Pyruvate kinase (Pyk1) levels influence both the rate and direction of carbon flux in yeast under fermentative conditions

Amanda K. Pearce,^{1†} Kay Crimmins,^{1‡} Evelyne Groussac,²
Michael J. E. Hewlins,³ J. Richard Dickinson,⁴ Jean Francois,²
Ian R. Booth¹ and Alistair J. P. Brown¹

Author for correspondence: Alistair J. P. Brown. Tel: +44 1224 273183. Fax: +44 1224 273144.
e-mail: al.brown@abdn.ac.uk

¹ Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

² Centre de Bioingenierie Gilbert Durand, UMR-CNRS 5504 UR-INRA 792, Département de Génie Biochimique et Alimentaire, Institut National des Sciences Appliquées, 31077 Toulouse Cedex 04, France

³ Department of Chemistry, Cardiff University, PO Box 912, Cardiff CF10 3TB, UK

⁴ Cardiff School of Biosciences, Cardiff University, PO Box 915, Cardiff CF10 3TL, UK

Yeast phosphofructo-1-kinase (Pfk1k) and pyruvate kinase (Pyk1) are allosterically regulated enzymes that catalyse essentially irreversible reactions in glycolysis. Both the synthesis and activity of these enzymes are tightly regulated. To separate experimentally the control of Pfk1k and Pyk1 synthesis from their allosteric regulation, a congenic set of PFK1, PFK2 and PYK1 mutants was constructed in which these wild-type coding regions were driven by alternative promoters. Mutants carrying PGK1 promoter fusions displayed normal rates of growth, glucose consumption and ethanol production, indicating that the relatively tight regulation of Pyk1 and Pfk1k synthesis is not essential for glycolytic control under fermentative growth conditions. Mutants carrying fusions to an enhancer-less version of the PGK1 promoter (PGK1_{Δ767}) expressed Pyk1 and Pfk1k at about 2.5-fold lower levels than normal. Physiological and metabolic analysis of the PFK1 PFK2 double mutant indicated that decreased Pfk1k had no significant effect on growth, apparently due to compensatory increases in its positive effector, fructose 2,6-bisphosphate. In contrast, growth rate and glycolytic flux were reduced in the PGK1_{Δ767}-PYK1 mutant, which had decreased Pyk1 levels. Unexpectedly, the reduced Pyk1 levels caused the flow of carbon to the TCA cycle to increase, even under fermentative growth conditions. Therefore, Pyk1 exerts a significant level of control over both the rate and direction of carbon flux in yeast.

Keywords: Glycolysis, metabolic flux, pyruvate kinase, phosphofructokinase, yeast physiology

INTRODUCTION

The glycolytic pathway is arguably the best characterized metabolic pathway in *Saccharomyces cerevisiae* (reviewed by Johnston & Carlson, 1992; Dickinson, 1999). The glycolytic enzymes can comprise up to 30–50% of total soluble protein during growth on a fermentable carbon source (Hess *et al.*, 1969; Fraenkel,

1982; Gancedo & Serrano, 1989). Most yeast glycolytic enzymes have been purified and their biochemical properties characterized, and many have had their crystal structures determined. All of the yeast glycolytic genes have been isolated and sequenced, and their regulation has been the subject of intense investigation. However, our understanding of the factors that control metabolic flux through this pathway *in vivo* under 'normal' physiological conditions remains relatively limited.

Once hexoses have been transported into the yeast cell and phosphorylated, they have several alternative metabolic routes. Hexose phosphates may be utilized for trehalose, glycogen or cell-wall biosynthesis, or catabolized via the glycolytic or pentose phosphate pathways.

[†] **Present address:** MRC Radiation and Genome Stability Unit, Harwell, Didcot, Oxfordshire OX11 0RD, UK.

[‡] **Present address:** Quest International, Menstrie, Clackmannanshire SK11 7ES, UK.

Abbreviations: MCA, metabolic control analysis; Pfk1k, 6-phosphofructo-1-kinase; Pyk1, pyruvate kinase.

The first glycolytic-specific reaction involves the generation of fructose 1,6-bisphosphate. Most glycolytic enzymes catalyse reversible reactions that are also utilized for the opposing pathway, gluconeogenesis. Therefore, early studies on glycolytic regulation focused on those enzymes that catalyse essentially irreversible reactions in this pathway and hence are specific to glycolysis such as 6-phosphofructo-1-kinase (Pfk1k) and pyruvate kinase (Pyk1). Both of these enzymes are subject to significant allosteric regulation (Hunsley & Suelter, 1969; Bañuelos *et al.*, 1977; Avigad, 1981; Bartrons *et al.*, 1982; Fraenkel, 1982; Arvantidis & Heinisch, 1994; Heinisch *et al.*, 1996). Hence, Pfk1k and Pyk1 were viewed as catalysing rate-limiting steps in glycolysis; this view still dominates conventional thinking. In addition, the genes encoding Pfk1k and Pyk1 appear to be regulated more tightly than other glycolytic genes. Pyk1 is a homotetramer, the monomer being encoded by *PYK1* (Burke *et al.*, 1983). Pfk1k is a heterooctamer, the α and β subunits being encoded by *PFK1* and *PFK2* (Heinisch, 1986). The *PYK1*, *PFK1* and *PFK2* genes appear to be regulated at both the transcriptional and translational levels (Moore *et al.*, 1990a, b, c, 1991). From a genetic viewpoint, it seemed attractive to suggest that these regulatory phenomena might contribute to the control of glycolysis.

Metabolic control analysis (MCA) suggests that all enzymes in a biochemical pathway contribute to some extent to metabolic flux through that pathway (Kacser & Burns, 1973, 1979; Fell, 1997). Under some specific circumstances a particular enzyme might exert a high degree of control over the flux through a pathway and hence be rate limiting with respect to this flux. However, this is unlikely to be the case for enzymes that are subject to allosteric regulation, because according to MCA theory, a consequence of allosteric regulation is the potential transfer of some flux control to other steps in the pathway (Kacser & Burns, 1973, 1979; Fell, 1984, 1997). This view is reinforced by the general observation that the overexpression of regulatory enzymes subject to feedback inhibition did not significantly increase flux through the corresponding pathways (Fell, 1997), and the specific observation that the overexpression of individual glycolytic enzymes in yeast did not increase fermentation rates significantly (Schaaff *et al.*, 1989; Davies & Brindle, 1992). Hence, from an MCA perspective, the Pfk1k and Pyk1 enzyme levels, controlled in part through *PYK1*, *PFK1* and *PFK2* gene regulation, are unlikely to exert a high level of control over glycolytic flux.

