Saccharomyces cerevisiae Null Mutants in Glucose Phosphorylation: Metabolism and Invertase Expression

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ABSTRACT

A congenic series of Saccharomyces cerevisiae strains has been constructed which carry, in all combinations, null mutations in the three genes for glucose phosphorylation: HXK1, HXK2 and GLK1, coding hexokinase 1 (also called PI or A), hexokinase 2 (PII or B), and glucokinase, respectively; i.e., eight strains, all of which grow on glucose except for the triple mutant. All or several of the strains were characterized in their steady state batch growth with 0.2% or 2% glucose, in aerobic as well as respiration-inhibited conditions, with respect to growth rate, yield, and ethanol formation. Glucose flux values were generally similar for different strains and conditions, provided they contained either hexokinase 1 or hexokinase 2. And their aerobic growth, as known for wild type, was largely fermentative with ca. 1.5 mol ethanol made per mol glucose used. The strain lacking both hexokinases and containing glucokinase was an exception in having reduced flux, a result fitting with its maximal rate of glucose phosphorylation in vitro. Aerobic growth of even the latter strain was largely fermentative (ca. 1 mol ethanol per mol glucose). Invertase expression was determined for a variety of media. All strains with HXK2 showed repression in growth on glucose and the others did not. Derepression in the wild-type strain occurred as ca. 1 mM glucose. The metabolic data do not support--or disprove—a model with HXK2 having only a secondary role in catabolite repression related to more rapid metabolism.

Saccharomyces cerevisiae has three enzymes for glucose phosphorylation: hexokinase 1 (also known as PI or A), hexokinase 2 (PII or B), and glucokinase. Their obvious function is in the first step of glucose metabolism, and it is known primarily through the work of MAITRA and LOBO with deficiency mutants that any one of these enzymes is sufficient for growth on glucose (e.g., LOBO and MAITRA 1977a,b; MAITRA and LOBO 1983). Even with regard to growth, however, the individual functions of the three enzymes in wild-type strains are not so clear. At least in some strains, hexokinase 2 predominates on glucose and hexokinase 1 in its absence (GANCEDO, CLIFTON and FRAENKEL 1977; MURATSUBAKI and KATSUNE 1979; R. B. WALSH, to be reported).

Aside from catalyzing the first step of glucose metabolism, the three kinases have a role in high affinity glucose uptake (BISSON and FRAENKEL 1983; LANG and CIRILLO 1987; MCELLLAN and BISSON 1988). Also, hexokinase 2 seems to have a special role in carbon catabolite repression, deficiency mutants having typically derepressed levels of a sensitive enzyme, such as invertase, in the presence of glucose concentrations which would normally repress its synthesis (e.g., ENTIAN and MECKE 1982; MICHELS, HAHENBERGER and SYLVESTRE 1983; MA and BOTSTEIN 1986; for reviews, see ENTIAN 1986; GANCEDO and GANCEDO 1986; CARLSON 1987; JOHNSTON 1987).

Thus, neither the common nor individual roles of the three kinases are yet understood. In the present paper we report construction of a set of congenic strains carrying null mutations hxx1::LEU2, hxx2::LEU2 and glk1::LEU2—eight strains, seven of which grow on glucose—and their characterization as to growth, cell yield, glucose flux, ethanol formation and expression of invertase. Many of these functions have, over the years, been assessed in a variety of wild type strains, and certain ones recently even in kinase null mutants [e.g., MA and BOTSTEIN (1986) on catabolite repression; see also MCCLELLAN and BISSON (1988), on glucose transport]. But a congenic complete set has not been evaluated in this way. Perhaps the most extensive previous study of the role of these enzymes in glucose metabolism is that of LOBO and MAITRA (1977b), and in part the present paper is an extension of that work to the new strains emphasizing metabolism in growth. The other emphasis is on invertase expression, and in this respect the present work complements, with the various combinations of null mutants, the detailed recent studies of hexokinase 2 point mutants (MA et al. 1989a,b).

