Induction of specific enzymes of the oxidative pentose phosphate pathway by glucono- δ -lactone in *Saccharomyces cerevisiae*

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Growth of *Saccharomyces cerevisiae* on D-glucono- δ -lactone (δ gl) was found to be associated with a specific co-ordinate induction of the synthesis of two enzymes of the oxidative pentose phosphate pathway – 6-phosphogluconate dehydrogenase and 6-phosphogluconolactonase – together with that of a third enzyme, gluconokinase. The *gnd1* mutation, responsible for an approximately 80% loss of 6-phosphogluconate dehydrogenase activity and the inability of the cells to grow on δ gl, completely abolished the induction of all three enzymes, while the *gnd2* mutation affected this only partially. One class of *gnd1* revertants, selected for growth on δ gl, was found to have recovered normal dehydrogenase activity and the ability to synthesize the three enzymes when induced by δ gl. Another class of δ gl-positive revertants possessed constitutively elevated levels of gluconokinase. In contrast, glucose-positive revertants of *gnd1*, with restored constitutive dehydrogenase activity, continued to remain deficient in induction of the three enzymes and also failed to grow on δ gl. Induction of 6-phosphogluconate dehydrogenase activity was associated with increased transcription of the gene coding for the major isoenzyme; the transcript remained undetectable in the *gnd1* mutant. Induction of these specific enzymes thus appears to be essential for growth of *S. cerevisiae* on δ gl.

Introduction

The pentose phosphate pathway has been traditionally considered a biosynthetic route to ribose 5-phosphate for nucleotide and nucleic acid synthesis, erythrose 4-phosphate, the precursor of the aromatic skeleton, and NADPH for reductive steps in cellular biosynthesis. It remains, however, perhaps the least studied of all the important metabolic pathways in *Saccharomyces cerevisiae*. This could possibly be attributed to this yeast being rather conservative in its ability to use pentoses, carbon sources which can be metabolized directly through this pathway, for growth (see Fraenkel, 1982, 1986). The functions and regulation of both the oxidative and non-oxidative branches of this pathway thus remain largely unknown.

Glucono- δ -lactone (δ gl), the 1–4 lactone of D-gluconic acid, can serve as a poor carbon source for *S. cerevisiae*

(Gancedo & Delgado, 1984). Since it is a hexose which could be catabolized exclusively through the pentose phosphate pathway, a study of its metabolism should help in studying the operation of this pathway in yeast. The reactions by which δ gl is metabolized to the level of the pathway intermediates have never been investigated. Two possible routes can be envisaged (Fig. 1).

1. The hydrolysis of δ gl by the enzyme gluconolactonase to gluconic acid, which can then be phosphorylated by a gluconokinase to 6-phosphogluconate. This shunt intermediate can then be further metabolized by the enzyme 6-phosphogluconate dehydrogenase in the oxidative branch of the pentose phosphate pathway.

2. A direct phosphorylation of δ gl by a lactone kinase to 6-phosphogluconolactone, followed by its hydrolysis to 6-phosphogluconate by 6-phosphogluconolactonase, another reaction of the oxidative pathway.

Although evidence exists for gluconolactonase and gluconokinase activities in yeast extracts *in vitro* (Brodie & Lipmann, 1955; Cohen, 1955), the physiological significance of these enzymes is unknown. The phosphorylation of δ gl has never been demonstrated either *in vitro* or *in vivo* and therefore remains only hypothetical.

This paper investigates the specific induction of synthesis of the three enzymes, 6-phosphogluconate

Abbreviations: δ gl, glucono- δ -lactone; GND, 6-phosphogluconate dehydrogenase; PGL, 6-phosphogluconolactonase; GNK, gluconokinase; GPD, glucose-6-phosphate dehydrogenase.

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Fig. 1. Possible routes of δ gl metabolism in *S. cerevisiae*.

dehydrogenase (EC 1.1.1.44), 6-phosphogluconolactonase (EC 3.1.1.31) and gluconokinase (EC 2.7.1.12) during growth of yeast on δ gl. The previously isolated *gnd1* mutant, which has reduced levels of GND (Lobo & Maitra, 1982), was further characterized and found to be affected in the induction of all three enzymes by δ gl. Revertants of the *gnd1* mutant with different 'induction' phenotypes have also been studied and reveal the relevance of this phenomenon, possibly under co-ordinate regulatory control, for the growth of yeast on δ gl.

