The relationship of growth rate and catabolite repression with *WHI2* expression and cell size in *Saccharomyces cerevisiae*

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Mixtures of D-glucosamine and glucose were used to slow the growth of wild-type and *whi2* mutant strains of *Saccharomyces cerevisiae* without affecting the level of catabolite repression. The following observations were made. Firstly, mutant cells were found to be partially resistant to the inhibitory effect of glucosamine. Secondly, slow growth induced by glucosamine resulted in cells becoming larger, in direct contrast to the effect of slowing growth by glucose limitation in a chemostat or by carbon source substitution. It is concluded that the level of repression/derepression, rather than absolute growth rate, is responsible for controlling cell size. Thirdly, when *WHI2* transcript levels were measured it was found that expression was correlated with growth rate rather than the level of repression. These results are interpreted in terms of a model which envisages that the *WHI2* product acts as a negative regulator of catabolite repression. A test of this model is reported: it is shown that mutant cells respired more actively in the presence of glucose and grew more rapidly on glycerol, whereas overexpression of *WHI2* from multicopy plasmids prevented growth on glycerol and depressed respiration.

Introduction

The *WHI2* gene of *Saccharomyces cerevisiae* is involved in coordinating cell proliferation, cell growth rate and cell size (Mountain & Sudbery, 1990; Rahman *et al.*, 1988; Saul *et al.*, 1985; Sudbery *et al.*, 1980). *whi2* mutants were first recognized by their failure to enter G0 upon carbon starvation (Saul *et al.*, 1985; Sudbery *et al.*, 1980). Subsequently it was shown that during growth on ethanol and other non-fermentable carbon sources *whi2* cells were smaller than wild-type cells, and had a higher specific growth rate. Moreover, in the absence of aerobic growth on ethanol the mutant phenotype is not observed in the stationary phase of a glucose batch culture (Rahman *et al.*, 1988). Presumably, therefore, the regulatory function of the *WHI2* gene is associated with either an aerobic pattern of growth or with slow-growing cells.

A cycle of batch culture growth on glucose consists of two distinct phases. In the first phase, cells grow rapidly, fermenting glucose to ethanol. Glucose represses the expression of a number of enzymes (Polakis & Bartley, 1965) and mitochondrial functions (Polakis *et al.*, 1965) required for respiratory growth, an effect commonly

referred to as catabolite repression. In the second phase, cells grow more slowly, respiring ethanol to carbon dioxide and water, which requires the derepression of the functions repressed by glucose in the first phase.

An investigation into the transcription of the *WHI2* gene during the growth cycle of glucose batch cultures revealed that the expression of the gene in both mutant and wild-type cells was higher during the glucose phase compared to the subsequent ethanol phase (Mountain & Sudbery, 1990). The down-regulation of *WHI2* expression is approximately coincident with the switchover between the two phases. This poses the question as to the nature of the signal regulating *WHI2* gene expression. It could be sensitive to the overall cell growth rate, which is different in the two phases, or it could be induced by glucose or sensitive to the level of catabolite derepression. In principle this is a difficult question to resolve since slowing the growth rate by changing the carbon source or glucose limitation in a chemostat will also affect the level of catabolite repression. One possible approach to this problem is the use of D-glucosamine as a glucose analogue.

The effect of glucose analogues, including glucosamine, on fungal metabolism has been reviewed by Moore (1981). Glucosamine is a competitive inhibitor of glucose fermentation (Woodward & Hudson, 1953). Its inhibitory action is relieved by the addition of glucose

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Fig. 1. Effect of D-glucosamine on the growth of (a) wild-type and (b) *whi2* cells. Cells from an exponentially growing YEPD starter culture were used to inoculate 15 ml medium in a 500 ml flask, to give approximately 4×10^5 cells ml^{-1} . For each curve the medium consisted of YEP (see Methods) plus 1% glucose plus the indicated concentration (% w/v) of D-glucosamine. At the points indicated by the arrows, samples were removed for RNA isolation.