To examine these contrasting views of glycolytic regulation in yeast, we have studied the consequences of (a) disturbing the transcriptional and post-transcriptional regulation of the *PYK1*, *PFK1* and *PFK2* genes and (b) altering Pyk1 and Pfk1k levels upon yeast physiology. The data suggest that gene regulation plays a minor role in the control of glycolytic flux under fermentative growth conditions, and that Pyk1 levels influence both the rate and direction of carbon flux in yeast.

METHODS

Growth conditions. Strains were grown in YPD (2%, w/v, glucose; 2%, w/v, bacto-peptone; 1%, w/v, yeast extract), YPL (3%, v/v, lactate; 2%, w/v, bacto-peptone; 1%, w/v, yeast extract), GYNB (2%, w/v, glucose; 0.65%, w/v, yeast nitrogen base without amino acids) or SC (Strathern *et al.*, 1994).

Strain construction. All of the glycolytic mutants constructed in this study were made in *S. cerevisiae* W303-1B (Thomas & Rothstein, 1989). To generate these mutants (Table 1), promoterless or promoter-containing 3'-truncated versions of the *PYK1*, *PFK1* and *PFK2* genes were integrated into the corresponding chromosomal locus (Fig. 1). To achieve this, nine plasmids were constructed in which the three glycolytic 3'-truncated ORFs lacked a promoter or were fused to the *PGK1* or *PGK1*_{Δ767} promoters (Ogden *et al.*, 1986). All of the necessary PCR amplifications were performed using *Pfu* polymerase (Stratagene). The nine plasmids were transformed

Table 1. Strains used in this study

Strain	Genotype
Parental strain	
W303-1B	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i>
<i>PGK1</i> promoter fusions	
YKC1	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1p-PYK1-URA3</i>
YKC2	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1p-PFK1-HIS3</i>
YKC3	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1p-PFK2-TRP1</i>
YKC4	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1p-PFK1-HIS3 PGK1p-PFK2-TRP1</i>
YKC5	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1p-PFK1-HIS3 PGK1p-PFK2-TRP1</i> <i>PGK1p-PYK1-URA3</i>
<i>PGK1</i>_{Δ767} promoter fusions	
YKC11	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1_{Δ767}-PYK1-URA3</i>
YKC12	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1_{Δ767}-PFK1-HIS3</i>
YKC13	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1_{Δ767}-PFK2-TRP1</i>
YKC14	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1_{Δ767}-PFK1-HIS3 PGK1_{Δ767}-PFK2-TRP1</i>
YKC15	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>PGK1_{Δ767}-PFK1-HIS3 PGK1_{Δ767}-PFK2-TRP1</i> <i>PGK1_{Δ767}-PYK1-URA3</i>
Promoterless fusions	
YKC21	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>Δpyk1-URA3</i>
YKC22	<i>MATα ade1 ade2 leu2 his3 ura3 trp1 Δpfk1-HIS3</i>
YKC23	<i>MATα ade1 ade2 leu2 his3 ura3 trp1</i> <i>Δpfk2-TRP1</i>
YKC24	<i>MATα ade1 ade2 leu2 his3 ura3 trp1 Δpfk1-HIS3</i> <i>Δpfk2-TRP1</i>
YKC25	<i>MATα ade1 ade2 leu2 his3 ura3 trp1 Δpfk1-HIS3</i> <i>Δpfk2-TRP1 Δpyk1-URA3</i>

Fig. 1. Construction of the YKC mutants. (a) Cartoon illustrating structure of the YKC mutations. The *PYK1*, *PFK1* or *PFK2* coding region is indicated by a white box; wild-type promoter, thick black line; *PGK1* promoter, black box with UAS marked; *PGK1* Δ 767, promoter with UAS deleted. Promoterless mutants were also created (not shown). The resultant strains are listed in Table 1. (b) Cartoon illustrating the construction of the YKC mutants. Ylp-based plasmids containing the appropriate promoter fused to a 3'-truncated version of the appropriate coding region were integrated at the corresponding chromosomal locus to generate a truncated gene with its wild-type promoter and a wild-type coding region (grey) with its new promoter (black). (c) Southern blot confirming correct single-copy integration of pPGK-PFK1, pPGK Δ 767-PFK1 and pPFK1 to generate YKC2, YKC12 and YKC22, respectively: WT, W303-1B; 2, YKC2; 12, YKC12; 22, YKC22. Genomic DNA was digested with *SacI* (S) or *EcoRI* (E) and probed with the *Bam*HI-*Sac*II *PFK1* insert from pPFK1. Positions of size markers (kb) are shown. Compared to W303-1B, each mutant has additional bands of the expected lengths. Southern analyses for the other mutants is presented in Crimmins (1995).

Table 2. PCR primers used in this study

Restriction sites are underlined.

Primer	Sequence (5'-3')	Restriction site	Coordinates	ORF length
5'PYK1	CAAGTAAAGGATCCCTAACAATGTCTAGATTAG	<i>Bam</i> HI	-5	-
3'PYK1	ACTGGCTTACCGCGGAAGTTAGACTTAG	<i>Sac</i> II	+882	1500
5'PFK1	TCTGAAACAAAATCGGATCCAAGATGCAATCTC	<i>Bam</i> HI	-3	-
3'PFK1	CAATTCATCAACCGCGGATGGCCATTTCG	<i>Sac</i> II	+987	2961
5'PFK2	CAAGAACTAAGGATCCACAATGACTGTTAC	<i>Bam</i> HI	-3	-
3'PFK2	CCGACAGTACCGCGGATATTCAAATG	<i>Sac</i> II	+1033	2877
5'PGK1	TTTAGCTTCCAAGCTTCAACTCAAGACGCACAG	<i>Hind</i> III	-	-
3'PGK1	AAGATAAAGACATAGATCTTATATTTGTTG	<i>Bgl</i> II	-	-

into *S. cerevisiae* (Gietz & Woods, 1998) and single-copy integration at the appropriate glycolytic locus confirmed by Southern analysis.