Methods

Strains. The strains used in this study are listed in Table 1.

Media. Synthetic minimal medium was prepared according to Sherman *et al.* (1986). The enriched YEP medium containing yeast extract, peptone and carbon source has been described previously (Lobo & Maitra, 1977). The synthetic medium was adjusted to pH 6.5 whenever δ gl was used as carbon source. All strains bearing the *gnd1* mutation were maintained on YEP medium containing 150 mM-ethanol.

Chemicals. δ gl, D-gluconic acid, glucose 6-phosphate and 6-phosphogluconate dehydrogenase were from Sigma. All other substrates and enzymes were from Boehringer Mannheim. Hybond-N membrane for Northern blotting was obtained from Amersham. Labelling of the DNA probe was done with the random primed DNA labelling kit from Boehringer Mannheim.

Enzyme assays. Enzymes were assayed fluorimetrically, either in cell-free extracts or in toluene lysates, as described previously (Maitra & Lobo, 1971). All cell-free extracts were made in 50 mM-HEPES buffer, pH 7.3, containing 5 mM-EDTA and 5 mM- β -mercaptoethanol. Toluene lysates were used for assaying 6-phosphogluconate dehydrogenase in experiments on the kinetics of its induced synthesis. For this purpose, a loopful of actively growing cells was suspended in 0.5 ml of the above-described HEPES buffer, a drop of toluene added and the mixture shaken on a reciprocating shaker at 37 °C for 30 min. About 50–100 μ l of this cell suspension was used for the assays.

All enzymes, except 6-phosphogluconolactonase, were assayed in 50 mM-triethanolamine buffer, pH 7.4, containing 10 mM-MgCl₂.

6-Phosphogluconate dehydrogenase was assayed by following the appearance of NADPH in a 0.8 ml reaction mixture containing 0.4 mM-6-phosphogluconate and 0.1 mM-NADP⁺.

Glucose 6-phosphate dehydrogenase was assayed similarly, except that 0.1 mM-glucose 6-phosphate was used as the substrate instead of 6-phosphogluconate.

Gluconokinase was measured in discontinuous assays by incubating cell-free extract in a 0.8 ml reaction mixture containing 5 mM-gluconate and 2 mM-ATP for 60 min at room temperature. The mixture was then heated in a boiling water bath for 5 min and the product (6-phosphogluconate) assayed fluorimetrically with 0.5 mM-NADP⁺ and 1 unit of 6-phosphogluconate dehydrogenase.

6-Phosphogluconolactonase was assayed by pre-incubating a 0.8 ml reaction mixture containing 50 μ M-glucose-6-phosphate, 0.5 mM-NADP⁺ and 0.5 units of glucose-6-phosphate dehydrogenase in 50 mM-MES buffer, pH 6.5, containing 25 mM-KCl and 10 mM-MgCl₂, until the reaction was complete. This was followed by addition of 1 unit of 6-phosphogluconate dehydrogenase which resulted in a slow increase in fluorescence due to the spontaneous hydrolysis of the lactone formed during the earlier reaction. The addition of cell-free extracts at this stage led to an enhanced rate of NADP reduction due to the lactonase reaction. The actual lactonase rate was calculated by subtracting the previous blank rate from this final rate.

Table 1. Yeast strains used in course of this work

Strain	Genotype	Source*	Remarks
MC3	<i>MATa ino1 ino4</i>	G. R. Fink	Strains used as the wild-type
Kn79	<i>MATa trp1 leu2</i>	B. Hall	
gnd T5A	<i>MATa gnd1 GND2</i>	Z. Lobo	gnd mutant strains used for the isolation of revertants
gnd T9A	<i>MATa gnd1 gnd2</i>	Z. Lobo	
gnd T10A	<i>MATa gnd1 GND2</i>	This work	Segregants of a tetrad type tetrad from a cross of the <i>gnd1 gnd2</i> double mutant (gnd T9A) with a wild-type strain (EG103)
gnd T10B	<i>MATa gnd1 gnd2</i>		
gnd T10C	<i>MATa GND1 gnd2</i>		
gnd T10D	<i>MATa GND1 GND2</i>		
R34		This work	Glucose-positive revertants of strain gnd T5A
R41			
R1		This work	δ gl-positive revertants of strain gnd T9A
R4			
R39			
R7			
CT4	<i>MATa gnd1, gnd2::GND1</i>	Z. Lobo	High-copy-number cDNA clone of <i>GND1</i>