(Furst & Michels, 1977), probably due to the competitive inhibition of glucosamine uptake. Glucosamine is also a gratuitous inducer of catabolite repression (Elliott & Ball, 1973; Furst & Michels, 1977; Hockney & Freeman, 1980), preventing growth on non-fermentable carbon sources at low concentrations. It can be used to isolate catabolite-derepressed mutants (Elliott & Ball, 1973; Hockney & Freeman, 1980). The properties of glucosamine, therefore, allow it to be used to regulate cell growth, through adjustment of the glucose:glucosamine ratio, whilst maintaining a constant level of catabolite repression. McGoldrick & Wheals (1989) have demonstrated its use in this fashion. In this paper we report experiments designed to exploit this system to determine whether the level of *WHI2* expression is sensitive to cellular growth rate or to catabolite derepression. The design of the experiments also provided the opportunity to investigate a separate problem of yeast proliferation. As the growth rate of yeast cells is slowed, either by carbon source substitution or glucose limitation in a chemostat, cell size at first also decreases. However, at slow growth rates cell size becomes independent of growth rate (Johnston *et al.*, 1979; Lord & Wheals, 1980; Lorincz & Carter, 1979). The break point in the size-growth rate curve is approximately coincident with the point at which catabolite derepression occurs, but it is not clear whether this is fortuitous (Walton, 1984). Our results suggest that cell size is sensitive to the level of derepression rather than to growth rate *per se*.

Methods

Media. Cells were grown on YEP, which consists of 1% (w/v) yeast extract, 2% (w/v) peptone and 0.04% adenine. This was supplemented with 1% (w/v) glucose plus glucosamine at concentrations described in the text. YEPD consists of YEP plus 2% glucose.

Strains. Diploid wild-type and mutant strains were those described in the accompanying paper (Mountain & Sudbery, 1990).

RNA extraction and Northern hybridization. RNA was extracted by breaking cells with glass beads in the presence of phenol as described in the accompanying paper (Mountain & Sudbery, 1990). Northern hybridizations were done by standard methods using GeneScreen (Dupont) membranes (Mountain & Sudbery, 1990). The probe used to detect the *WHI2* transcript was pSG6 (Saul & Sudbery, 1985), which consists of the *WHI2* gene cloned in pAT153 (Twigg & Sherratt, 1980). Apart from the *WHI2* gene it contains no other yeast sequences.

Cell size. Cell volumes were determined using a Coulter counter and channelyser as described previously (Saul *et al.*, 1985).

Reproducibility. The experiments reported in this paper have been repeated and identical results obtained.

Results

Preliminary experiments established that the growth rate could be conveniently adjusted by growth on YEP medium plus 1% glucose and a range of glucosamine concentrations between 0.5% and 5%. Fig. 1(a) shows the growth of wild-type cells on such glucose/glucosamine mixtures after inoculation from an exponential YEPD culture to a density of 4×10^5 cells ml^{-1} . The

Fig. 2. Effect of glucosamine on levels of *WHI2* and *CDC7* mRNA in wild-type and *whi2* cells. Total cellular RNA was extracted at the points indicated in Fig. 1, and 20 µg from each sample was used in a Northern transfer experiment. The membrane was hybridized sequentially to probes for *WHI2*, *CDC7* and 18S rRNA, the last serving as a control for loading variation. The figures above each lane show the percentage of glucosamine used in the culture from which the RNA was isolated. YEPE refers to RNA isolated from cultures using ethanol as a carbon source. Lanes labelled 'mRNA' are positive controls using samples of the polyadenylated RNA fraction which was known from previous experiments to give a good signal. EH refers to λ DNA digested with both *EcoRI* and *HindIII*, used as molecular mass markers (Kelly *et al.*, 1988).