The first 882 bp of the *PYK1* ORF, 987 bp of the *PFK1* ORF and 1033 bp of the *PFK2* ORF were PCR amplified using the primers described in Table 2, cloned as *Bam*HI-*Sac*II fragments into pRS306, pRS303 and pRS304, respectively (Sikorski & Hieter, 1989) and resequenced. This generated the promoterless constructs pPYK1, pPFK1 and pPFK2. The *PGK1* promoter was isolated as a 1.5 kb *Eco*RI-*Bgl*II fragment from pMA91 (Mellor *et al.*, 1985). This fragment was cloned into pPYK1, pPFK1 and pPFK2 to generate the *PGK1* fusions in the plasmids, pPGK-PYK1, pPGK-PFK1 and pPGK-PFK2, respectively. The *PGK1* Δ 767 promoter was PCR amplified from pMA767 (Ogden *et al.*, 1986) using the primers 5'PGK1 and 3'PGK1 (Table 2) and cloned as a 0.78 kb *Hind*III-*Bgl*II

fragment into pLH1 (Amersham). This *Hind*III-*Bgl*II fragment was then cloned into pPYK1 to generate the plasmid pPGK Δ 767-PYK1. To avoid multiple *Hind*III restriction sites in pPFK1 and pPFK2, the *PGK1* Δ 767-PFK fusions were created in two stages. First, the *Hind*III-*Bgl*II *PGK1* Δ 767 fragment and the *Bam*HI-*Sac*II *PFK1* fragment were cloned between the *Hind*III and *Sac*II sites in pRS306 using a three-way ligation. Then the resultant *PGK1* Δ 767-PFK1 fusion was cloned as a *Xho*I-*Sac*II fragment into pRS303 to make pPGK Δ 767-PFK1. The same approach was used to generate pPGK Δ 767-PFK2 from pRS304.

The three *PYK1* plasmids, pPYK1, pPGK-PYK1 and pPGK Δ 767-PYK1, were linearized within the 3'-truncated ORF using *Bgl*II and transformed into W303-1B, selecting for the *URA3* marker. The three *PFK1* plasmids were linearized with *Xba*I and W303-1B transformants selected using the *HIS3* marker. The three *PFK2* plasmids were linearized with *Stu*I

and W303-1B transformants selected using *TRP1*. Double and triple mutants were generated by transforming single and double mutants with the appropriate plasmids.

Southern, Northern and Western blotting. *S. cerevisiae* DNA was isolated according to the protocol of Hoffman & Winston (1987) and subjected to Southern blotting (Church & Gilbert, 1984; Wicksteed *et al.*, 1994). Standard methods were used for yeast RNA preparation and Northern analysis (Brown, 1994; Planta *et al.*, 1999). Signals were quantified by phosphor-imaging, and mRNA levels were measured relative to the *ACT1* mRNA which acted as an internal control for variations in RNA loading on gels (Moore *et al.*, 1991; Planta *et al.*, 1999). Hybridization probes were as follows: *PFK1*, the 3.2 kb *EcoRI* fragment from pPFK1 (Heinisch, 1986); *PFK2*, the 3 kb *EcoRI* fragment from pPFK2 (Heinisch, 1986); *PYK1*, the 0.89 kb *BamHI*–*SacII* fragment from pPYK1; *ACT1*, the 1.5 kb *HindIII*–*EcoRI* fragment from pSPACT9 (Moore *et al.*, 1991). Random-primed labelling of DNA fragments was carried out using [α - 32 P]dCTP with the Pharmacia Ready-To-Go DNA Labelling Kit, which is based on the method of Feinberg & Vogelstein (1983).

For extraction of total soluble protein from yeast, 50 ml cells was grown to mid-exponential phase in YPD or SC at 30 °C. Cells were harvested at 3000 g for 5 min, washed twice in 1 M sorbitol and resuspended in 200–300 μ l extraction buffer [20 mM HEPES, pH 7.5, 0.2 mM EDTA, 1.5 mM MgCl₂, 25 % (w/v) glycerol, 0.5 mM DTT, Complete protease inhibitor cocktail tablets (Boehringer Mannheim)]. Samples were vortexed with glass beads six times for 1 min with 1 min intervals on ice between vortexing and centrifuged at 14000 g for 15 min at 4 °C. Supernatants were stored at –80 °C in extraction buffer containing 2 mM fructose 2,6-bisphosphate (Reibstein *et al.*, 1986). Protein extracts were electrophoresed on SDS-polyacrylamide gels and Western analysis performed using published protocols (Laemmli, 1970; Towbin *et al.*, 1979). Nitrocellulose membranes were probed using a polyclonal antiserum against yeast Pfk (Heinisch *et al.*, 1996). The second antibody was anti-rabbit IgG–alkaline phosphatase conjugate (Boehringer Mannheim).

Enzyme assays. Pyruvate kinase assays were carried out as described previously (Hunsley & Suelter, 1969; Yun *et al.*, 1976). Phosphofructo-1-kinase assays were carried out in duplicate using previously published procedures (Racker, 1947; Reibstein *et al.*, 1986). Protein determinations were performed using the Bradford assay (Bradford, 1976).

Metabolite assays. Glucose, ethanol and glycerol assays were carried out in triplicate using Boehringer Mannheim kits. Methylglyoxal assays were performed in triplicate using the procedure of Ferguson *et al.* (1995). Measurements of intracellular metabolites (glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate and ATP) after a glucose pulse to yeast cells were performed by coupling with NADH production or consumption as described by Bergmeyer (1986). Fructose 2,6-bisphosphate was measured according to Van Schaftingen *et al.* (1982). For the glucose-pulse experiment, yeast strains were grown to an OD₆₀₀ of 2.0 in GYNB, harvested by centrifugation, washed once with water and resuspended at an OD₆₀₀ of 5.0 in 25 mM potassium phosphate buffer, pH 6.0. After 30 min pre-incubation, glucose was added to 1 %, and 5 ml samples were collected at various times and quenched in cold methanol. Metabolites were extracted in buffered ethanol as described in Gonzalez *et al.* (1997). Errors for metabolite assays were between 5 and 10 %.

NMR. Briefly, cells were grown to an OD₆₀₀ of 0.6 and labelled with 20 mg [1- 13 C]glucose ml^{–1} (99 atom %; Sigma) in SC

medium for 45 min at 30 °C. The cells were harvested by centrifugation and perchloric acid extracts were prepared for 13 C NMR as described previously (Dickinson & Hewlins, 1988, 1991). The spectra were recorded as described by Dickinson *et al.* (1997) using a Bruker AMX360 spectrometer operating at 90.5 MHz using 32 K data points over 22 kHz with Waltz-16 1 H decoupling and by the DEPT method to determine the number of protons attached to each carbon atom. Signals were identified by comparison with spectra of standard compounds recorded under identical conditions. Standard Bruker software (UXNMR) was used throughout. Chemical shifts were reported relative to external tetramethyl silane in C 2 HCl₃. Addition of sodium trimethylsilylpropane-sulphonate gave a methyl signal at –2.6 p.p.m. under the conditions used here.