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The specific activities of all the enzymes in the cell-free extracts were expressed in mU, (i.e. nmol substrate utilized min⁻¹) per mg total protein (mU mg⁻¹), and that of 6-phosphogluconate dehydrogenase in toluene lysates as mU per OD₆₅₀ unit, i.e. the optical density of the cell-suspension in a 1 cm pathlength cuvette at a wavelength of 650 nm [mU (OD₆₅₀ unit)⁻¹].

Total protein was estimated by the method of Bradford according to Peterson (1983), using BSA as standard.

Genetic techniques. The genetic analysis of all the mutants followed standard techniques of crossing, sporulation and tetrad dissection as described by Sherman *et al.* (1974).

Isolation of revertants. Mutant strains bearing the *gnd1* mutation were grown to late-exponential phase on YEP medium containing 150 mM-ethanol. The cells were then washed free of the medium and plated on synthetic medium, containing either 50 mM-glucose or 50 mM- δ gl, at approximately 5×10^7 – 10^8 cells per plate. The glucose plates were incubated at 30 °C and the δ gl plates at 36 °C. Not more than two or three visibly different revertant colonies were picked from a particular culture. This was done to increase the possibility of selecting revertants which arose from separate events. The colonies were purified and maintained on the appropriate restrictive medium.

Northern blot analysis. Total yeast RNA was prepared according to Carlson & Botstein (1982) with certain modifications. About 4 g of sterile glass beads was added to a pellet of 100–150 mg cells (collected during the exponential phase of growth) in 2 ml LETS buffer containing 0.1 M-LiCl, 0.1 M-Tris (pH 7.5), 10 mM-EDTA, 1% (w/v) SDS and 0.1% diethylpyrocabonate. About 2 ml of a mixture of freshly distilled phenol, chloroform and isoamyl alcohol (25:25:11, by vol.) was added immediately and the suspension vortexed vigorously for 15–30 min, with 30 s alternate breaking and cooling cycles. Following low-speed centrifugation for 10 min, the upper aqueous layer was removed and the remainder extracted again with 2 ml LETS buffer and 2 ml of the phenol mixture. The pooled aqueous supernatant was then mixed with 0.1 vol. 5 M-potassium acetate, pH 4.8, and 2 vols chilled absolute ethanol and allowed to stand at –20 °C overnight to precipitate the RNA. After centrifugation at 12000 *g* for 15 min, the precipitate was finally resuspended in 10 mM-Tris, 1 mM-EDTA, pH 8,

to a final concentration of 8–10 mg ml⁻¹, as measured by the absorbance at 260 nm.

The RNA was electrophoresed on a 1.2% formaldehyde/agarose gel in MOPS buffer exactly as described in Sherman *et al.* (1986). The gel was then equilibrated in 20 $\times$ SSPE, containing 3.6 M-NaCl, 0.2 M-NaH₂PO₄ and 20 mM-EDTA, pH 7.4, for 20 min and transferred to Hybond-N membrane as recommended by the supplier. Hybridization to the DNA probes was done under high stringency conditions, again using a protocol suggested by Amersham.

Results and Discussion

Growth on glucono- δ -lactone

Ethanol-grown wild-type MC3 cells, when inoculated into minimal medium containing 50 mM- δ gl, grew with a doubling time of about 10 h as compared to 5 h on 150 mM-ethanol or 1.75 h on 50 mM-glucose. The cell yield obtained, in terms of wet weight, was also significantly less than those obtained on ethanol or glucose (Table 2). Since δ gl can readily hydrolyse to D-gluconic acid in solution, the low cell yields could be due to the low amounts of unhydrolysed lactone and/or the low pH of the medium.

