Regulation of the *Saccharomyces cerevisiae* *WHI2* gene

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(Received 3 October 1989; accepted 11 December 1989)

***WHI2* mRNA levels were followed through the growth cycle in *WHI2* mutant and wild-type cells of *Saccharomyces cerevisiae*. Levels were high during the first (glucose) phase of growth, and were reduced sharply during the second (ethanol) phase of growth. Transcript levels of the glycolytic genes *PDC1* and *PYK1* were also measured; they each showed a pattern similar to that of *WHI2*, whereas transcript levels of the *CDC7* gene remained constant throughout the cycle, showing that a decrease in transcription is not a general feature of genes. These results make it unlikely that the *WHI2* product acts as an inhibitor of cell proliferation which is activated upon carbon starvation. No difference was observed between the pattern of expression of mutant and wild-type strains, showing that the mutant phenotype was not the result of a change in regulation at the transcriptional level.**

Introduction

When the yeast *Saccharomyces cerevisiae* is inoculated and cultured in glucose batch culture a well-characterized growth cycle ensues. Initially the cells proliferate rapidly, fermenting glucose to ethanol, which accumulates in the culture medium (Lagunas, 1986). Upon exhaustion of available glucose, ethanol is utilized aerobically, the rate of proliferation and mass increase slows, and cell size at commitment to division decreases (Lord & Wheals, 1980; Lorincz & Carter, 1979). Finally, upon ethanol limitation, cell proliferation ceases, and cells accumulate in the G1 phase of the cell cycle (Hartwell, 1974) and undergo complex physiological changes which include the accumulation of the reserve carbohydrates glycogen and trehalose (Lillie & Pringle, 1980) and result in the cells becoming resistant to environmental stresses such as heat shock (Parry *et al.*, 1976; Saul *et al.*, 1985; Schenberg-Frascino & Moustacchi, 1972), nystatin treatment (Saul *et al.*, 1985; Snow, 1966) and cell wall digestion with zymolyase (Deutch & Parry, 1973; Saul *et al.*, 1985). The concerted changes in metabolic activity, growth rate, cell proliferation and cell size that occur through this growth cycle in response to changing environmental conditions show that all these cell functions must be finely integrated with respect to each other and to the environment.

This coordination is disturbed in *whi2* mutants. Such mutants are apparently normal in the glucose phase, but

an abnormal pattern of growth and proliferation is exhibited during the ethanol phase and upon carbon exhaustion (Rahman *et al.*, 1988; Saul *et al.*, 1985; Sudbery *et al.*, 1980). During growth on ethanol, *whi2* cells are smaller and grow faster than wild-type cells, while upon carbon exhaustion the cells continue to attempt proliferation, becoming arrested randomly in the cycle and retaining the properties of exponentially growing cells. It seems clear that the regulatory network controlling cell growth and proliferation has become disturbed in *whi2* mutants. The mode of action of the *WHI2* gene may be reflected in a change in the pattern of expression throughout the course of the cycle, or its activity may be modulated by post-translational modification. This paper reports experiments designed to monitor mRNA levels to discover if any significant changes do occur through the cycle.

Methods

Media. Cells were grown on YEPD medium, consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 0.04% adenine.

Strains. These were as follows.

Wild-type: $\frac{a}{\alpha} \frac{adel}{adel} \frac{his4}{his4} \frac{leu2}{+} \frac{ura3}{+} \frac{met3}{+} \frac{trp5}{trp5}$

Mutant: $\frac{a}{\alpha} \frac{whi2}{whi2} \frac{adel}{+} \frac{his4}{his4} \frac{ura3}{+} \frac{met3}{+} \frac{trp5}{trp5}$

Apart from the indicated markers the strains are at least 87.5% isogenic.