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MATERIALS AND METHODS

Strains: The strains (see Table 1) are DFY1 and seven kinase mutants congenic with it. Each strain also carries lysI-1 and SUCG and, except DFY1, leu2-1 at normal chromosomal position; strains DFY1, -566, -567, and -581 are mating type a and the others a. Their construction employed subcloning of clones pBY11-1-115 (Walsh, KawasakI and Fraenkel 1983) and insertion of a ca. 4-kbp NcoI fragment (Sedivy and Fraenkel 1985) containing LEU2. The genes have been sequenced (Frohlich, Entian and Mecke 1985; Kopetzki, Entian and Mecke 1985; Stachelek et al. 1986; Albig and Entian 1988), and the mutations are hskl::LEU2, a ca. 0.9-kbp substitution extending from codon 15 in the 5' direction; hsk2::LEU2, an insertion at codon 15; and gsk1::LEU2, a ca. 1-kbp substitution from codon 105 in the 5' direction. Each mutation abolished complementation of the original triple mutant strain DFY437. Strains containing hskl::LEU2 were crossed with hskl::Ade5, hsk2::Ade5, or both (from DFY87, Walsh, KawasakI and Fraenkel 1983) were obtained by five or more backcrosses, and the null mutations substituted into the chromosome by transplacement with Ueu2-1 selection: galactose was permissive carbon source. The chromosomal substitutions were confirmed by gel transfer hybridization (Southern analyses) and genetic mapping (linkage of hsk1::LEU2 with met10 and hsk2::LEU2 with ade5). Further crosses and assays allowed identification of the three single gene null mutants. The double mutant, hskl::leuZ hsk2::LEU2, was then obtained, crossed with the gsk1::LEU2 strain and the seven mutant strains identified among segregants.

Growth and enzyme assays: Medium R61 (Fraenkel 1983) was supplemented with the indicated carbon sources (1% galactose, etc.). For "anaerobic" metabolism the respiration inhibitor antimycin A was included at 1 μg/ml. All cultures originated from small inocula (e.g., AS80 values of 0.002) and, for enzyme assays (unless indicated otherwise) were harvested long before glucose exhaustion: e.g., at AS80 values of ca. 2 for cells from medium originally with 2% glucose, and of 0.5 for cultures from 0.2% glucose (glucose exhaustion typically occurs at AS80 of ca. 2 in the latter medium, except for strain DFY568). Cycloheximide, 20 μg/ml, was added just before harvest and also included in washing and resuspension buffer (YEB, 50 mM KH2PO4, 2 mM Na2EDTA, 2 mM 2-mercaptoethanol, pH 7.4); as was 2 mM phenylmethylsulfonyl fluoride in the final resuspension, which was usually to ca. 1/100 of initial culture volume. Hexokinase or glucokinase, and glucose 6-P dehydrogenase assay used extracts made with a French press and 30-min centrifugation at 15,000 X g; similar values were obtained without centrifugation. The assay employed 5 mM triethanolamine with 10 mM MgCl2, pH 7.4, 0.5% NADP, and was supplemented with 1 mM ATP, 5 mM glucose or fructose, and 5 μl of a 1:1 mixture of phosphoglucone isomerase (Boehringer-Mannheim 127-396, 2 mg/ml) and glucose 6-P dehydrogenase (Boehringer-Mannheim 127-035, 1 mg/ml) or, for glucose 6-P dehydrogenase, 1 mM glucose 6-P. Activities at 30° were calculated from increase in absorbance at 340 nm. For Table 1, specific activities were calculated according to protein assay (Bradford 1976) with bovine plasma albumin as standard or, values in parentheses, according to AS80 values in cell suspension before treatment with the French press.

For invertase, cells prepared as above were treated with toluene (1 drop per ml cell suspension), 30 sec on a vortex mixer and 30-min vigorous shaking in a 37° bath. (Similar activities were obtained with French press extraction, about twice the values with glass bead extracts.) The assay mixture contained 75 μl 0.2 m sodium acetate, pH 4.7, 25 μl 0.5 M sucrose (or water), and cells plus YEB to 140 μl. After 60 min at 30°, 100 μl 0.5 M potassium phosphate, pH 7.0, was added and the reaction stopped by 3 min at 100°. Glucose in the supernatant was assayed with hexokinase and glucose 6-P dehydrogenase. Activities were normalized to AS80 in the cell suspension before toluene treatment and, in some cases, protein was measured (Lowry et al. 1951) after precipitation of cells (before toluene treatment) with 0.5 M trichloroacetic acid and resuspension in 1 M NaOH. Protein values were between 80 and 120 μg per AS80.

Yield: Cultures grown from low AS80 were sampled over a range of ca. 0.5 to 2 AS80. For cultures with initial glucose concentration 0.2%, glucose was measured by enzymatic assay (as above), and yield values obtained from the (linear) slopes of plots of glucose vs. AS80. Yields were calculated according to protein assay (Bradford 1976) in the first order growth rate constant, hr -1 , i.e., μmol per hr per AS80. Yields of ethanol were determined from the same samples, with ethanol assay according to Sigma technical bulletin 351-UV.