RESULTS

Construction of *PYK1*, *PFK1* and *PFK2* mutants

Previously, one of our groups had reported that the expression of the *PYK1* and *PFK2* genes is more tightly regulated than other glycolytic genes. *PYK1* and *PFK2* mRNA levels are induced to greater extents by glucose, and these mRNAs also appear to be co-regulated at the translational level (Moore *et al.*, 1990a, b, c, 1991). Therefore, we were interested in establishing the importance of this regulation to the control of glycolysis. Hence in this study, our first aim was to examine the effects of disrupting the normal regulation of the *PYK1*, *PFK1* and *PFK2* genes. The transcriptional and translational regulation of *PYK1* is dependent upon its promoter and 5' leader sequence (Moore *et al.*, 1990a, b, c, 1991). Therefore these sequences were replaced with those of the *PGK1* gene since this gene is not subject to the same degree of regulation as *PYK1* (Moore *et al.*, 1991). Analogous mutations were also made for *PFK1* and *PFK2* (Fig. 1).

A second aim was to establish the effects of down-regulating Pyk1 and Pfk1 levels. To address this aim, a

Table 3. *PYK1* expression levels during growth on glucose

Numbers in parentheses are percentage of wild-type.

Strain	Relevant genotype	Relative <i>PYK1</i> mRNA level*	Pyruvate kinase specific activity†
W303-1B	Wild-type	3.0 (100)	3.2 ± 0.09 (100)
YKC1	<i>PGK1p</i> – <i>PYK1</i>	4.5 (150)	3.6 ± 0.04 (125)
YKC11	<i>PGK1</i> _{Δ767} – <i>PYK1</i>	1.3 (43)	0.77 ± 0.25 (24)
YKC21‡	<i>Δpyk1</i>	NM (–)	NM (–)

* *PYK1* mRNA levels were measured relative to the *ACT1* mRNA in arbitrary units.

† Units are μ mol min^{–1} (mg protein)^{–1}.

‡ The *pyk1* null mutant was not viable on glucose. NM, Not measurable.

Fig. 2. Pfk1 expression in YKC mutants. (a) Northern analysis of *PFK1/2* mRNA levels compared to the *ACT1* mRNA. Membranes were probed with the *EcoRI* *PFK2* fragment from pPFK2 (which cross-hybridizes with the *PFK1* mRNA), stripped and reprobed for *ACT1*. Relative *PFK1* mRNA levels are presented in Table 4. Similar data were obtained with the *PFK1* probe (not shown). (b) Western analysis of Pfk1 subunits using polyclonal antiserum against yeast Pfk1. The upper panel shows the Coomassie stained gel, and the lower panel shows the Western blot. Pfk1 and Pfk2 subunits are marked. WT, W303-1B; 4, YKC4 (*PGK1p-PFK1 PGK1p-PFK2*); 11, YKC11 (*PGK1_{Δ767}-PYK1*); 14, YKC14 (*PGK1_{Δ767}-PFK1 PGK1_{Δ767}-PFK2*); 15, YKC15 (*PGK1_{Δ767}-PFK1 PGK1_{Δ767}-PFK2 PGK1_{Δ767}-PYK1*); 22, YKC22 (*Δpfk1*); 23, YKC23 (*Δpfk2*).

second set of mutants was made in which the *PYK1*, *PFK1* and *PFK2* loci were placed under the control of the *PGK1_{Δ767}* promoter and 5' leader sequences. This promoter lacks the *PGK1* upstream activator sequence required for efficient and regulated expression (−389 to −473 with respect to the ATG; Ogden *et al.*, 1986; Piper *et al.*, 1988).

The strategy for the construction of these mutants involved the integration of 3'-truncated ORFs fused to the new promoter region (Fig. 1). Single-copy integration at the correct glycolytic locus was confirmed for each mutant by Southern blotting (e.g. Fig. 1c). This strategy generated a tandemly repeated locus where the native glycolytic promoter drives a 3'-truncated version of the gene and the new promoter drives the synthesis of the wild-type glycolytic enzyme.

Expression of *PYK1*, *PFK1* and *PFK2* in the mutants

For this strategy to be successful, the *PYK1*, *PFK1* and *PFK2* genes had to be inactivated by the 3'-truncation event (Fig. 1b). To test this presumption, promoterless mutants were also made. As expected, the promoterless *pyk1* and *pfk1 pfk2* mutants (YKC21 and YKC24) were unable to grow on glucose. Furthermore, the *PYK1* mRNA was not detectable by Northern analysis in the *pyk1* mutant, and the *PFK1* and *PFK2* mRNAs were not detectable in the *pfk1 pfk2* double mutant, following growth of these strains on YPL (not shown). Therefore, 3' truncation did inactivate each of these loci, and the synthesis of Pyk1 and Pfk1 was dependent on the *PGK1* or *PGK1_{Δ767}* promoters in the mutants.

PYK1, *PFK1* and *PFK2* expression levels were examined in the mutants during growth on glucose. Relative to their congenic parent (W303-1B), *PYK1* mRNA levels were slightly elevated in the mutant carrying the *PGK1-PYK1* fusion (YKC1; 150%) and were reduced in the strain with the *PGK1_{Δ767}-PYK1* fusion (YKC11; 43%; Table 3). Analogous observations were made for the corresponding double *PFK* mutants: *PFK1* and *PFK2* mRNA levels were elevated in YKC4 (240%) and reduced in YKC14 (40%; Table 4, Fig. 2b). Pyk1 and Pfk1 assays confirmed that the reductions in mRNA levels had led to roughly equivalent changes in the levels of these enzymes (Tables 3 and 4). In addition, decreased levels of each Pfk1 subunit (Pfk1p and Pfk2p) were

Table 4. *PFK1* and *PFK2* expression levels during growth on glucose

Numbers in parentheses are percentage of wild-type.

Strain	Relevant genotype	Relative <i>PFK1/2</i> mRNA level*	Phosphofructokinase specific activity†
W303-1B	Wild-type	0.1 (100)	1.3 ± 0.1 (100)
YKC4	<i>PGK1p-PFK1 PGK1p-PFK2</i>	0.22 (220)	ND
YKC14	<i>PGK1_Δ-PFK1 PGK1_Δ-PFK2</i>	0.04 (40)	0.8 ± 0.01 (62)
YKC24‡	<i>Δpfk1 Δpfk2</i>	NM (−)	NM (−)

* The *PFK1* probe detected both the *PFK1* and *PFK2* mRNAs, which were measured relative to the *ACT1* mRNA in arbitrary units.