Measurement of cell size, cell number, glucose and ethanol concentration, and cellular carbohydrates. Cell size and cell number were determined

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with a Coulter counter model ZB1 with a C1000 channelyser. Glucose concentrations were determined with a Sigma diagnostic kit, using the glucose oxidase assay. The manufacturer's instructions were followed except that the enzyme reaction was conducted at 30 °C for 30 min and the absorbance measured at 420 nm. Ethanol was determined by gas-liquid chromatography (GLC). Trehalose was extracted from a known number of cells by extraction with ethanol at 80 °C, deionized with Amberlite mixed-bed resin (IR-120H), derivitized and measured by GLC, all as described by Holligan & Drew (1971). The pellet which remained after ethanol extraction was used for glycogen estimations as described by Gunja-Smith *et al.* (1977) and modified by Lillie & Pringle (1980). No distinction was made between the soluble and insoluble glycogen pools as described by Gunja-Smith *et al.* (1977).

RNA extraction and Northern hybridization. RNA was extracted by breaking cells with glass beads in the presence of phenol as described previously (Kelly *et al.*, 1988). Northern hybridizations were done on Gene Screen (Dupont) membranes according to the method of Thomas (1980), using glyoxylated RNA, and with glyoxylated λ DNA digested with *EcoRI* and *HindIII* as molecular mass standards. Probes were labelled *in vitro* by nick-translation as previously described (Saul & Sudbery, 1985) and hybridizations were done at 42 °C in the presence of formamide. Autoradiograms were prepared using intensifying screens at -70 °C.

Probes. These were as follows. *WHI2*: whole pSG6 (Saul & Sudbery, 1985) was used. This consists of the *WHI2* gene cloned into the *HindIII*-*BamHI* site of pAT153. 18S rRNA: pY1rG1 (Petes *et al.*, 1978), received from S. Oliver. *PYK1* (Burke *et al.*, 1983): probe received from G. D. Searle Ltd. *PDC1* (Schmitt *et al.*, 1983): probe received from T. Wright. *CDC7* (Patterson *et al.*, 1986): probe received from J. Rosamund.

Results

In order to follow the expression of the *WHI2* gene throughout a cycle of batch growth the following experiment was done. Cells from a YEPD starter culture were inoculated into a fermenter and grown on YEPD with vigorous aeration (so that the oxygen concentration was kept above 95% saturation). At intervals samples were withdrawn. Each sample was divided into two. From one portion total RNA was extracted and the *WHI2* transcript level determined by Northern hybridization. In order to provide a physiological context for these measurements the other portion was used to measure cell number per ml, proportion of cells budded, cellular glycogen and trehalose levels, and glucose and ethanol levels in the culture medium. Both *whi2* mutant and wild-type strains were used in separate experiments to ascertain whether differences in the pattern of expression were responsible for the mutant phenotype. These experiments employed diploid rather than haploid strains because we found that this reduced clumping and thus allowed greater accuracy in cell number determination. The results presented below show that the diploid strains behaved identically to the haploid strains we have used in previous studies.

Fig. 1 shows the growth characteristics for both wild-

type and mutant cells. Cell number increased exponentially with time in both cases. Glucose was utilized and ethanol accumulated exponentially in the medium. Upon exhaustion of the glucose, ethanol was consumed, and there was a corresponding change in the rate of cell number increase. Growth was therefore diauxic. Immediately after inoculation there was a sharp increase in size (median cell volume), which was probably due to the synchronous division cycle as cells grew out from G1 arrest, the high peak volume representing the end of the first cell cycle with a high proportion of cells with large buds prior to cell separation. The subsequent gradual decrease in size reflects the increased asynchrony. In the mid-exponential phase of glucose growth the wild-type had a median volume of 80 μm^3 and the mutant of 67 μm^3 , so in this experiment a difference between mutant and wild-type was evident even in the glucose phase. During the ethanol phase of growth, cell size decreased in both strains, as would be expected with the decreased growth rate (Lord & Wheals, 1980; Lorincz & Carter, 1979). However, the reduction in volume was greater in the mutant strain, so that it is in this phase of growth that the marked difference in size becomes apparent (60 μm^3 and 33 μm^3).