Table 1. Effect of glucosamine on growth rate and cell size

Glucosamine concn (%, w/v)	Wild-type		<i>whi2</i>	
	Doubling time (h)	Size* (µm ³)	Doubling time (h)	Size* (µm ³)
0	2.39	66	2.1	51
0.5	3.22	67	3.6	65
1.0	3.78	76	4.9	70
2.0	5.35	82	4.8	65
5.0	9.15	88	4.3	65

* Median cell volume.

resulting doubling times are shown in Table 1. Increasing concentrations of glucosamine increased doubling time and produced increasing lag periods after inoculation.

A similar experiment was carried out with *whi2* mutant cells. The resulting growth curves are shown in Fig. 1(b) and the doubling times in Table 1. While 1.0% glucosamine had a similar effect to that in wild-type cells, higher concentrations of glucosamine did not further inhibit growth. *whi2* cells are therefore partially resistant to the inhibitory effects of glucosamine.

At the times indicated by arrows in Fig. 1, samples were withdrawn from each culture used in the two experiments described above. Total cellular RNA was extracted and 20 µg used in a Northern hybridization

experiment using pSG6 (Saul & Sudbery, 1985) as a probe for the *WHI2* transcript. After the resulting autoradiogram had been developed, the *WHI2* probe was stripped from the membrane and the membrane rehybridized successively to pY1rG1, a probe for 18S rRNA (Petes *et al.*, 1978), and a probe for the *CDC7* gene (Patterson *et al.*, 1986). The autoradiogram showing the effect of increasing glucosamine concentrations on the expression of these genes is shown in Fig. 2. In wild-type cells, increasing concentrations of glucosamine decreased the expression of the *WHI2* gene, and this decrease was correlated with the decrease in growth rate. In the mutant cells there was also a drop in the level of *WHI2* expression when 1.0% glucosamine was present. However, higher concentrations of glucosamine did not reduce the expression further. Thus in mutant cells cellular growth rate and *WHI2* expression were both sensitive to 1.0% glucosamine but higher concentrations had no further effect. While the expression of the *WHI2* gene was reduced in wild-type cells by glucosamine, the expression of the *CDC7* transcript was relatively unaffected, demonstrating that the observed effect of glucosamine on *WHI2* transcription is not the result of a general inhibitory effect on mRNA transcription. Table 1 shows the effect of different glucosamine concentrations on cell size and doubling times. In the case of wild-type cells, size increased with increasing concentrations of glucosamine. So, whereas both ethanol and glucos-

amine slow growth, the former causes a reduction in cell size and the latter causes an increase. This suggests that growth rate is not the critical factor in determining size. In mutant cells, glucosamine increased cell size at concentrations of 1% or less; higher concentrations had no further effect.

The partial resistance of mutant cells to glucosamine suggested that the cells may be catabolite derepressed, at least partly, in a constitutive fashion. A possible explanation for this, which would be consistent with the observation that the product is more highly expressed in the glucose phase, is that *WHI2* acts as a negative regulator of repressible functions. Upon glucose exhaustion the down-regulation would allow derepression, the residual levels still providing some modulation in wild-type strains; the mutant phenotype would arise through loss of this modulation.

In order to test this conclusion, triphenyltetrazolium was used (Ogur *et al.*, 1957) to indicate the level of respiration in the presence of glucose. Mutant and wild-type strains were used together with strains carrying the *WHI2* gene on the multicopy plasmids pSG1 and pSG3 (Saul & Sudbery, 1985), which results in overexpression as judged by mRNA levels. The rationale of this experiment was that glucose should repress respiration, although some would occur after glucose exhaustion and subsequent ethanol metabolism. Lack of effective control in the mutant strain should result in a higher level of respiration and deeper staining with triphenyltetrazolium. Conversely, *WHI2* overexpression should depress respiration and so decrease staining. The mutant strain did indeed stain more darkly than the wild-type, while strains which were overexpressing the *WHI2* gene stained much more lightly than either non-transformed strain. This was not due to the vector or to changes induced by transformation, as a mutant strain transformed with YEp13 (a 2 µm-based vector lacking the *WHI2* gene) stained as darkly as the non-transformed mutant strain. As a further test, the ability of these strains to grow on glycerol was examined, as respiration is obligatory for growth on this substrate. The mutant strain grew slightly better than the wild-type, while transformed strains grew less well than the respective non-transformed parent strain. There was thus a striking difference between the non-transformed mutant strain and the wild-type transformed with pSG3. We conclude that *WHI2* overexpression causes a marked reduction in respiration as monitored by triphenyltetrazolium staining, resulting in a reduced ability to grow on glycerol – a non-fermentative growth substrate. Although of a smaller magnitude, differences between mutant and wild-type strains were also observable in these respects, confirming that the *WHI2* product exerts a negative effect on respiratory capacity.

