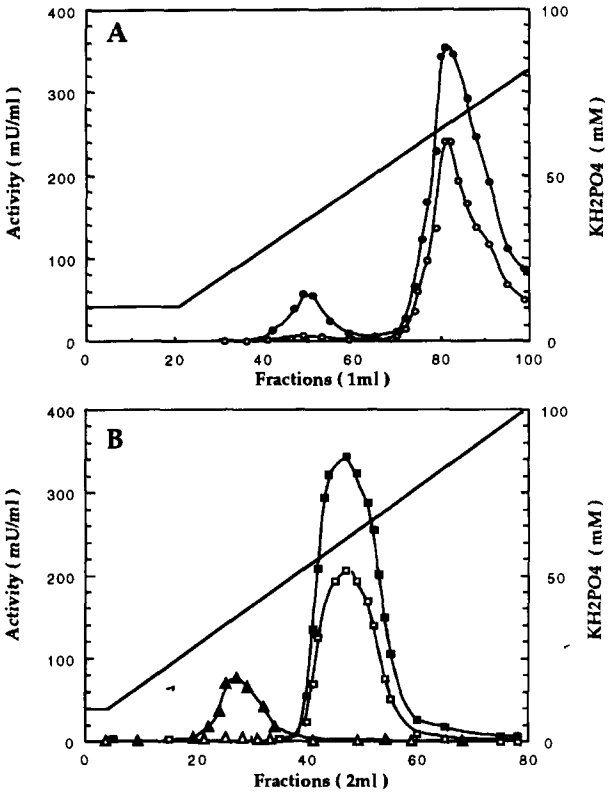

Fig. 1. Elution profile from *S. pombe* hexokinases. A protamine sulfate fraction (see section 2) from wild type, *hxk1* or *hxk2* disruptants was chromatographed on a (6 cm × 2.5 cm^2) column of hydroxylapatite and eluted with a gradient of potassium phosphate. Initial amount of phosphorylating activity on fructose was 10 units (wild type), 7 units (*hxk1* disruptant), and 2 units (*hxk 2* disruptant). The wild type was grown in glucose and the disruptants in fructose; all were harvested in the exponential phase of growth. (A) Wild type. (B) *hxk1* or *hxk2*disruptants. Symbols: circles, wild type; squares, *hxk1* disruptant; triangles, *hxk 2* disruptant. Full symbols, activity on fructose; void symbols, acitivity on glucose.

