Multiple copies of the pyruvate kinase gene affect yeast cell growth

PAUL A. MOORE, ANDREW J. E. BETTANY† and ALISTAIR J. P. BROWN*

Department of Molecular and Cell Biology, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, UK

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The Saccharomyces cerevisiae pyruvate kinase gene (PYKI) was transformed into yeast using the multicopy vector pJDB207. Growth rates and PYKI gene expression levels varied considerably amongst the transformants. Yeast transformants expressing the PYKI gene at high levels formed small colonies compared with those expressing the gene at relatively low levels. Slow-growing transformants 'reverted' at high frequency to more rapid growth, and this correlated with decreases in PYKI gene copy number and PYKI mRNA abundance. This apparent selection against PYKI over-expression was disrupted by the introduction of a stop codon at the 5'-end of the PYKI coding region, thus confirming that the growth effects were mediated by the PYKI gene. However, massive overproduction of pyruvate kinase in yeast, using multiple copies of a PGK:PYK gene fusion, had no significant effect upon cell growth. This suggests that the deleterious effect upon the host yeast cell is mediated by abnormally high levels of the wild-type gene or PYKI mRNA, rather than by increased pyruvate kinase levels.

Introduction

During fermentative growth, the glycolytic enzymes can comprise over 30% of soluble cell protein in the yeast Saccharomyces cerevisiae (Fraenkel, 1982). The glycolytic genes are among the most efficiently expressed genes in this organism, and most have been isolated and sequenced (Holland & Holland, 1979; Holland et al., 1981; Alber & Kawasaki, 1982; Bennetzen & Hall, 1982; Hitzeman et al., 1982; Watson et al., 1982; Burke et al., 1983; Kopetzki et al., 1985; Kellermann et al., 1986; Aguilera & Zimmerman, 1986; Stachelek et al., 1986; Tekamp-Olson et al., 1988; White & Fothergill-Gilmore, 1988; Heinisch et al., 1989; McNally et al., 1989; Schwelberger et al., 1989). It is frequently assumed that glycolytic gene expression is increased when yeast cultures are transferred from nonfermentative to fermentative carbon sources, but this is not the case for all strains of Saccharomyces (Clifton & Fraenkel, 1981).

Pyruvate kinase catalyses the last energy-conserving reaction in the glycolytic pathway. The enzyme converts phosphoenolpyruvate and ADP to pyruvate and ATP, and this is one of the irreversible steps on the pathway. The yeast enzyme, which is a tetramer of four identical 55 kDa subunits, responds to a number of allosteric effectors, including fructose-1,6-diphosphate and NH₄⁺ (Hunsley & Suelter, 1969; Yoshino & Murakami, 1982). Pyruvate kinase is one of the first glycolytic enzymes to be induced when glucose is added to yeast cultures growing on acetate (Maitra & Lobo, 1971). These observations suggest that under some conditions pyruvate kinase contributes significantly to the regulation of glycolytic flux.

Owing to the high efficiency of yeast glycolytic gene expression, promoters from several of these genes, including PYKI, have been used to drive the expression of a large number of heterologous proteins in yeast (for reviews see Kingsman et al., 1985; Goodey et al., 1987; King et al., 1989). In several cases, the expression of a particular heterologous sequence has been compared with that of the appropriate wild-type glycolytic gene on a similar multicopy plasmid (e.g. Chen et al., 1984; Mellor et al., 1985). These experiments have demonstrated that the expression of some glycolytic genes can be markedly increased without affecting yeast growth or viability. In this paper we describe the effects of over-expressing the PYKI gene upon the yeast cell. These effects set the PYKI gene apart from most other glycolytic genes.

Methods

Yeast strains and plasmids. The S. cerevisiae strains DBY746 (MATα his3 leu2 trpl1 ura3[cir+]) and X4003-5B (MATα ade1 gal1 his4 leu2 met2 trp5 ura3[cir+]) were used throughout. The plasmids used in this study

† Present address: AFRC Institute for Grassland and Environmental Research, Welsh Plant Breeding Station, Aberystwyth, Dyfed SY23 3EB, UK.

Abbreviations: PYKI, pyruvate kinase gene; PGKI, phosphoglycerate kinase gene; HXK1, HXK2 hexokinase genes; PGI1, phosphoglucone isomerase gene; PFK2, phosphofructokinase gene; TPI1, triosephosphate isomerase gene; PGM1, phosphoglyceromutase gene.
Fig. 1. PYK1 constructs used in this study. The 2-micron-based plasmids pLD1(35), pLD1(35)IC and pLD1(35)2D (Bettany et al., 1989), and pMA91 (Mellor et al., 1983) have been described previously. PGK1 sequences are hatched, wild-type PYK1 sequences are in upper case, and insertions are in lower case. pLD1(35) carries the wild-type PYK1 gene. pLD1(37) carries a 4 bp insertion that introduces a stop codon at the 5'-end of the coding region.