For growth in media initially containing 2% glucose, the method (adapted from Roengstads, Clark and Katz 1973; Katz and Roengstad 1976) was to measure transfer of radioactivity from 2H1-glucose (initially 10 μCi/ml, TRK361, Amersham) to medium. 

RESULTS AND DISCUSSION

Kinase levels: Table 1 shows enzyme activities for cells obtained from exponential growth on glucose. The three strains carrying just a single kinase gene showed fructose/glucose phosphorylation ratios in extracts in line with knowledge of the individual pure enzymes: values of ca. 3 for hexokinase 1, 1 for hexokinase 2, and less than 0.1 for glucokinase. For strains with more than one intact kinase gene interpretation is less easy. However, as mentioned, experiments where the enzymes have been individually assessed show hexokinase 2 to predominate in growth on glucose, which fits with the present data, both for the wild type and for any strain with HXK2. Also, as
Yeast Hexokinase Mutants

TABLE 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>HxK-Glu</th>
<th>HxK-Fru</th>
<th>G6PDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFY1 (+++)</td>
<td>1.15 (5.81)</td>
<td>1.70</td>
<td>0.13</td>
</tr>
<tr>
<td>DFY566 (+++)</td>
<td>1.30 (7.00)</td>
<td>1.63</td>
<td>0.13</td>
</tr>
<tr>
<td>DFY567 (+++)</td>
<td>0.47 (2.07)</td>
<td>1.43</td>
<td>0.19</td>
</tr>
<tr>
<td>DFY581 (+++)</td>
<td>1.50 (7.92)</td>
<td>1.70</td>
<td>0.13</td>
</tr>
<tr>
<td>DFY568 (+++)</td>
<td>0.17 (0.68)</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td>DFY582 (+++)</td>
<td>1.25 (7.57)</td>
<td>1.25</td>
<td>0.12</td>
</tr>
<tr>
<td>DFY585 (+++)</td>
<td>0.40 (2.29)</td>
<td>1.29</td>
<td>0.15</td>
</tr>
<tr>
<td>DFY570 (----)</td>
<td>0.12 (0.63)</td>
<td>1.15</td>
<td>0.18</td>
</tr>
<tr>
<td>DFY646 (++ ++ ++)</td>
<td>0.56 (3.17)</td>
<td>0.60</td>
<td>0.12</td>
</tr>
</tbody>
</table>

The strains were harvested (see MATERIALS AND METHODS) from growth in medium with 2% glucose and extracts assayed for glucose and fructose phosphorylation activities (HxK-Glu and HxK-Fru, respectively), as well as glucose-6-P dehydrogenase (G6PDH).

1. The notation in parentheses is +, wild type allele, and -, null allele, for HXK1, HXK2 and GLK1, respectively. Strains 9 and 10 are diploids, wild type, and heterozygous for HXK2.

2. Enzyme activities are expressed as units (μmol/min) per mg protein; in the HxK-Glu column values are also given, in parentheses, as μmol/hr/A600, see text.

3. This strain does not grow on glucose, hence no entries in the table. Assays from growth in a permissive medium revealed no glucose or fructose phosphorylation activities.

seen in the three strains with single intact kinase genes, putatively maximal hexokinase 1 activity was lower than hexokinase 2, and glucokinase activity was even lower (relative values of ca. 0.3 and 0.1, respectively).

Growth on various sources: The two enzymes hexokinase 1 and hexokinase 2 have been long studied (Colowick 1973) and known to phosphorylate glucose, fructose and mannose. Yeast glucokinase is less well known (Maitra 1975). Contrary to its name, it does phosphorylate, but with lower Vmax and higher Km, other sugars and derivatives, including mannose, but fructose is barely employed. Earlier work on undefined deficiency mutants showed that growth of yeast on fructose required either hexokinase 1 or hexokinase 2; that a strain lacking both still grew on glucose, as did strains with only hexokinase 1 or hexokinase 2; and that a strain lacking all three did not grow on glucose (Lobo and Maitra 1977a; Gancedo, Clifton and Fraenkel 1977).

The present strains with null mutations grow in accord with findings from previous mutants. Thus, as judged on plates (Table 2), all the mutant strains grew normally on galactose and all except the triple mutant grew well on glucose. Neither the triple mutant DFY570 nor the double mutant DFY568 (hxk1 hxk2) grew on fructose, and the double mutant was intermediate in its growth on mannose. And, obviously, the triple null mutant strain is clearly viable (as also reported by Albic and Entian 1988). DFY570 did not notably differ in its growth from the triple mutant previously employed, DFY437 (Walsh, Kawasaki and Fraenkel 1983). Tests with antiserum showed cross reacting material to hexokinase 2 but not to hexokinase 1 in the latter strain, but not when the allele was hxk2::LEU2 instead of hxk2-1 (Vojtek 1988).