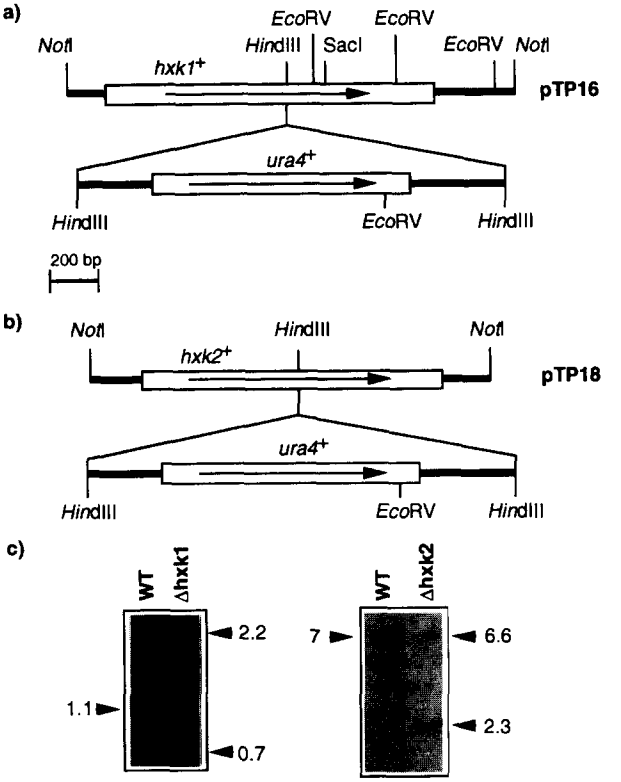

Fig. 2. Structure of the *hxk1*$^+$ and *hxk2*$^+$ regions from *S. pombe* and Southern blot analysis of *hxk1*$^+$ and *hxk2*$^+$ disruptants. The inserts of pTP3 and pTP5 were cloned to give pTP16 (a) and pTP18 (b) (for details see section 2). The coding regions of *hxk1*$^+$ and *hxk2*$^+$ are enclosed in a box. Both *hxk1*$^+$ and *hxk2*$^+$ were interrupted with *ura4*$^+$ (see section 2). Southern analysis of the interruptions is shown in (c). DNA was digested with *Eco*RV and probed with the 1.08 kb *Not*I–*Eco*RV fragment of pTP16 (*hxk1*$^+$) and with the 1.68 kb *Not*I–*Not*I fragment of pTP18 (*hxk2*$^+$). Size of the bands is indicated near the gels. Horizontal arrows indicate direction of transcription.

Table 1
Kinetic properties of the hexokinases from *Schizosaccharomyces pombe*

Enzyme	Compound	Apparent K_m (mM)	Relative maximal rate[1]
Hexokinase 1	Fructose	1.5	100
	Glucose	8.4	11
	Mannose	0.13	23
	2 deoxyglucose	4[2]	–
	Glucosamine	11[2]	–
	ATP	0.6[3]	–
		3.2[4]	–
Hexokinase 2	Fructose	1.5	100
	Glucose	0.16	59
	Mannose	0.07	42
	2 deoxyglucose	0.5[2]	–
	Glucosamine	2.1[2]	–
	ATP	0.4[3]	–

A partially purified fraction of each enzyme eluted from a hydroxylapatite column (see section 2) was used for the determinations. Activity was measured spectrophotometrically as described in section 2.
[1]Determined with 1 mM ATP. [2]Calculated as K_i with fructose as substrate. [3]Determined with 10 mM fructose. [4]Determined with 10 mM glucose.

pTP33, respectively. The *Not*I–*Not*I fragment from pTP32 was integrated into the chromosomal locus of *hxk1*$^+$ in strain PB003, and the *Not*I–*Not*I fragment from pTP33 was integrated into the chromosomal locus of *hxk2*$^+$ in strain PB004. The correctness of the integration was checked by Southern analysis (Fig. 2).

3. Results and discussion

3.1. Biochemical characterization of hexokinases from S. pombe

Chromatography of an extract from *S. pombe* through an hydroxylapatite column yielded two peaks of enzymatic phosphorylating activity when fructose was used as substrate, but only one of them showed significant activity on glucose (Fig. 1A). The kinetic characteristics of both activities are shown in Table 1. Both are hexokinases as they phosphorylate glucose, fructose and mannose; however, hexokinase 1 is clearly different from other hexokinases studied [20,21] as it presents a low affinity for glucose and some of its analogues. Hexokinase 2 behaves kinetically as a conventional hexokinase [20]. None of the activities was inhibited by trehalose-6-P thus confirming an earlier observation by Blázquez et al. [6]. In gel filtration experiments, hexokinase 1 showed a molecular weight of ca. 53 kDa and hexokinase 2 one of ca. 50 kDa.