Results

PYK1 transformants differ in their growth rates

The plasmid pLD1(35) comprises a 6-3 kb yeast genomic fragment cloned between the HindIII and NdeI sites of the multicopy vector pJDB207 (Parent et al., 1985; Bettany et al., 1989). The wild-type PYK1 gene on this plasmid (Fig. 1) complements the conditionally-defec-
Excess PYK1 inhibits yeast growth

Fig. 2. Growth of transformants carrying multicopy PYK1 or pykl plasmids. (a, b) DBY746 was transformed with pLD1(35). Fresh transformants were patched onto minimal agar lacking leucine and incubated at 30°C for 5 d (a). This plate was then stored for 1 week at 4°C, after which each transformant was then repatched onto fresh minimal agar minus leucine, and grown at 30°C for a further 5 d (b). (c) Fresh DBY746:pLD1(37) transformants treated identically to (a).

Slow growth correlates with high PYK1 expression levels

The abundance of PYK1 mRNA was measured relative to actin mRNA in more than thirty DBY746:pLD1(35) transformants. Actin mRNA was used as an internal control for minor variations in RNA yield. Data were only used if hybridizations were proven experimentally to have taken place under conditions of probe-excess (see Methods). The abundance of the PYK1 mRNA was 0-6% of total mRNA in the untransformed host strain. PYK1 mRNA levels varied considerably amongst transformants, from less than 1-0% up to 10% of total mRNA. These quantitative measurements of PYK1 mRNA abundance obtained by dot-blotting were confirmed qualitatively by Northern analysis (Fig. 3). Significantly, slow-growing transformants contained relatively high levels of PYK1 mRNA (typically more than 1-5% of total mRNA) compared with fast-growing transformants (typically less than 1-5% of total mRNA).

Abnormally high PYK1 transcript levels 'wind down' during growth

As described above, pLD1(35), pAYE4(34), pLD1(35)1C or pLD1(35)2D transformants with high PYK1 mRNA levels grew relatively slowly, and these slow-growing transformants quickly 'reverted' to rapid
Table 1. Effect of storage upon PYK1 mRNA levels in DBY746 transformants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Before storage*</th>
<th>After storage†</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY746</td>
<td>–</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>WM4</td>
<td>pLD1(35)</td>
<td>8.9</td>
<td>1.0</td>
</tr>
<tr>
<td>WM5</td>
<td>pLD1(35)</td>
<td>4.0</td>
<td>1.1</td>
</tr>
<tr>
<td>1CM2</td>
<td>pLD1(35)IC</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>1CM6</td>
<td>pLD1(35)IC</td>
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<td>1.1</td>
</tr>
<tr>
<td>2DN1</td>
<td>pLD1(35)2D</td>
<td>4.6</td>
<td>0.6</td>
</tr>
<tr>
<td>2DN2</td>
<td>pLD1(35)2D</td>
<td>1.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*PYK1 mRNA abundance determined immediately following transformation.
†PYK1 mRNA abundance redetermined after each transformant had been stored on minimal plates for about 8 weeks at 4°C.

Fig. 3. Northern analysis of the PYK1 mRNA in pLD1(35) transformants. RNA isolated from DBY746 and three slow-growing pLD1(35) transformants was subjected to Northern analysis. The filter was probed for PYK1 mRNA (a), the probe was stripped from the filter, and the filter then probed for actin mRNA (b). At each stage the amount of bound radioactivity in each band was determined using an AMBIS 2D-Radioimaging System. Note that the amount of total RNA loaded in each lane differs for each sample.

Fig. 3. Northern analysis of the PYK1 mRNA in pLD1(35) transformants. RNA isolated from DBY746 and three slow-growing pLD1(35) transformants was subjected to Northern analysis. The filter was probed for PYK1 mRNA (a), the probe was stripped from the filter, and the filter then probed for actin mRNA (b). At each stage the amount of bound radioactivity in each band was determined using an AMBIS 2D-Radioimaging System. Note that the amount of total RNA loaded in each lane differs for each sample.

For about 8 weeks at 4°C on minimal agar lacking leucine. The abundance of the PYK1 mRNA in each RNA sample was then measured (Table 1). In all cases where the abundance of the PYK1 mRNA was initially greater than 1.5% of total mRNA, the PYK1 mRNA was found to have decreased following storage. This correlated with 'reversion' to rapid growth. No significant changes in PYK1 mRNA abundance were observed where the initial level was less than 1.5% of total mRNA (Table 1).