† Units are 10³ μmol min^{−1} (mg protein)^{−1}. ND, Not done.

‡ Not viable on glucose. NM, Not measurable.

Fig. 3. Effect of the *PGK1* promoter fusions upon growth in SC containing 2% glucose. Growth was monitored using OD_{600} (a), and samples were taken to measure glucose consumption (b) and ethanol production (c) per unit biomass (relative to OD_{600}). W303-1B (wild-type), ○; YKC1 (*PGK1p-PYK1*), ◆; YKC4 (*PGK1p-PFK1 PGK1p-PFK2*), ■; YKC5 (*PGK1p-PFK1 PGK1p-PFK2 PGK1p-PYK1*), ▲.

Fig. 4. Effect of the *PGK1*_{Δ767} promoter fusions upon growth in SC containing 2% glucose. Growth was monitored using OD_{600} (a), and samples were taken to measure glucose consumption (b) and ethanol production (c) per unit biomass (relative to OD_{600}). W303-1B (wild-type), ○; YKC11 (*PGK1*_{Δ767}-*PYK1*), ◆; YKC14 (*PGK1*_{Δ767}-*PFK1 PGK1*_{Δ767}-*PFK2*), ■; YKC15 (*PGK1*_{Δ767}-*PFK1 PGK1*_{Δ767}-*PFK2 PGK1*_{Δ767}-*PYK1*), ▲.

Fig. 5. Glycerol and methylglyoxal assimilation by *PGK1*_{Δ767} mutants during growth on SC containing 2% glucose. W303-1B (wild-type), ○; YKC11 (*PGK1*_{Δ767}-*PYK1*), ◆; YKC14 (*PGK1*_{Δ767}-*PFK1 PGK1*_{Δ767}-*PFK2*), ■; YKC15 (*PGK1*_{Δ767}-*PFK1 PGK1*_{Δ767}-*PFK2 PGK1*_{Δ767}-*PYK1*), ▲.

Fig. 6. Metabolite levels in $PGK1_{\Delta 767}$ mutants. Strains were grown in SD to an OD_{600} of 0.2, harvested and resuspended in buffer. Glucose was then added (1%) and cells harvested to measure metabolite levels at various times thereafter. G6P/F6P, glucose 6-phosphate plus fructose 6-phosphate; F1,6BP, fructose 1,6-bisphosphate; F2,6BP, fructose 2,6-bisphosphate; T6P, trehalose 6-phosphate. W303-1B (wild-type), ○; YKC11 ($PGK1_{\Delta 767}-PYK1$), ◆; YKC14 ($PGK1_{\Delta 767}-PFK1$), ■.

confirmed in YKC14 by Western blotting (Fig. 2b). The decreases in *PYK1*, *PFK1* and *PFK2* expression levels observed for the chromosomally integrated $PGK1_{\Delta 767}$ fusions (about 2.5-fold) were not as great as for those observed previously for a multicopy $PGK1_{\Delta 767}-PGK1$ gene (Ogden *et al.*, 1986; Piper *et al.*, 1988). Nevertheless, mutants containing the $PGK1_{\Delta 767}$ fusions did display decreased Pyk1 and Pfk1k levels, and those carrying the $PGK1$ promoter fusions had slightly elevated *PYK1* and *PFK* expression levels.

Physiological effects of elevated *PYK1*, *PFK1* or *PFK2* expression

The strains carrying the $PGK1$ fusions were compared with their congenic parent during growth on glucose (Fig. 3). None of these strains displayed significant differences in doubling time, glucose consumption or ethanol production per unit biomass. Furthermore, they all progressed through diauxie at similar rates and displayed similar growth patterns on lactate (not

shown). Therefore, the relatively acute transcriptional and translational controls that normally operate on the *PYK1* and *PFK1/2* genes (Moore *et al.*, 1990a, b, c, 1991) are not essential for proper growth under these experimental conditions.

Physiological effects of reduced *PYK1*, *PFK1* or *PFK2* expression

The mutants carrying the $PGK1_{\Delta 767}$ fusions were compared with W303-1B during growth on glucose (Fig. 4). Decreasing *PFK1* and *PFK2* expression levels had no significant effect on growth rate, glucose consumption or ethanol production, either in the single or double mutants (YKC12, YKC13 and YKC14). However, reducing the level of Pyk1p led to a decrease in the specific growth rate from 0.419 ± 0.021 (for W303-1B) to $0.287 \pm 0.011 \text{ h}^{-1}$ (for YKC11). This mutant consumed a similar amount of glucose per unit biomass to W303-1B (Fig. 4c), but during mid-exponential growth phase, the rate of glucose consumption by YKC11 was about 65%

Fig. 7. ^{13}C NMR spectra of perchloric acid extracts from (a) W303-1B (wild-type) and (b) YKC11 ($PGK1_{\Delta 767}$ - $PYK1$). Cells were incubated in the presence of $[1-^{13}\text{C}]$ glucose for 45 min. The resonances marked are: $\beta 1$, C-1 of β -D-glucose; $\alpha 1$, C-1 of α -D-glucose; 1,3, C-1,3 of glycerol; G2-G4, C2-C4 respectively of glutamate; N2, C-2 of glutamine; A2, C-2 of acetate; E2, C-2 of ethanol; L3, C-3 of alanine.

that of W303-1B. Therefore, the $PGK1_{\Delta 767}$ - $PYK1$ mutation caused a decrease in glycolytic flux.

Compared to W303-1B, YKC11 consumed normal amounts of glucose per unit biomass, but produced about one-third of the ethanol per unit biomass (Fig. 4b, c). Therefore, in this strain carbon flux was being redirected from biomass and ethanol production. It was possible that reduced flux through Pyk1p might have led to increased accumulation of glycerol or methylglyoxal (generated by dephosphorylation of dihydroxyacetone phosphate). Therefore, we examined the accumulation of glycerol and methylglyoxal in the medium of W303-1B and YKC11 cultures (Fig. 5). No difference in methylglyoxal accumulation was observed, and YKC11 produced significantly lower amounts of glycerol than its parent. Therefore, the excess carbon utilized by YKC11 was not excreted as glycerol or methylglyoxal.

A reduced specific growth rate ($0.25 \pm 0.02 \text{ h}^{-1}$) was also observed for the triple mutant that carries reduced Pyk1p and Pfk1k levels (YKC15; Fig. 4a). YKC15 also accumulated glycerol more slowly than W303-1B (Fig. 5a). However, this mutant displayed normal rates of glucose consumption, and ethanol and methylglyoxal production (Figs 4b, c and 5b).