There are other observations on these growth patterns:

1. An enriched medium was employed, so there was some growth in the absence of added carbon source; this was reduced by use of minimal medium or by the respiration inhibitor antimycin A. Thus, on a minimal plate with glucose and 5 days of incubation, colonies of the triple mutant strain were of ca. 0.1-mm diameter vs. 0.8-mm colonies for the double mutant strain DFY568. However, prolonged incubation of the triple mutant on a minimal glucose plate showed slight growth (0.7-mm colonies after 6 days), and there were similar indications on enriched medium that the double and triple mutant strains DFY568 and DFY570 are not completely inert to fructose, or fructose and glucose, respectively. The possibility of marginal metabolism of these sugars, by whatever pathway, is not excluded.

2. The strains unable to phosphorylate glucose, or fructose, were indifferent to these sugars in their residual growth on the enriched medium. In data not presented, the same result of no apparent inhibition of the triple mutant by glucose was seen on plates containing both pyruvate and glucose. Glucose was somewhat inhibitory on a plate containing galactose, direct interference with galactose uptake being a likely explanation.

3. In spite of lack of glucose toxicity in the triple mutant, maltose and sucrose were inhibitory. Maltose even inhibited growth of the double mutant strain DFY568, a surprising finding in view of its growth on...
phorylation strain whose growth consistently was slower than the others, doubling time of in the presence of antimycin assayed, and the determinations also done for growth adequate for relatively normal growth.

Although growth on glucose, as judged by colony sizes (Table 3), did not differ much between the seven strains with one or more kinase, actual growth rates might significantly differ and metabolic flux of glucose might differ even more. After all, S. cerevisiae has a relatively inefficient glucose metabolism which is largely fermentative even aerobically (Fraenkel 1982), so large differences in glucose flux in mutants might be compensated by respiration.

We therefore determined (see MATERIALS AND METHODS) growth rate, yield, and glucose flux values (Table 3). For some of the strains ethanol was also assayed, and the determinations also done for growth in the presence of antimycin A. The following points may be made.

1. All seven strains grew well on glucose in liquid medium, as expected from growth on plates. The only strain whose growth consistently was slower than the others, doubling time of ca. 125 min instead of ca. 95 min, was the strain containing just glucokinase, DFY568, which has the lowest value for glucose phosphorylation in vitro (Table 1). That level is clearly adequate for relatively normal growth.

2. Each strain grew at similar rate (and yield) on glucose. Experiments on this matter will be reported elsewhere.

**Rate and yield of glucose metabolism in growth:**
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and decrease cell yield and growth rate (Table 3), as if this strain has significant respiratory metabolism in these conditions. The same pattern was observed for mutant strains containing or HXK2 or HXK1. Mere absence of hexokinase 2, therefore, has a marginal effect on fermentation itself.

For the strain with just glucokinase, DFY568, aerobic ethanol yield was 0.9 mol per mol glucose—something less than the other strains—and antimycin A markedly slowed growth, decreased cell yield and doubled ethanol yield. Thus, the contribution of respiratory metabolism is larger in this strain than in the others.

**Invertase:** Table 4 reports total cell associated invertase in the same conditions used for glucose flux, as well as some other ones. In spite of differences in methodology, these data are in many ways confirmatory of the known picture of hexokinase 2 involvement in catabolite repression. Thus (i), in the four strains with HXK2 invertase was repressed in growth on 2% glucose (values of ca. 0.02 unit/A500) while hxxk2 mutants were not repressed (ca. 1 unit/A500). (ii) About the same result as with 2% glucose was obtained for medium with 0.2% glucose (column 2), but with somewhat higher repressed levels. (iii) For growth in the presence of antimycin A (column 3), repressed values were higher than in normal aerobic growth, with, again, typical derepressed values in the haxk2 strains. (iv) Invertase was derepressed in HXK2 strains after glucose exhaustion from 0.2% glucose cultures (column 4), with similar levels to cultures in exponential growth on galactose (column 5). Derepressed levels of invertase were similar throughout the set of strains. (v) A pair of congenic diploids of the same series, HXK2/HXK2 and hxxk2/hxxk2, showed normal repressed level of invertase on 2% glucose and normal derepressed level on galactose. (vi) For the wild-type strain, as cultures depleted glucose from 0.2% concentration, half-maximal invertase level was observed at ca. 1 mM glucose (data not shown).