In an attempt to confirm that PYK1 over-expression causes the deleterious effect upon the growth of the host yeast cell, and that the 'wind-down' in PYK1 over-expression correlates with the 'reversion' of slow-growing transformants to more rapid growth, a slow-growing transformant was studied. Strain X4003-5B was transformed with pLD1(35) (Fig. 1), and a transformant that had formed a small colony, and was therefore presumed to have an abnormally slow growth rate, was picked for further analysis. The doubling time of the transformant was initially twice that of the untransformed strain (which has a doubling time of about 2 h), and decreased by approximately 45% during growth (Fig. 2). However, amongst different pLD1(35) transformants the relationship between PYK1 mRNA abundance and the doubling time of the culture was not linear. For example, in Fig. 3 the transformants containing PYK1 mRNA levels of 1.6% and 1.8% of total mRNA had doubling times of 2.7 and 4.1 h, respectively, and yet the transformant with a PYK1 mRNA level of 2.7% of total mRNA had a doubling time of 3.3 h. Nevertheless, we consistently observed that transformants containing significantly elevated PYK1 mRNA levels grew more slowly than the untransformed host strain.

To confirm the generality of the relationship between slow growth and elevated PYK1 mRNA levels, a range of transformants were analysed in detail. RNA was prepared from the transformants before and after storage for about 8 weeks at 4°C in selective broth for 75 generations (Fig. 4a).
Excess PYKI inhibits yeast growth

Fig. 4. Abnormally high levels of PYKI expression decrease during growth. PYKI copy number and mRNA abundance were measured during serial batch growth of a slow-growing X4003-5B:pLD1(35) transformant (a; closed symbols), and a X4003-5B:pLD1(37) transformant (b; open symbols). Fresh cultures were inoculated from cultures that had reached late-exponential growth phase. Doubling times, \( t_d \) (\( A, A \)) were measured as OD600. PYKI mRNA abundance (a) is expressed as a percentage of total mRNA, and PYKI copy number (+, 0) is expressed per haploid genome.

4a). The copy number of the PYKI gene approached normality and the abundance of the mRNA approached wild-type levels. Southern blot analysis confirmed the existence of both chromosomal and plasmid-encoded PYKI sequences throughout the experiment and confirmed the changes in the relative abundance of these sequences (not shown). No new plasmids (which might have formed via recombination with the endogenous 2-micron plasmid) were detected.

Inactivation of PYKI disrupts the effect upon growth rate

The previous experiments confirmed that increasing growth rates of pLD1(35) transformants correlate with decreasing levels of PYKI gene expression. However, they failed to exclude the possibility that the growth rate effect is mediated by sequences other than PYKI in pLD1(35). The CYC3 and FUNII genes are located 5' and 3' to the PYKI gene on chromosome 1 (Coleman et al., 1986). Comparing the restriction map of this region of chromosome 1 (Coleman et al., 1986) with that of the genomic insert of pLD1(35) (Burke et al., 1983) suggests that while the entire CYC3 gene is probably present in pLD1(35), it is likely that only part of the FUNII gene is present. Therefore, these genes may have contributed at least partially to the growth effects described above. The plasmid pLD1(37) was constructed to exclude this possibility. pLD1(37) is identical to pLD1(35) except for the insertion of a premature termination codon at the N-terminus of the PYKI coding region (Fig. 1).

Fresh X4003-5B:pLD1(37) transformants generally showed strong growth on plates, in contrast to fresh pLD1(35) transformants (Fig. 2c). A pLD1(37) transformant was then subjected to detailed analysis during prolonged growth over about 75 generations in a similar experiment to that described above for pLD1(35). As before, pykl copy number and mRNA abundance were quantified by dot-blotting, and the doubling time of the culture was measured using the optical density at 600 nm. During prolonged growth there was no significant change in the abundance of the pykl mRNA above the level of the untransformed host strain (0-6% of total mRNA). In contrast to the X4003-5B:pLD1(35) transformant, the copy number of the pykl gene increased about fivefold to over 20 copies per haploid genome in cells carrying pLD1(37) while the doubling time of the culture decreased by 23% (Fig. 4b). Hence the negative effects upon growth are mediated by PYKI and are not due to other plasmid-borne sequences since plasmid copy number increased during the experiment. Therefore, high levels of PYKI gene expression inhibit growth of the host yeast cell, thus creating a strong selection against this over-expression.

The growth effects are not due to high intracellular levels of pyruvate kinase

It seemed likely that the effects of high levels of PYKI gene expression upon growth were mediated by excess pyruvate kinase. This was tested using the plasmid pMA91/PYK (a generous gift from Jane Mellor, University of Oxford, UK). This multicopy plasmid carried a PGK:PYK gene fusion in which the PGKl promoter and 5'-untranslated region were fused to the PYKI coding and 3'-untranslated regions to regenerate the correct PYKI coding sequence (Fig. 1). pMA91/PYK transformed DBY746 more efficiently than plasmids which carried the wild-type PYKI gene.