Metabolite levels in $PGK1_{\Delta 767}$ mutants

YKC11 metabolism was examined further by measuring the concentrations of intracellular metabolites following a pulse of glucose (Fig. 6). Glucose 6-phosphate, fructose 6-phosphate and ATP levels were increased in YKC11 compared to W303-1B, and fructose 2,6-bisphosphate and trehalose 6-phosphate levels were decreased. Fructose 1,6-bisphosphate and pyruvate levels were not affected significantly. Hence the reduction in Pyk1 levels had altered the levels of some glycolytic intermediates, effectors and ATP. However, the observed reduction in trehalose 6-phosphate levels, the maintenance of normal pyruvate levels and the increase in ATP were not consistent with a simple model involving a metabolic bottleneck at Pyk1.

The changes in metabolite levels in YKC14 ($PGK1_{\Delta 767}$ - $PFK1$ $PGK1_{\Delta 767}$ - $PFK2$) were consistent with the reduction in Pfk1k. As expected (Teusink *et al.*, 1998), glucose 6-phosphate, fructose 6-phosphate and initially trehalose 6-phosphate levels were increased, and fructose 1,6-bisphosphate was reduced compared to the wild-type strain (W303-1B; Fig. 6). Interestingly, the levels of fructose 2,6-bisphosphate, an allosteric activator of Pfk1k, were also elevated. This was consistent

with previous reports which have shown that compensatory changes in fructose 2,6-bisphosphate levels accompany alterations in Pfk1 levels (Davies & Brindle, 1992).

¹³C NMR analysis of glucose utilization

Glucose consumption per unit biomass was normal in YKC11, but ethanol and glycerol production was significantly decreased (Figs 4 and 5). Therefore, ¹³C NMR was used to examine the metabolic fate of the carbon utilized by YKC11. YKC11 and W303-1B cells were labelled with [1-¹³C]glucose, and cell extracts subjected to NMR (Fig. 7). In addition to the C-1 peaks for α -D-glucose (α 1) and β -D-glucose (β 1), three prominent signals were observed for W303-1B. Peaks E2 and 1,3 correspond to the expected glycolytic labelling of the C-2 of ethanol and C-1,3 of glycerol. Peak T1 corresponds to C-1,1' of trehalose, labelled through flux from glucose 6-phosphate. Most other peaks are indicative of glutamine and glutamate labelling via flux through the TCA cycle.

The ¹³C NMR spectra for YKC11 and W303-1B showed significant differences (Fig. 7). Firstly, peaks E2 and 1,3 were significantly reduced for YKC11, consistent with the decreased production of ethanol and glycerol by this mutant (Figs 4c and 5a). Acetate (A2) and trehalose (T1) were both also reduced. Most significantly, peak G3 is significantly increased, relative to peaks G2 and G4 (Fig. 7). These correspond to C-3, C-2 and C-4 of glutamate, respectively. Entry of [2-¹³C]acetyl CoA into the TCA cycle leads to the labelling of glutamate in the C-4 position. The C-2 position also becomes labelled in the subsequent cycle, and the C-3 position is labelled in the following cycle. Therefore, the elevated level of 3-labelled glutamate in YKC11 indicates that increased TCA cycling has taken place in this strain.

Increased TCA cycling might indicate that, even during growth under fermentative conditions, YKC11 is more dependent upon mitochondrial metabolism than its wild-type parent. Also, Arvantidis & Heinisch (1994) have shown previously that a *pfk2* deletion mutant is respiration dependent. Therefore, we compared the effects of ethidium bromide (an inhibitor of mitochondrial function) upon YKC11 and W303-1B. The zone of growth inhibition caused by ethidium bromide was significantly greater for YKC11 (45 ± 7 mm) than for W303-1B (12 ± 1.5 mm), YKC14 (11 ± 1.5 mm) or YKC15 (13 ± 2 mm). Furthermore, unlike these other strains, YKC11 did not yield any petite colonies as a result of ethidium bromide treatment. These observations suggest that mitochondrial metabolism is required for growth of YKC11 on glucose.

DISCUSSION

In this study we have examined firstly the significance of *PYK1*, *PFK1* and *PFK2* gene regulation, and secondly the effects of reducing levels of Pyk1 and Pfk1 upon

fermentative metabolism in yeast. To achieve this we generated a congeneric set of mutants in which the *PYK1*, *PFK1* and *PFK2* coding regions were placed under the control of *PGK1* or *PGK1* _{Δ 767} promoters (Fig. 1). Wild-type Pyk1, Pfk1 and Pfk2 proteins, which are subject to their normal allosteric controls, are synthesized in these strains. Therefore, we have separated the genetic regulation of Pyk1, Pfk1 and Pfk2 synthesis from the biochemical regulation of their activities. Physiological analyses of these strains have revealed their status following growth and adaptation to the mutations they harbour, rather than the direct metabolic effects of the mutations. Also, we cannot exclude the possibility that other glycolytic enzymes are affected by the mutations. Nevertheless, significant conclusions regarding the role of Pfk1 and Pyk1 in glycolytic regulation can be drawn from our data.

Placing *PYK1*, *PFK1* and/or *PFK2* under the control of the *PGK1* promoter did not have any significant effect upon growth rate on glucose or lactate, or glucose or ethanol consumption (Fig. 3). Therefore, the relatively acute genetic regulation of these loci (Moore *et al.*, 1990a, b, c, 1991) is not essential for growth under fermentative or gluconeogenic conditions. Tight transcriptional and translational control of, for example, *PYK1* might increase the competitiveness of yeast under some conditions. Alternatively, controls at the genetic and biochemical levels may be partially redundant with respect to glycolytic regulation.

Pfk1 and Pyk1 levels were elevated in YKC1 and YKC4 (Tables 3 and 4), but no significant increase in glycolytic flux was observed (Fig. 3). These data are consistent with those of Schaaf *et al.* (1989) who reported that glycolytic flux remains unaffected by overexpression of hexokinase, Pfk1, phosphoglycerate kinase, phosphoglycerate mutase, Pyk1, pyruvate decarboxylase or alcohol dehydrogenase. Davies & Brindle (1992) have also shown that Pfk1 overexpression does not affect glycolytic flux significantly. All of these studies have examined engineered yeast cells following a lengthy period of adaptation. Nevertheless, the data all suggest that none of these enzymes exert a high degree of control over glycolytic flux under fermentative conditions. Most of this control is thought to lie at the level of glucose import (Ye *et al.*, 1999).