Respiration appeared to be essential for the utilization of δ gl; wild-type strains failed to grow on δ gl anaerobically (data not shown).

Induction of specific enzymes by glucono- δ -lactone

Addition of δ gl to ethanol-grown cells led to a significant rise in the activities of three enzymes, 6-phosphogluconate dehydrogenase (GND), 6-phosphoglucono-

Table 2. *Growth parameters of wild-type yeast on δ gl, as compared to those on ethanol and glucose*

Cells of strain MC3 were grown to early exponential phase in minimal ethanol medium, washed, and inoculated into minimal media containing the respective carbon sources. Growth was monitored by recording the optical density at 650 nm at periodic intervals. Cell yields were measured after 3 d growth at 30 °C when the cells had reached stationary phase. Colony diameters were measured following 4 d growth of plated cells on minimal agar medium, containing the respective carbon sources, at 30 °C. The growth parameters of yeast in liquid media were estimated twice; the values shown are representative. Colony diameters were measured from 50 randomly chosen colonies in two experiments.

Carbon source	Doubling time (h)	Cell yield (mg wet wt ml ⁻¹)	Colony diameter (mm)
δ gl (50 mM)	10	0.78	2.0–2.5
Ethanol (150 mM)	5	13.10	3.5–4.0
Glucose (50 mM)	1.75	14.80	5.0–6.0

Table 3. *Induction by δ gl of the synthesis of enzymes of the oxidative pentose phosphate pathway and gluconokinase in wild-type yeast*

All cultures, of strain Kn79, were grown initially on YEP ethanol medium to mid-exponential phase prior to addition of the test carbon sources. The cultures were induced for 3 h before cell-free extracts were prepared and the enzymes assayed, as described in Methods. Cycloheximide was added to a final concentration of 10 μ g ml⁻¹ to a culture of cells grown to mid-exponential phase in YEP ethanol medium and the culture shaken for 30 min. δ gl was then added to a concentration of 50 mM and the culture induced for 3 h before extracts were prepared. The experiment on induction of the enzymes with different carbon sources was repeated six times, cycloheximide being included in two of them; only representative results are shown.

Carbon Source	Doubling time (h)	Enzyme specific activity [mU (mg protein) ⁻¹]			
		GND	PGL	GNK	GPD
Ethanol	3.0	59.5	5.5	17.0	144.0
Ethanol + glucose	1.25	74.2	7.4	20.0	148.0
Ethanol + δ gl	2.5	616.0	40.2	58.0	163.0
Ethanol + δ gl + cycloheximide	6.0	125.0	10.0	15.0	150.0

lactonase (PGL) and gluconokinase (GNK), as measured *in vitro* (Table 3). The increase in activity of glucose-6-phosphate dehydrogenase (GPD) was not consistently observed. The co-ordinate rise in activity of all these enzymes was evident within 15 min of addition of δ gl and reached its peak in about 1–2 h. Although there was some variation in the basal levels of enzyme

activity, GND usually showed a tenfold increase, PGL and GNK approximately fivefold (Fig. 2).

The remarkably high δ gl-induced GND specific activity of 1.4 U (mg protein)⁻¹ can be compared to the specific activity – 70 U (mg protein)⁻¹ – of the pure enzyme (unpublished data); GND thus constitutes approximately 2% of the total cellular protein when cells are grown on δ gl.

The specificity of induction was restricted to these three enzymes only, since none of the glycolytic, gluconeogenic or non-oxidative pentose phosphate pathway enzymes were similarly induced. Glucose, glycerol, xylulose and gluconate, on the other hand, failed to induce the δ gl-inducible enzymes (data not shown).

The rise in activity of all three enzymes was sensitive to the addition of cycloheximide to a growing culture, implicating *de novo* protein synthesis during the process of induction (Table 3). The growth rate of the culture was, however, only slightly reduced during this treatment.

Induction of 6-phosphogluconate dehydrogenase: kinetics

Since the specific activity of GND is fairly high, the enzyme can be detected easily in permeabilized yeast cells. An investigation was therefore made into the kinetics of induction of this enzyme.