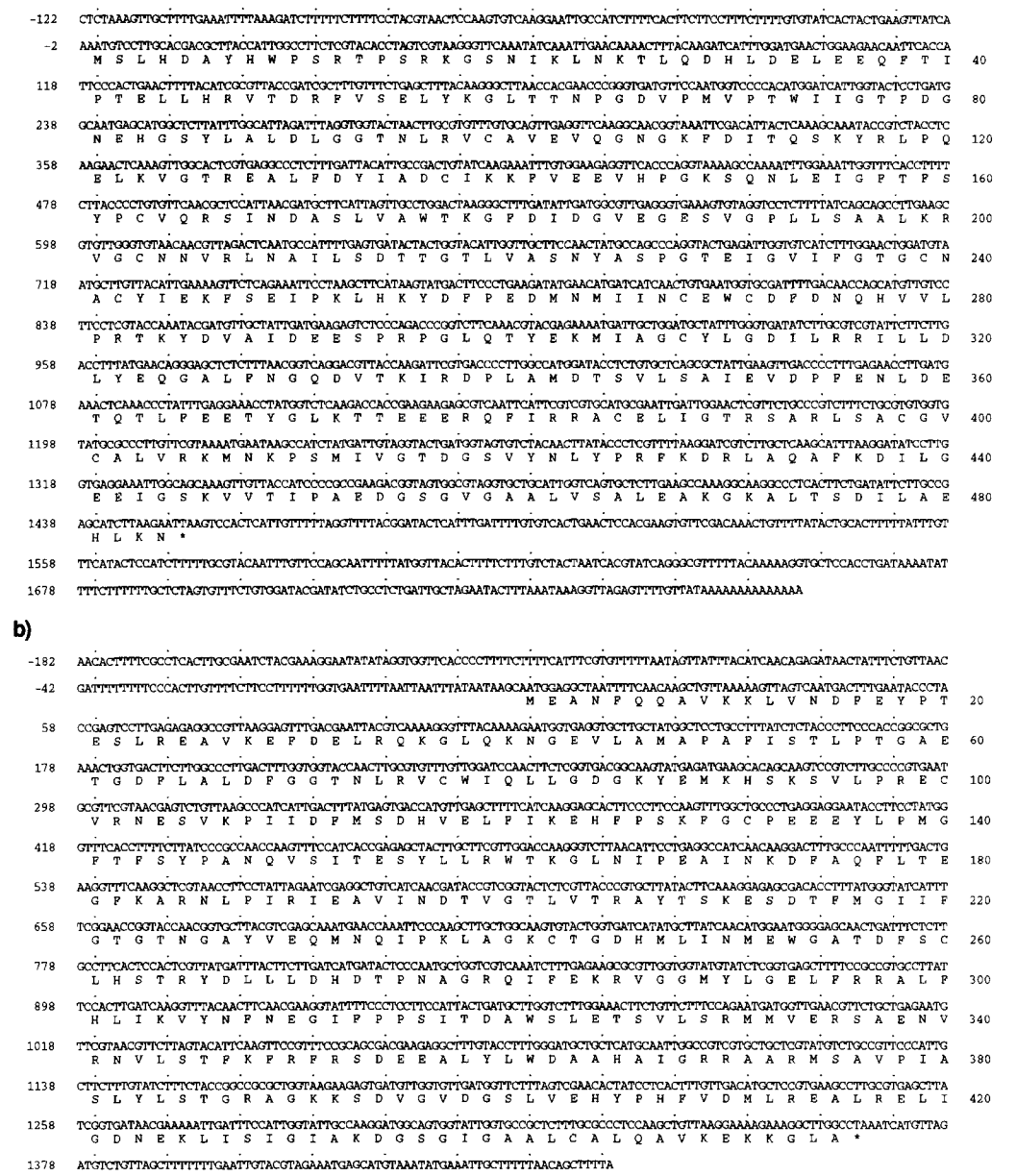

Fig. 3. Nucleotide sequences of genes *hxk1*$^+$ and *hxk2*$^+$ from *S. pombe* and predicted amino acid sequences. Accesion numbers in EMBL data bank: *hxk1*$^+$, X92894; *hxk2*$^+$, X92895.

3.2. *Cloning and sequencing of the genes encoding hexokinase 1 and hexokinase 2*

Functional complementation for growth on fructose of a *S. cerevisiae hxk1 hxk2* mutant by a *S. pombe* cDNA library was used to isolate DNA fragments encoding hexokinases 1 and 2. Plasmids pTP3 or pTP5 containing the two different inserts obtained (see section 2 and Fig. 2) were used to retransform *S. cerevisiae* THG1 carrying disruptions in the *HXK1*, *HXK2* and *GLK1* genes. The kinetic characteristics and the chromatographic behaviour of the phosphorylating activities of the transformed yeasts were studied. The one encoded by the DNA fragment in pTP3 had the same characteristics as hexokinase 1 and that encoded by pTP5 behaved as hexokinase 2. Therefore these plasmids carry the genes *hxk1*$^+$ and *hxk2*$^+$, respectively.