Discussion

The interpretation of experiments which use an inhibitor to probe aspects of cell proliferation must clearly be tentative. The inhibitor may act in unknown ways on the cell, and cell proliferation is likely to be sensitive to many aspects of the metabolic state. Nevertheless, a clear pattern of results emerged from the experiments, which have interesting implications for the integration of cell metabolism and cell proliferation.

Furst & Michels (1977) showed that in *Saccharomyces carlsbergensis* 0.15% glucosamine was sufficient to repress a range of glucose-repressible functions such as O₂ uptake, cytochrome *c* oxidase and maltase levels. Hockney & Freeman (1980) showed that *S. cerevisiae* responded in a closely similar fashion. The levels of glucosamine used in the present study were considerably higher. This, together with the presence of glucose, ensured that reduction in growth rate caused by D-glucosamine would not be accompanied by catabolite derepression. In wild-type cells this results in the level of expression of the *WHI2* gene being markedly reduced. It seems likely, therefore, that the expression of the *WHI2* gene is sensitive to growth rate itself rather than to derepression, which is also normally associated with growth rate reductions.

These experiments are also relevant to the problem of whether growth rate or derepression is responsible for the reduction in cell size that occurs as cells grow more slowly. As the growth rate slows with increasing glucosamine concentrations, cell size increases rather than decreases. In contrast, cells whose growth rate is slowed by the substitution of ethanol for glucose become smaller. These results suggest that cell size is sensitive to the level of derepression rather than to growth rate. The increase in cell size caused by glucosamine may be due to glucosamine being a more potent inducer of catabolite repression than glucose (Furst & Michels, 1977). This conclusion is at variance with that of Bugeja & Carter (1982), who concluded that cell size is a growth-rate-related phenomenon on the basis that petite mutants showed the same break-point in the size-growth rate curve as wild-type strains. However, as pointed out by Walton (1984), Perlman & Mahler (1974) showed that petite mutants become derepressed for non-mitochondrial enzymes upon glucose limitation. Therefore it is clear that petite strains still retain the physiological mechanisms responsible for catabolite derepression and that the observation of Bugeja & Carter, (1982) would still be consistent with size changes being dependent on the degree of derepression.

whi2 cells are partially resistant to glucosamine, showing a normal response to 1.0% glucosamine but being resistant to higher concentrations. As outlined in

the Introduction, glucosamine acts at several different stages in glucose metabolism and ATP generation. Our results suggest that the *whi2* mutant has become resistant at one or more of these stages but remains sensitive at others. Resistance to glucosamine is a method of obtaining catabolite-derepressed mutants (Elliott & Ball, 1973; Hockney & Freeman, 1980). Similar mutants obtained using 2-deoxyglucose have an altered hexokinase PII (Entian *et al.*, 1984), one of the potential targets for glucosamine inhibition. We conclude that the *WHI2* product is expressed during glucose growth and acts to repress functions normally only expressed in the absence of glucose. It is possible to speculate that this may be due to an interaction between the *WHI2* product and hexokinase PII, since both are apparently involved in glucose repression of other functions and *whi2* mutants are partially resistant to glucosamine, one target of which is hexokinase.

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