The differential rates of synthesis of GND during growth on ethanol, in the presence and absence of the inducer, are shown in Fig. 3. Enzyme synthesis on δ gl, as sole carbon source, began to fall off as the cells reached stationary phase, usually within 24 h of growth. The sustained synthesis when ethanol was also present indicated a role for the latter more as an easily available energy source; by itself, ethanol supported only the constitutive level of synthesis.

The initial and final rates of enzyme synthesis, as well as the final levels of enzyme activity reached, were dependent on the inducer concentration used (Fig. 4). Thus, the rate of GND synthesis increased between 5- and 25 mM- δ gl, while the steady-state enzyme level was even higher at 50 mM- δ gl. Withdrawal of δ gl during the course of induction halted further enzyme synthesis, suggesting that the continued presence of the inducer is essential for maintenance of the induced state (data not shown).

*Pleiotropic effects of the *gnd1* and *gnd2* mutations*

The *gnd1gnd2* double mutant, isolated previously in this laboratory as a glucose-negative, GND-deficient strain (Lobo & Maitra, 1982), exhibited extremely slow growth in minimal medium with 50 mM- δ gl at 30 °C, while

Fig. 2. Kinetics of the increases in activity of GND, GPD, PGL and GNK on induction with δ gl. Two cultures of strain MC3 were grown in YEP ethanol medium. δ gl was added at time zero to a final concentration of 50 mM to one culture (filled symbols), growing exponentially (cell density about 10^7 cells ml^{-1}). No additions were made to the other, control culture (open symbols). Samples of the cultures were removed at the indicated time intervals and the specific activity of the enzymes measured in cell-free extracts, as described in Methods. This experiment was repeated three times; the results shown are representative. (a) GND ($\circ$, $\bullet$) and GPD ($\square$, $\blacksquare$); (b) PGL ($\circ$, $\bullet$) and GNK ($\square$, $\blacksquare$).

Fig. 3. Differential rate of the increases in activity of GND in fully induced cultures. Cells of strain MC3, growing exponentially in YEP media with 150 mM-ethanol and 50 mM- δ gl, individually and in combination, were re-inoculated into fresh media at a density of about $3\text{--}6 \times 10^7$ cells ml^{-1} . Samples of the cultures ($\bullet$, ethanol; $\circ$, δ gl; $\square$, ethanol + δ gl) were then removed at intervals and GND activity was assayed in toluene lysates, as described in Methods. Representative results for the two experiments performed are shown.

Fig. 4. Effect of inducer concentration on the kinetics of increases in GND activity. A culture of strain MC3, grown to early exponential phase (about 10^7 cells ml^{-1}) on YEP ethanol medium, was divided into five equal portions, and to four of these δ gl was added at 5 ($\bullet$), 10 ($\square$), 25 ($\blacksquare$) and 50 ($\triangle$) mM, respectively. One portion was maintained without inducer ($\circ$). Samples of the cultures were then removed at the indicated time intervals and GND activity was assayed in toluene lysates, as described in Methods. This experiment was repeated twice; representative results are shown.

growth was completely inhibited at 36 °C (Table 4). A segregant bearing the *gnd1* mutation alone retained 20% of wild-type GND activity but showed the same phenotype as that of the double mutant. The glucose-positive *gnd2* mutant, with approximately 80% wild-type activity, was, however, comparable to the wild-type strain in its growth on δ gl.

The *gnd1* mutant was found, rather surprisingly, to be totally lacking in the δ gl-induced synthesis of all three enzymes, GND, PGL and GNK (Table 4). The *gnd2* mutant was only partially affected, the inducibility of the enzymes being approximately 25% of that in the wild-type strain.