Pfk1 appears to exert a relatively small amount of control over glycolytic flux under the conditions tested (Figs 3 and 4; Schaaf *et al.*, 1989; Davies & Brindle, 1992), and yet this enzyme is subject to tight allosteric regulation (Bañuelos *et al.*, 1997; Avigad, 1981; Bartrons *et al.*, 1982; Fraenkel, 1982; Arvantidis & Heinisch, 1994; Heinisch *et al.*, 1996). How can this be rationalized? In fact, the principles of MCA (Kacser & Burns, 1973, 1979; Fell, 1984, 1997) predict that enzymes that display tight allosteric regulation may exert a low degree of flux control with respect to the pathway as a whole. In the case of yeast Pfk1, compensatory changes in fructose 2,6-bisphosphate may act to buffer the system under metabolic duress to maintain essential glycolytic

flux (Davies & Brindle, 1992). A relatively modest reduction in the level of Pfk1k was achieved in this study (~40% decrease). The elasticity of this system, achieved by compensatory modulation of fructose 2,6-bisphosphate levels, might have limits. In other words, larger changes in Pfk1k level might have more significant effects upon glycolytic flux.

Decreasing Pyk1 levels had unexpected effects upon the physiology of YKC11. Rates of growth and glucose consumption were reduced significantly (approx. 65% of wild-type), indicating that glycolytic flux was reduced in this strain. Hence, Pyk1 exerts some degree of control over glycolytic flux under fermentative growth conditions. Although glucose consumption per unit biomass was normal in YKC11 (Fig. 4), ethanol and glycerol production were reduced (Figs 4 and 5), and TCA cycling was increased (Fig. 7). It seems counterintuitive to suggest that a decrease in Pyk1 caused increased TCA cycling. Nevertheless, the data clearly indicate that both the rate and direction of carbon flux was affected in this strain (YKC11).

It is not clear how a decrease in Pyk1 caused this apparent switch from fermentative to respiratory metabolism. The switch from fermentative to respiratory metabolism in yeast has been correlated with decreased growth rate (Postma *et al.*, 1989) or glucose concentration (Meijer *et al.*, 1998). Neither can account for the phenotypic differences observed for YKC11, YKC15 and W303-1B. YKC11 and YKC15 grew more slowly than W303-1B under conditions of glucose excess, yet only YKC11 displayed decreased ethanol production and increased mitochondrial dependence. Whatever the mechanism involved, our data suggest that Pyk1 exerts a significant level of control over both the rate and direction of carbon flux in yeast during growth on glucose.

ACKNOWLEDGEMENTS

We thank Susan Budge for excellent technical assistance. We also thank Jurgen Heinisch for the Pfk1k antisera and Sue Kingsman for the *PGK1* and *PGK1*_{Δ767} promoters. A.K.P. and K.C. were supported by studentships from the Biotechnology and Biological Sciences Research Council.

REFERENCES

- Arvantidis, A. & Heinisch, J. J. (1994). Studies on the function of yeast phosphofructokinase subunits by *in vitro* mutagenesis. *J Biol Chem* **269**, 8911–8918.
- Avigad, G. (1981). Stimulation of yeast phosphofructokinase activity by fructose 2,6-bisphosphate. *Biochem Biophys Res Commun* **102**, 985–991.
- Bañuelos, M., Gancedo, C. & Gancedo, J. M. (1977). Activation of phosphate by yeast phosphofructokinase. *J Biol Chem* **252**, 6394–6398.
- Bartrons, R., Van Schaftingen, E., Vissers, S. & Hers, H.-G. (1982). The stimulation of yeast phosphofructokinase by fructose-2,6-bisphosphate. *FEBS Lett* **143**, 137–140.
- Bergmeyer, H. U. (1986). *Methods in Enzymatic Analysis*, 3rd edn. Weinheim: Verlag Chemie.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brown, A. J. P. (1994). Measurement of mRNA stability. In *Molecular Genetics of Yeast: a Practical Approach*, pp. 147–159. Edited by J. R. Johnston. Oxford: Oxford University Press.
- Burke, R. L., Tekamp-Olson, P. & Najarian, R. (1983). The isolation, characterisation and sequence of the pyruvate kinase gene of *Saccharomyces cerevisiae*. *J Biol Chem* **258**, 2193–2201.
- Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991–1995.
- Crimmins, C. (1995). *The significance of genetic regulation in the control of glycolysis in Saccharomyces cerevisiae*. PhD thesis, University of Aberdeen.
- Davies, S. E. C. & Brindle, K. M. (1992). Effects of overexpression of phosphofructokinase on glycolysis in the yeast *Saccharomyces cerevisiae*. *Biochemistry* **31**, 4729–4735.
- Dickinson, J. R. (1999). Carbon metabolism. In *The Metabolism and Molecular Physiology of Saccharomyces cerevisiae*, pp. 23–55. Edited by J. R. Dickinson & M. Schweizer. London: Taylor & Francis.
- Dickinson, J. R. & Hewlins, M. J. E. (1988). A study of the role of the hexose monophosphate pathway with respect to fatty acid biosynthesis in sporulation of *Saccharomyces cerevisiae*. *J Gen Microbiol* **134**, 333–337.
- Dickinson, J. R. & Hewlins, M. J. E. (1991). ¹³C NMR analysis of a developmental pathway mutation in *Saccharomyces cerevisiae* reveals a cell derepressed for succinate dehydrogenase. *J Gen Microbiol* **137**, 1033–1037.
- Dickinson, J. R., Lanterman, M. M., Danner, D. J., Pearson, B. M., Sanz, P., Harrison, S. J. & Hewlins, M. J. E. (1997). A ¹³C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 26871–26878.
- Feinberg, A. P. & Vogelstein, B. (1983). A technique for radio-labelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**, 6–13.
- Fell, D. A. (1984). Phosphofructokinase and glycolytic flux. *Trends Biochem Sci* **9**, 515–516.
- Fell, D. A. (1997). *Understanding the Control of Metabolism*. London: Portland Press.
- Ferguson, G. P., McLaggan, D. & Booth, I. R. (1995). Potassium channel activation by glutathione-S-conjugates in *Escherichia coli*: protection against methylglyoxal is mediated by cytoplasmic acidification. *Mol Microbiol* **17**, 1025–1033.
- Fraenkel, D. G. (1982). Carbohydrate metabolism. In *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Biosynthesis*, pp. 1–37. Edited by J. N. Strathern, E. W. Jones & J. R. Broach. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Gancedo, C. & Serrano, R. (1989). Energy yielding metabolism. In *The Yeasts*, vol. 3: *Metabolism and Physiology of Yeasts*, 2nd edn, pp. 205–259. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- Gietz, R. D. & Woods, R. A. (1998). Transformation of yeast by the lithium acetate/single-stranded carrier DNA/PEG method. In *Yeast Gene Analysis: Methods in Microbiology*, vol. 26, pp. 53–66. Edited by A. J. P. Brown & M. F. Tuite. London: Academic Press.
- Gonzalez, B., François, J. & Renaud, M. (1997). A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. *Yeast* **13**, 1347–1356.