In the wild-type, glycogen content was initially high in the stationary-phase cells of the inoculum and then fell to minimum levels until the glucose was exhausted, at which point it was accumulated. Trehalose levels fell to near zero shortly after inoculation and only increased after glucose exhaustion. The resolution of the experiment is insufficient to determine whether glycogen increased just before glucose exhaustion and trehalose just after, as described previously (Lillie & Pringle, 1980). The mutant had a lower glycogen content in the initial stationary-phase inoculum than the wild-type; glycogen content increased in exponential growth, reaching a similar level to the wild-type, but then dropped to a lower level in the stationary phase. The trehalose content of mutant cells was approximately half that of the wild-type cells in stationary phase, but it showed the same pattern of degradation and accumulation. The results show that the growth characteristics of these diploid strains are similar to those reported previously in haploid strains.

Figs 2 and 3 show that *WHI2* transcript levels underwent large changes through the growth cycle. The initial stationary-phase cells of the inoculum had a very low level of transcript, which showed a large increase during the glucose phase of growth but decreased markedly during the ethanol phase. The disappearance of the transcript was coincident with glucose exhaustion and the onset of ethanol growth. The results showed no significant differences between mutant and wild-type strains.

Fig. 1. Growth characteristics of (a–c) the wild-type strain and (d–f) the *whi2* mutant during growth on YEPD batch culture. Stationary-phase cells from a starter culture were inoculated into a 5 litre fermenter using a 2 litre working volume. At intervals samples were withdrawn and measurements made of cell number (shown in each panel for comparison), cell size (median cell volume) and percentage of cells budded (a, d), glucose and ethanol concentrations in the culture medium (b, e) and cellular glycogen and trehalose content (c, f). The figures beside the cell number curves in (a) and (d) refer to samples used to prepare RNA used for the Northern hybridization experiments described in Figs 2 and 3.

Fig. 2. Levels of *WHI2*, *PDC1*, *PYK1* and *CDC7* transcripts, and 18S rRNA, during the growth cycle of wild-type cells. The samples used to obtain the results in Fig. 1 were also used to prepare total cellular RNA; 20 µg from each sample was hybridized to pSG6 (which contains the complete *WHI2* sequence but no other yeast sequences) and the resulting autoradiogram is shown. The probe was then stripped from the membrane and the membrane hybridized in turn to probes for each of the above sequences. The numbers above each lane refer to the sample in Fig. 1 from which the RNA was prepared. Exposure times: *WHI2*, 3 d; *PDC1*, 2 d; *PYK1*, 4 d; *CDC7*, 4 d; 18S rRNA 7 h. M, molecular mass standards, which were visualized in the *WHI2* hybridisation but not in the others. pA, poly(A) mRNA fraction from glucose-grown cells used as a positive control.

Fig. 3. Levels of *WHI2*, *PYK1* and *CDC7* transcripts, and 18S rRNA, during the growth cycle of *whi2* mutant cells. Autoradiograms showing the level of transcripts for the above genes were prepared as described in Fig. 2. Exposure times: *WHI2*, 14 d; *PYK1*, 2 d; *CDC7*, 9 d; 18S rRNA, 7 h.

To reveal any loading variation which may have occurred, the membranes were stripped of the *WHI2* probe and rehybridized to pY1rG1 (Petes *et al.*, 1978), a probe for 18S rRNA. The resulting autoradiograms are also shown in Figs 2 and 3 for mutant and wild-type cells respectively. It is clear that such variation is not responsible for the change in the *WHI2* signal observed upon glucose exhaustion.

In order to assess the significance of the pattern of expression observed, the same membranes were hybridized to a panel of probes for other yeast genes. *PYK1* (pyruvate kinase) and *PDC1* (pyruvate decarboxylase) were chosen as genes involved in glycolysis which show a similar pattern of expression, being high in the presence of glucose and low in its absence (Schmitt *et al.*, 1983; Burke *et al.*, 1983). Conversely, *CDC7* is a gene required for cell division and may be expected to be constitutively expressed in dividing cells (Patterson *et al.*, 1986). The resulting autoradiograms are shown in Figs 2 and 3 for the wild-type and mutant respectively. Although *PDC1* is only shown for wild-type cells, other experiments have shown that the pattern of expression shown is similar in the *whi2* mutant. The pattern of expression of the *WHI2* gene was very similar to that of the two representative glycolytic genes, which had high mRNA levels in the presence of glucose, falling sharply upon glucose exhaustion (Figs 2 and 3). *CDC7* transcript levels remained approximately constant throughout the growth cycle, showing that the decrease in transcript levels described above is not a general feature of mRNA levels at this point.