Table 4. Comparison of the constitutive and δ gl-induced levels of enzymes of the oxidative pentose phosphate pathway and gluconokinase in the *gnd1* and *gnd2* mutants, different revertants of the *gnd1* mutant and a strain carrying the cDNA clone which complements the *gnd1* mutation

The doubling time of the strains was measured in minimal medium containing either 150 mM-ethanol (EtOH) at 30 °C or 50 mM- δ gl at 36 °C. This experiment was repeated twice; representative values are shown. For the measurement of induced enzymes, the cultures were grown to early exponential phase in YEP ethanol media and induced with 50 mM- δ gl for 3 h at 30 °C. Cell-free extracts were made and the enzymes assayed as described in Methods. The results shown are representative of the four experiments performed. The genotypes of the *gnd* mutants are listed in Table 1, while the phenotypes of the revertants and the cDNA clone are described in the text.

Strain	Doubling time (h)		Enzyme specific activity [mU (mg protein) ⁻¹]							
			GND		PGL		GNK		GPD	
	EtOH	δ gl	EtOH	δ gl	EtOH	δ gl	EtOH	δ gl	EtOH	δ gl
<i>gnd</i> T10A	6.5	38	19	23	6	10	30	32	245	247
<i>gnd</i> T10B	9	>45	11	12	7	9	19	19	208	222
<i>gnd</i> T10C	6.5	15	47	226	7	15	16	38	210	173
<i>gnd</i> T10D	4.5	13	69	814	6	35	15	70	200	212
R34	5	19.5	51	49	9	7	27	27	213	194
R41	5.5	>24	41	45	7	10	15	13	173	171
R39	7	10	90	800	10	56	32	86	209	235
R7	5.5	9.5	18	22	9	9	64	71	364	324
CT4	6	>24	3777	4102	45	31	15	14	272	273

It was important to examine the possibility that the *gnd1* mutant may harbour yet another mutation responsible for its inability to be induced for the enzymes PGL and GNK. A *gnd1* segregant, *gnd* T5A, was therefore crossed to the wild-type strain Kn79, and 15 complete tetrads were analysed for glucose-negativity, the ability to grow on δ gl, and constitutive as well as induced GND activity. The characteristic glucose-negativity of the *gnd1* lesion co-segregated 2⁺:2⁻ with the phenotypic lack of induction of GND and δ gl-negativity in all tetrads, indicating that these three phenotypes are very tightly linked.

Reversion studies of the *gnd1* mutant

Spontaneous revertants of the *gnd1* mutant were obtained using two independent selection schemes: the ability to grow on glucose at 30 °C and on δ gl at 36 °C. Both the *gnd1* mutant (strain *gnd* T5A) and the *gnd1 gnd2* double mutant (strain *gnd* T9A) yielded similar kinds of revertants – an indication that the *gnd2* lesion does not by itself exert any selection pressure for revertants to arise.

Glucose-positive revertants of the *gnd1* mutant

The majority of glucose-positive revertants of *gnd1* have been reported to be deficient in GPD activity (Lobo & Maitra, 1982). All of the revertants selected were thus assayed for GPD, and 30 back-revertants showing constitutive GPD and GND activity were analysed for

growth on δ gl. None of these revertants could grow on δ gl at 30 °C or at 36 °C and were also completely deficient in their ability to be induced for all three enzymes, GND, PGL and GNK, by δ gl. The activities of these enzymes in two such revertants (R34 and R41) of strain *gnd* T5A are shown in Table 4.

To determine the nature of the reversion events, the two revertants R34 and R41 were subjected to tetrad analysis in crosses with the wild-type strain Kn79. Twenty complete tetrads were dissected in each cross. All the tetrads of the R34 cross segregated 4⁺:0⁻ for growth on glucose, implicating a locus tightly linked to the *gnd1* mutation for the reversion. The tetrads of the R41 cross, on the other hand, segregated differently for glucose-positivity, the numbers of parental ditype (4⁺:0⁻), non-parental ditype (2⁺:2⁻) and tetratype (3⁺:1⁻) tetrads being five, one and fourteen respectively. R41 had clearly reverted at an extragenic locus.

Glucono- δ -lactone revertants of the *gnd1* mutant

Approximately 200 revertants of the strains *gnd* T5A and *gnd* T9A, capable of growth on δ gl at 36 °C, were analysed for the inducibility of GND by δ gl. Since the loss of GPD activity did not enable the *gnd1* mutant to grow on δ gl (unpublished observations), such mutants were never selected for. All the revertants obtained by this selection fell into either of the two following phenotypic classes.