The sequences of the coding regions of genes *hxk1*$^+$ and *hxk2*$^+$ are shown in Fig. 3. For *hxk1*$^+$, an open reading frame of 1452 bp encoding a protein of a predicted molecular weight of 53 kDa was identified. For *hxk2*$^+$, the open reading frame had 1365 bp and could encode a protein with a predicted molecular weight of 50 kDa. From the molecular weights found by gel filtration it may be concluded that both hexokinases exist in the cell as monomers.

The amino acid sequences presented a high overall homology with the hexokinases of *S. cerevisiae*: 50% in the case of *hxk1*$^+$, and 38% in the case of *hxk2*$^+$. There was also a 40% homology

with glucokinase. A phylogenetic analysis (Fig. 4) indicated that the hexokinases from *S. pombe* were not derived from a recent duplication of a common ancestral gene in contrast to what is suggested for the *S. cerevisiae* isoenzymes. More likely, *S. pombe* hexokinase 2 diverged from the rest of yeast hexokinases as early as *S. cerevisiae* glucokinase did.

3.3. *Expression of* hxk1$^+$ *and* hxk2$^+$

The steady state levels of the mRNA corresponding to genes *hxk1*$^+$ and *hxk2*$^+$ were determined in cells grown in different carbon sources and harvested during the exponential and stationary phases of growth (Fig. 5). Only one band was detected in all cases. Both *hxk1*$^+$ and *hxk2*$^+$ were highly expressed during growth in glycerol. *hxk1*$^+$ was also highly expressed during the exponential phase of growth in fructose but the signal decreased strongly during the stationary phase. Thus glucose represses maximal expression of both genes. This – apparently illogical – result parallels the findings of Herrero et al. [22] who reported that the expression of *HXK1* and *GLK1* in *S. cerevisiae* is glucose repressible.

3.4. *Effects of disruption of the* hxk1$^+$ *and* hxk2$^+$ *genes*

Southern blot analysis showed that only one chromosomal copy of *hxk1*$^+$and *hxk2*$^+$ is present in the genome of the *S. pombe* strain used (result not shown). We disrupted the genes *hxk1*$^+$ and *hxk2*$^+$ by insertion of the *S. pombe ura4*$^+$ gene (Fig. 2). Chromatography on hydroxylapatite of extracts from strains carrying one of the disruptions produced the expected elution profile (Fig. 1B). Disruption of *hxk1*$^+$ did not affect growth in fructose but increased the generation time in glucose from 230 to 520 min. Disruption of *hxk2*$^+$ did not affect growth in glucose or fructose. These results are in agreement with the kinetic properties found in vitro for the corresponding enzymes.

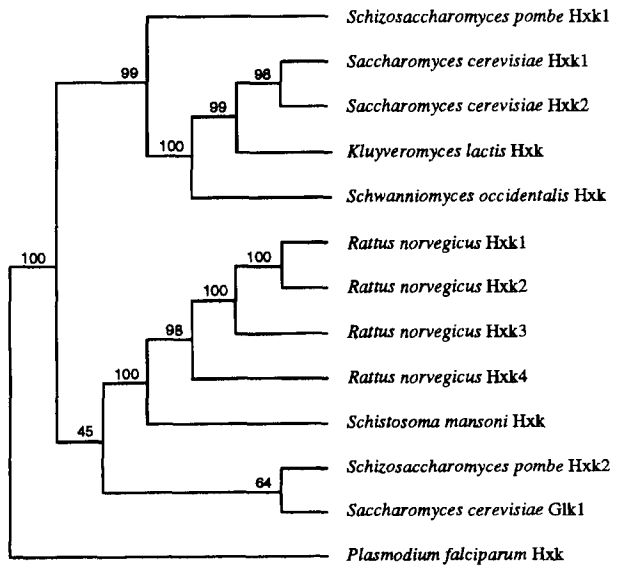

Fig. 4. Sequence relationships from several hexokinases. The amino acid sequences of the various enzymes were aligned with the CLUSTAL V program [24]. A maximum parsimony consensus tree (861 bootstrap resamplings) was obtained with the PHYLIP 3.5 package [25]. The sequences were retrieved from the SWISSPROT database. In the case of the rat enzymes only the C-terminal moiety of the sequences was used. Numbers at the intersections indicate the bootstrap value in percentage.