**Comments: invertase expression and glucose metabolism:** Comparing invertase with glucose flux (Tables 3 and 4), cultures with high rates of glucose metabolism were repressed and ones with low rates were derepressed. One interpretation might be that repression is a secondary consequence of high catabolic rate: perhaps some ordinary metabolite whose amount differs in the two situations governs gene expression. The partial restoration of catabolite repression in a hxxk2 mutant strain by additional copies of HXK1 (MA and BOTSTEIN 1986) might fit this model. However, since strains with similar Vglu values, ca. 2.2, may be repressed (HXK2) or derepressed (hxxk2), differences in flux beyond the accuracy of our data would have to be significant. The same comment applies to metabolite levels, found as relatively normal (ENTIANT and FRÖHLICH 1984). But it should be remarked that if a function of catabolite repression were, say, maintenance of ATP, then levels of a signaling metabolite (e.g., ATP itself) might indeed be similar in the two steady states.

**TABLE 4**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>2% G</th>
<th>0.2% G</th>
<th>0.2% G + A</th>
<th>After 0.2% G</th>
<th>1% Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFY1 (+++)</td>
<td>0.02</td>
<td>0.05</td>
<td>0.14</td>
<td>0.63</td>
<td>0.87</td>
</tr>
<tr>
<td>DFY566 (-++)</td>
<td>(0.16)</td>
<td>(0.34)</td>
<td>(0.92)</td>
<td>(6.7)</td>
<td>(9.8)</td>
</tr>
<tr>
<td>DFY567 (+++)</td>
<td>1.00</td>
<td>0.67</td>
<td>—</td>
<td>1.07</td>
<td>1.09</td>
</tr>
<tr>
<td>DFY581 (+++)</td>
<td>(6.9)</td>
<td>(7.3)</td>
<td>(16.5)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DFY568 (-++)</td>
<td>0.01</td>
<td>0.14</td>
<td>—</td>
<td>1.12</td>
<td>0.78</td>
</tr>
<tr>
<td>DFY582 (-++)</td>
<td>1.38</td>
<td>1.17</td>
<td>2.03</td>
<td>1.12</td>
<td>0.74</td>
</tr>
<tr>
<td>DFY583 (-++)</td>
<td>(18.1)</td>
<td>(14.1)</td>
<td>(10.1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DFY570 (----)</td>
<td>0.02</td>
<td>0.05</td>
<td>0.16</td>
<td>0.58</td>
<td>0.47</td>
</tr>
<tr>
<td>DFY646 (++ ++++)</td>
<td>1.07</td>
<td>0.94</td>
<td>0.65</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>DFY647 (++ ++ ++)</td>
<td>(9.4)</td>
<td>(9.7)</td>
<td>(11.5)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DFY648 (++ ++ ++)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*As in Table 1.

1 Invertase activity is expressed as units (μmol glucose formed from sucrose per min) per A500, as well as for many samples, units per mg protein (values in parentheses); see MATERIALS AND METHODS.

The media contained glucose (G, or, last column, galactose) at the initial concentrations indicated; in column 3, A means that antimycin was also present. Cells were harvested (see MATERIALS AND METHODS) from a low absorbance, so that most of the sugar was still unused, except for column 4 samples, which were taken after glucose exhaustion ("After 0.2%.")

"-" means not done. Note that strain DFY570 does not grow on glucose.
A different model, not contradicted by the present results, has been emphasized (Entian 1986): a special function for hexokinase 2 separate from its metabolic role. An important line of evidence has been mutants with adequate catalysis but defective in repression (Entian and Fröhlich 1984). However, proof that the altered enzyme is normal in catalysis (Entian et al. 1985) is incomplete. Indeed, an extensive study of hexokinase 2 mutants showed a correlation between glucose phosphorylation activity and catabolic repression (Ma et al. 1989a,b). Perhaps, as suggested by S. Kuchin (personal communication), there is a catalytic activity of hexokinase 2 which is different from normal glucose phosphorylation. Hexokinase 2 has been reported as a protein kinase (Herrero, Fernandez and Moreno 1989), and both hexokinases as being phosphoproteins in vivo (Vojtek and Fraenkel 1990).

Finally, it need be emphasized that our data are only for invertase, and other systems subject to catabolite repression and/or hxxk2 derepression might differ in sensitivity or mechanism. One example is high affinity glucose uptake (Bisson and Fraenkel 1984; Bisson 1988), which, considering the Km values and that metabolism may be limited at uptake (Becker and Betz 1972) is likely in some of the present experiments to be derepressed when invertase is repressed.

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