- Heinisch, J. (1986). Isolation and characterisation of the two structural genes coding for phosphofructokinase in yeast. *Mol Gen Genet* **202**, 75–82.
- Heinisch, J. J., Boles, E. & Timpel, C. (1996). A yeast phosphofructokinase insensitive to the allosteric activator fructose-2,6-bisphosphate. *J Biol Chem* **271**, 15928–15933.
- Hess, B., Boiteux, A. & Kruger, J. (1969). Cooperation of glycolytic enzymes. *Adv Enzyme Regul* **7**, 149–169.
- Hoffman, C. S. & Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267–272.
- Hunsley, J. R. & Suelter, C. H. (1969). Yeast pyruvate kinase: kinetic properties. *J Biol Chem* **244**, 4819–4822.
- Johnston, M. & Carlson, M. (1992). Regulation of carbon and phosphate utilization. In *The Molecular and Cellular Biology of the Yeast Saccharomyces*, vol. 2: *Gene Expression*, pp. 193–281. Edited by E. W. Jones, J. R. Pringle & J. R. Broach. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Kacser, H. & Burns, J. A. (1973). The control of flux. *Symp Soc Exper Biol* **27**, 65–104.
- Kacser, H. & Burns, J. A. (1979). Molecular democracy: who shares the controls? *Biochem Soc Trans* **7**, 1149–1160.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Meijer, M. M. C., Bostra, J., Verkleij, A. J. & Verrips, C. T. (1998). Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. *J Biol Chem* **273**, 24102–24107.
- Mellor, J. E., Dobson, M. J., Roberts, N. A., Kingsman, A. J. & Kingsman, S. M. (1985). Factors affecting heterologous gene expression in *Saccharomyces cerevisiae*. *Gene* **33**, 215–226.
- Moore, P. A., Bettany, A. J. E. & Brown, A. J. P. (1990a). The expression of a yeast glycolytic gene is subject to dosage compensation. *Gene* **89**, 85–92.
- Moore, P. A., Bettany, A. J. E. & Brown, A. J. P. (1990b). Multiple copies of the pyruvate kinase gene inhibit yeast cell growth. *J Gen Microbiol* **136**, 2359–2366.
- Moore, P. A., Bettany, A. J. E. & Brown, A. J. P. (1990c). The yeast pyruvate kinase gene is regulated at multiple levels. In *Post-Transcriptional Control of Gene Expression*, pp. 421–432. Edited by J. E. G. McCarthy & M. F. Tuite. Berlin: Springer.
- Moore, P. A., Sagliocco, F. A., Wood, R. C. M. & Brown, A. J. P. (1991). Yeast glycolytic mRNAs are differentially regulated. *Mol Cell Biol* **11**, 5330–5337.
- Ogden, J. E., Stanway, C., Kim, S., Mellor, J., Kingsman, A. J. & Kingsman, S. M. (1986). Efficient expression of the *Saccharomyces cerevisiae* PGK gene depends on an upstream activation sequence but does not require TATA sequences. *Mol Cell Biol* **6**, 4335–4343.
- Piper, P. W., Curran, B., Davies, M. W., Hirst, K., Lockheart, A., Ogden, J. E., Stanway, C., Kingsman, A. J. & Kingsman, S. M. (1988). A heat shock element in the phosphoglycerate kinase gene promoter of yeast. *Nucleic Acids Res* **16**, 1333–1348.
- Planta, R., Brown, A. J. P., Cadahia, J. L. & 13 other authors (1999). Yeast functional analysis reports: transcript analysis of 250 novel yeast genes from chromosome XIV. *Yeast* **15**, 329–350.
- Postma, E., Verduyn, C., Scheffers, W. A. & van Dijken, J. P. (1989). Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **55**, 468–477.
- Racker, E. (1947). Spectrophotometric measurement of hexokinase and phosphohexokinase activity. *J Biol Chem* **167**, 843–854.
- Reibstein, D., den Hollander, J. A., Pilkis, S. J. & Shulman, R. G. (1986). Studies on the regulation of yeast phosphofructo-1-kinase: its role in aerobic and anaerobic glycolysis. *Biochemistry* **25**, 219–227.
- Schaaff, I., Heinisch, J. & Zimmermann, F. K. (1989). Overproduction of glycolytic enzymes in yeast. *Yeast* **5**, 285–290.
- Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.
- Strathern, J. N., Mastrangelo, M., Rinckel, L. A. & Garfinkel, D. J. (1994). Ty insertional mutagenesis. In *Molecular Genetics of Yeast: a Practical Approach*, pp. 111–119. Edited by J. R. Johnston. Oxford: Oxford University Press.
- Teusink, B., Baganz, F., Westerhoff, H. V. & Oliver, S. G. (1998). Metabolic control analysis as a tool in the elucidation of the function of novel genes. In *Yeast Gene Analysis: Methods in Microbiology*, vol. 26, pp. 297–336. Edited by A. J. P. Brown & M. F. Tuite. London: Academic Press.
- Thomas, B. J. & Rothstein, R. (1989). The genetic control of direct-repeat recombination in *Saccharomyces*: the effect of *rad52* and *rad1* on mitotic recombination at *GAL10*, a transcriptional regulation gene. *Genetics* **123**, 725–738.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.
- Van Schaftingen, E., Lederer, B., Bartrons, R. & Hers, H.-G. (1982). A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. *Eur J Biochem* **129**, 191–195.
- Wicksteed, B. L., Collins, I., Dershowitz, A., Stateva, L. I., Green, R. P., Oliver, S. G., Brown, A. J. P. & Newlon, C. S. (1994). A physical comparison of chromosome III in six strains of *Saccharomyces cerevisiae*. *Yeast* **10**, 39–57.
- Ye, L., Kruckelberg, A. L., Berden, J. A. & van Dam, K. (1999). Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter. *J Bacteriol* **181**, 4671–4675.
- Yun, S. L., Aust, A. E. & Suelter, C. H. (1976). A revised preparation of yeast (*Saccharomyces cerevisiae*) pyruvate kinase. *J Biol Chem* **251**, 124–128.

Received 7 July 2000; revised 6 November 2000; accepted 7 November 2000.