One class of revertants, typically represented by strain R39, was capable of limited growth on glucose and had regained the normal constitutive levels of GND as well as inducibility of all three enzymes by δ gl (Table 4). Three such randomly chosen revertants, R1, R4 and R39, were subjected to tetrad analysis in crosses with the wild-type strain Kn79. Twenty tetrads, dissected in each of two crosses (R1 and R4), and 17 tetrads, dissected in the third cross (R39), segregated $4^+ : 0^-$ for growth on δ gl and for δ gl-induced GND synthesis, indicating that all three strains had reverted in loci tightly linked to the original *gnd1* lesion.

The other class of revertants, typically represented by strain R7, grew slowly on δ gl at 30 °C and at 36 °C but remained glucose-negative. These revertants possessed constitutively high levels of GNK although their GND and PGL levels remained comparable to the *gnd1* parent (Table 4). Nine tetrads, obtained in a cross between strain R7 and the wild-type Kn79, segregated $2^+ : 2^-$ for growth on glucose. However, they segregated differently for growth on δ gl, the number of parental ditype ($4^+ : 0^-$), non-parental ditype ($2^+ : 2^-$) and tetratype ($3^+ : 1^-$) tetrads being one, three and four respectively. The recovery of δ gl-negative progeny in this cross confirmed the extragenic nature of this reversion. The occurrence of two δ gl-positive spores in the non-parental ditype tetrads also showed that the revertant locus, by itself, did not affect growth on δ gl. Moreover, these segregants had high levels of GNK activity, clearly establishing that this effect was mediated by the revertant locus independent of the *gnd1* allele.

Direct evidence that the induction process has a meaningful role in δ gl metabolism comes from the *gnd1* mutant. Glucose-positive revertants of this mutant, with restored constitutive GND levels, do not show induction of any of the enzymes by δ gl and also fail to grow on this compound. In contrast, revertants selected for growth on δ gl regained the 'induction' phenotype to wild-type levels. There is thus a clear correlation between the ability of δ gl to mediate induction and the ability of cells to use this compound as a carbon source.

The δ gl-positive revertants of the *gnd1* mutant with elevated constitutive levels of GNK were able to grow slowly on δ gl, although GND and PGL were not induced to normal levels. GNK activity *in vivo* may thus be the rate-limiting step in δ gl metabolism, with the need for its induction being more critical than those of GND and PGL.

Analysis of a cDNA clone complementing the gnd1 mutation

A multicopy 1.65 kb cDNA clone, complementing the *gnd1* mutation, had been isolated previously and shown

to correspond to the structural gene for the major GND isoenzyme (Z. Lobo & P. K. Maitra, unpublished results). This clone (CT4) had high constitutive GND levels, though its GNK activity was comparable to that in the *gnd1gnd2* double mutant (Table 4). The PGL levels were also remarkably elevated in this clone. It was, however, totally deficient in the inducibility of all the three enzymes and failed to grow on δ gl at 36 °C, although growth on glucose was restored to the wild-type level.

The inability of cells carrying *GND1* cDNA to grow on δ gl in spite of elevated constitutive GND and PGL levels is not surprising, since they lack the 'induction' phenotype and consequently have a low level of GNK, comparable to that of the *gnd1gnd2* double mutant. This further supports the view that GNK activity levels may be most critical in the metabolism of δ gl, at least in the oxidative part of the pathway.

Identification of the importance of GNK activity for growth of yeast on δ gl could indicate that the metabolism of this compound proceeds preferentially by an initial conversion to gluconate rather than its direct phosphorylation to 6-phosphogluconolactone (Fig. 1). If this is true, more attention should be paid to the gluconolactonase activity reported by Brodie & Lipmann (1955). Does this enzyme also exhibit induction by δ gl? Our efforts to study this enzyme failed due to the instability of its substrate in solution, making the enzyme difficult to assay.

Molecular expression of the GND1 gene on induction with glucono- δ -lactone

The synthesis of most inducible proteins is governed at the level of transcription. Since a functional *GND1* gene appears to be essential for induction of GND synthesis, it was of interest to ascertain whether transcription from this gene was involved in induction. A 0.65 kb *SalI* internal fragment from the above-mentioned cDNA clone was therefore used in a Northern blot to probe total RNA prepared from the wild-type and the *gnd1* and *gnd2* mutants, as well as the different revertants, grown on ethanol with and without inducer.