The experiments described above have been repeated and identical results obtained; in addition, similar results were obtained in preliminary experiments when samples from the mid-exponential phase were compared with samples from the stationary phase.

Although the pattern of expression was similar for *WHI2* and the two glycolytic genes, absolute levels were different. The exposure time required for the autoradiogram with the probes for the glycolytic genes was less than that for the *WHI2* gene, while the density of the resulting bands was greater. Clearly *WHI2* is not so highly expressed as the glycolytic genes. Exposure times for the *CDC7* probe were slightly longer. During the glucose phase of growth *WHI2* transcript levels were much greater, but they fell to as low as or lower than *CDC7* levels during ethanol growth. *CDC7* transcript levels correspond to approximately one molecule per cell (Sclafani *et al.*, 1988), so during growth on ethanol *WHI2* transcript levels are clearly very low. This comparison of absolute levels of transcript is necessarily only approximate since comparison between autoradiograms is subject to variation of probe specific activity and other

variables of the hybridization process for which there is no internal control available for standardization.

Discussion

In this study we have been concerned to discover the pattern of *WHI2* gene activity by measurement of transcript levels. Transcript levels are, of course, only an indirect indication of gene activity, since activity will be affected by translation rates and by protein modifications and other interactions. A further difficulty was the problem of standardizing RNA loadings. The results presented in this paper used 18S rRNA as a standard and therefore measured the level of *WHI2* mRNA relative to 18S rRNA, and not the level of *WHI2* mRNA per cell or per unit mass. Nevertheless we observed a large reduction of transcript levels as growth changed from the glucose phase to the ethanol phase. Such variation may be due to an altered rate of transcription or mRNA stability or both. This result is of interest because many genes which are involved in regulating cell proliferation and passage through the cell cycle have been found not to be regulated at the transcriptional level.

We have also shown that a decrease in transcriptional activity is not a general feature of yeast genes during the ethanol phase of growth, since the *CDC7* gene maintains its level of transcription during this phase. Furthermore the validity of the methodology is shown by the fact that glycolytic genes, which are expressed more strongly during growth on glucose (Schmitt *et al.*, 1983), do show such a pattern of expression in our experiments.

The observation that the *WHI2* gene is more strongly expressed during growth on glucose is surprising in view of previously reported results. These showed that disruption of the gene produced the mutant phenotype (Kelly *et al.*, 1988) and that the mutant allele is complemented by the wild-type allele (Sudbery *et al.*, 1980; Saul & Sudbery, 1985). Furthermore, whereas the mutant allele allows more rapid proliferation on non-fermentative carbon sources (Rahman *et al.*, 1988), overexpression of the wild-type gene prevents growth on glycerol (Mountain & Sudbery, 1990). These results show that the product of the gene has a negative effect on cell proliferation. The simplest model would be that of an inhibitor which was synthesized or activated upon carbon starvation. However, the present results are in direct conflict with the pattern of synthesis which would be expected, and show that the mode of action does not easily fit into a simple model. One possibility that is ruled out by the present study is that the mutant phenotype arises out of an altered pattern of expression.

The intracellular signal controlling the transcription of the *WHI2* gene is of interest. The down-regulation is coincident with glucose exhaustion and the start of ethanol consumption. This switch-over results in both catabolite derepression and a reduction of growth rate. Either of these two changes could be responsible for the change in transcription. This problem is addressed in the accompanying paper (Mountain & Sudbery, 1990).

We are grateful to the following for supplying plasmids: S. Oliver, Searle Ltd, T. Wright and J. Rosamund. We thank Wynne Ellis for his guidance with the GLC. H. A. M. was supported by an SERC research studentship.

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