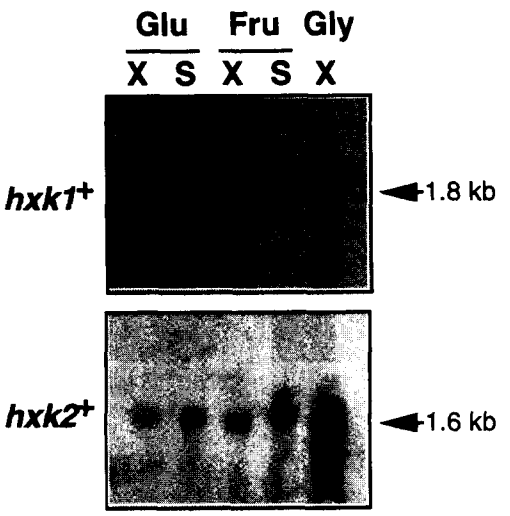

Fig. 5. Expression of *hxk1*$^+$ and *hxk2*$^+$ during growth in different carbon sources. Wild type cells were grown in glucose (Glu), fructose (Fru) or glycerol (Gly) and harvested in the exponential (X) or stationary (S) phase of growth. RNA was extracted as indicated in section 2. 10 μg of RNA were applied to each lane. The probes used were the 0.2 kb *Hin*dIII–*Sac*I fragment of pTP16 (*hxk1*$^+$) and the 1.68 kb *Not*I–*Not*I fragment of pTP18 (*hxk2*$^+$). The similarity in RNA amount present in each lane was checked by visual inspection of ethidium bromide stained rRNAs.

We tried to obtain a double *hxk1 hxk2* disruptant by crossing a *hxk1::ura4*$^+$ and a *hxk2::ura4*$^+$ strain. If the double disruptant grew in glucose, a $4^+:0^-$ segregation for growth on glucose would be expected. If it did not, the segregations expected would be: $4^+:0^-$ (parental ditype, PD), $3^+:1^-$ (tetratype, T) and $2^+:2^-$ (non-parental ditype, NPD). Taking into account the *ura4*$^+$ marker in both disruptions, all spores from PD tetrads should be prototrophic for uracil and one of the positive spores in T tetrads should be auxotrophic for uracil. This pattern was observed in 10 complete tetrads analyzed. One week after micromanipulation no growth was seen from spores that according to genetic analysis carried the double disruption. This shows that the double disruptant does not grow significatively on glucose.

3.5. *Detection of a NADP dependent glucose dehydrogenase*

During our assays of hexokinase in extracts using the phosphoglucose isomerase and glucose-6-P dehydrogenase coupled method (see section 2) we noticed a reduction of NADP in the presence of glucose that was not dependent of the addition of ATP or glucose-6-P dehydrogenase. This reduction disappeared after boiling and was not observed when fructose or NAD were used. These results indicated the existence of a NADP dependent glucose dehydrogenase. While this work was in progress Tsai et al. [23] published the purification of a glucose dehydrogenase from *S. pombe* with similar characteristics as the ones observed by us. We could not detect this activity in extracts of *S. cerevisiae* or *Kluyveromyces lactis*.

The results presented show that in *S. pombe* only the two hexokinases reported here are physiologically significant for growth in glucose or fructose. The physiological role of the

NADP dependent glucose dehydrogenase remains to be established as the yeast did not grow significantly in glucose or fructose in the absence of hexokinases. It was also shown that no glucokinase exists in the fission yeast. The differences in kinetic and regulatory properties of the *S. pombe* hexokinases and those of *S. cerevisiae* indicate that deep differences in the regulation of glycolysis could exist between these two yeast species.

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