Induction of GND synthesis correlates with the simultaneous appearance of a specific 1.6 kb mRNA species at fairly high levels during the growth of cells on δ gl (Fig. 5). This transcript was, however, absent in strains bearing the *gnd1* mutation, both constitutively and on induction. The effect, if any, of the *gnd2* mutation on the transcript level was not apparent in this experiment.

The δ gl-positive revertant R39 was comparable to the wild-type in the induction of its transcript by δ gl. The glucose-positive revertants, R34 and R41, however,

Fig. 5. Northern blot analysis of wild-type yeast, the *gnd1gnd2* mutants and different *gnd1* revertants, probed with a *GND1* cDNA. Total yeast RNA was isolated from wild-type yeast (*gnd* T10D), the *gnd* mutants (*gnd* T10A, *gnd* T10B and *gnd* T10C) and the *gnd1* revertants R34, R41 and R39, as described in Methods. A 0.65 kb *Sa*/I fragment of the DNA clone CT4 was used as the probe for hybridization. The 18S and 23S ribosomal RNA served as size standards. (a) Northern blot of yeast cultures grown in YEP ethanol medium (lane 1) and YEP ethanol medium containing δ gl (lane 2). (b) Northern blot after washing and re-probing the same filter with a genomic clone for the enzyme fructose biphosphate aldolase (1.2 kb) as a control.

failed to induce the GND mRNA to the same level under the same conditions. These were also the revertants that did not induce the δ gl-metabolizing enzymes, as assayed *in vitro*. Although R34 did show a low level of the transcript on induction, R41 lacked the message completely. These two strains had reverted at different loci, only one of which (that in R34) was linked to the original *gnd1* mutation.

Attention can now be focussed on the role of the *GND1* gene in induction. That the *GND1* gene-product *per se* is not involved in this process is evident from the failure of the cDNA clone of this gene to induce these enzymes. Recent work in this laboratory has established that the original *gnd1* mutant contains an extensive deletion (upward of 6 kb), covering almost two-thirds of the 5'-end of the *GND1* gene (Z. Lobo, unpublished results). This deletion thus spans at least 5 kb of the 5' upstream region of this gene. It is therefore quite likely that the induction could actually be mediated by the product of some other gene linked to *GND1* and covered by this

deletion, an implicit assumption being that the *GND1* gene itself codes for the major GND isoenzyme.

Although these results indicate a key role for an intact regulatory locus, possibly upstream of the *GND1* gene, neither the nature of its product nor the step at which it acts have been defined by these experiments. It could, for example, be involved in the initiation of transcription or in the stabilization of constitutively produced but rapidly degraded transcripts. One cannot distinguish between these possibilities at present. Determination of the exact role of the putative regulatory locus must await the isolation and analysis of the specific sequences upstream of the *GND1* gene, and the identification and characterization of the inducing factor(s).

Induction of all the three enzymes involves *de novo* protein synthesis. Northern blot analysis indicates that the induction of GND enzyme activity correlates with levels of GND transcript greater than those occurring constitutively in ethanol-grown cells. Although it has not yet been possible to investigate the regulation of the

genes coding for PGL and GNK, they may be regulated co-ordinately by the above-mentioned putative locus at the level of transcription.

What is the exact role of this catabolic pathway? *δgl* is a most unlikely natural substrate. Has this pathway evolved to remove metabolic intermediates, the accumulation of which might prove toxic to the cell? The *gnd1* mutant fails to grow on glucose while a *zwf1gnd1* double mutant, which also lacks GPD, can (Lobo & Maitra, 1982). This could imply that the intermediates of the oxidative pathway (6-phosphogluconolactone and/or 6-phosphogluconate) inhibit glucose metabolism if accumulated to non-physiological levels. Thus, the importance of the *δgl*-induced enzymes may be to prevent the deleterious accumulation of these otherwise-necessary metabolites in the cell.

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