Protein extracts prepared from DBY746, or DBY746 transformants carrying pMA91/PYK or pLD1(35), were subjected to SDS-PAGE (Fig. 5). Only limited over-production of pyruvate kinase was apparent in the pLD1(35) transformants which had PYKI mRNA.
Fig. 5. Synthesis of high levels of pyruvate kinase using a PGK:PYK gene fusion. Total soluble protein extracts from DBY746 (1), two DBY746:pLD1(35) transformants (2, 3), and a DBY746:pMA91/PYK transformant (4) were electrophoresed on an 8% (w/v) polyacrylamide/SDS gel and stained with Coomassie blue. The abundances of the PYKI mRNA (or PGKIPYK mRNA in the case of the DBY746:pMA91/PYK transformant) were 0.6%, 0.9%, 1.6% and 5.5% of total mRNA, respectively, as measured by dot-blotting and confirmed by Northern analysis (not shown). The loading for DBY746 is greater than for the transformants. The arrow indicates the pyruvate kinase band.

Discussion

We have demonstrated that abnormally high levels of the wild-type PYKI gene or mRNA have a deleterious effect upon the growth of yeast, which creates a strong selection for reduced PYKI copy numbers. Presumably the decrease in the average PYKI gene and mRNA levels during prolonged growth or storage of high-copy-number pLD1(35) transformants was mediated by unequal partitioning of this pJDB207-based vector during cell division (Futcher & Cox, 1984) followed by more rapid growth of cells carrying reduced PYKI copy numbers. This selection is disrupted by inactivating the PYKI gene with a premature stop codon, allowing the copy number to drift upwards during prolonged growth in medium lacking leucine, presumably owing to selection for increased copy number of the partially active LEU2d allele carried on the vector pJDB207 (Futcher & Cox, 1984). pJDB207 contains only one half of the 2-micron inverted repeat, and therefore is unable to use the natural mechanism for 2-micron amplification, which depends upon recombination between the two halves of the repeated sequence (Murray et al., 1987). In summary, the selection for a reduction in PYKI copy number dominates over that for an increase in LEU2d copy number.

The observations that high PYKI copy numbers affect cell growth, and that the over-expression of PYKI is limited by at least two dosage compensation mechanisms (Moore et al., 1990), appear to set this gene apart from most other glycolytic genes. It has become clear that yeast can tolerate extremely high levels of expression from most other glycolytic genes with little apparent effect upon cell growth or viability. These include the HXK2, PGII, TPII, PGKI and PGM1 genes (Alber & Kawasaki, 1982; Kawasaki & Fraenkel, 1982; Mellor et al., 1985; Aguilera & Zimmerman, 1986; Schaaf et al., 1989).

It is important to note that excess pyruvate kinase itself does not inhibit growth, and in this respect the enzyme is similar to hexokinase, phosphoglucose isomerase, triosephosphate isomerase, phosphoglycerate kinase and phosphoglycerate mutase (Alber & Kawasaki, 1982; Kawasaki & Fraenkel, 1982; Chen et al., 1984; Mellor et al., 1985; Aguilera & Zimmerman, 1986; Schaaf et al., 1989). The relatively normal behaviour of the pLD1(37) transformants, in which the plasmid-borne pykl locus carries a nonsense mutation, appears to implicate the enzyme in the effects on growth. However, the levels of pykl mRNA remained low (0.6% of total mRNA). Therefore, these effects appear to be mediated either by high wild-type PYKI copy numbers, high PYKI mRNA levels, or a combination of both. One possible explanation is that excess PYKI sequences may inhibit the expression of other essential genes by saturating a factor

abundances of 0.9% and 1.6% of total mRNA. Massive overproduction of pyruvate kinase was observed in the pMA91/PYK transformant which had a PGK:PYKI mRNA abundance of 5.5% of total mRNA. However, this pMA91/PYK transformant grew as strongly as the untransformed strain (as did pMA91/PYK transformants in general). Therefore, the deleterious effects of multicopy plasmids carrying the wild-type PYKI gene were not due to the synthesis of excess pyruvate kinase.
which is required for their expression, but which is present in limiting concentrations. The inhibition of PFK2 mRNA translation by excess PYKI mRNA (Moore et al., 1990) is consistent with this hypothesis.

Clearly, the PYKI gene is subject to more levels of regulation than most other yeast glycolytic genes. The physiological relevance of this remains an intriguing question, but may relate to the ability of some pykI alleles to confer cell cycle arrest (cde19; Fraenkel, 1982).

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