Changes in Gene Expression in the Ras/Adenylate Cyclase System of *Saccharomyces cerevisiae*: Correlation with cAMP Levels and Growth Arrest

Marijane Russell, Judith Bradshaw-Rouse, David Markwardt, and Warren Heideman

School of Pharmacy, University of Wisconsin, Madison, Madison, Wisconsin 53706

Submitted April 15, 1993; Accepted May 24, 1993

Levels of cyclic 3',5'-cyclic monophosphate (cAMP) play an important role in the decision to enter the mitotic cycle in the yeast, *Saccharomyces cerevisiae*. In addition to growth arrest at stationary phase, *S. cerevisiae* transiently arrest growth as they shift from fermentative to oxidative metabolism (the diauxic shift). Experiments examining the role of cAMP in growth arrest at the diauxic shift show the following: 1) yeast lower cAMP levels as they exhaust their glucose supply and shift to oxidative metabolism of ethanol, 2) a reduction in cAMP is essential for traversing the diauxic shift, 3) the decrease in adenylate cyclase activity is associated with a decrease in the expression of *CYR1* and *CDC25*, two positive regulators of cAMP levels and an increase in the expression of *IRA1* and *IRA2*, two negative regulators of intracellular cAMP, 4) mutants carrying disruptions in *IRA1* and *IRA2* were unable to arrest cell division at the diauxic shift and were unable to progress into the oxidative phase of growth. These results indicate that changes cAMP levels are important in regulation of growth arrest at the diauxic shift and that changes in gene expression plays a role in the regulation of the Ras/adenylate cyclase system.

INTRODUCTION

As yeast grow and divide in glucose medium, the culture shows a characteristic growth pattern (Pringle and Hartwell 1981). Initially, the cells grow rapidly, utilizing glucose as a carbon source, until the supply of glucose becomes depleted. The cells then temporarily arrest growth in the G1 phase of the mitotic cycle while shifting metabolism from fermentation of glucose to oxidation of ethanol (the diauxic shift). After a period of several hours, the cells then resume proliferative growth. This pause in growth produces a plateau in the growth curve, separating fermentative from oxidative growth. The diauxic shift is therefore a complex process, involving both exit from the mitotic cycle, as well as major changes in the pattern of metabolic enzymes that are synthesized (Wills, 1990). Intracellular levels of cyclic 3',5'-cyclic monophosphate (cAMP) have been implicated in controlling the decision to enter the mitotic cycle (Matsumoto *et al.*, 1985). Mutations that elevate cAMP produce cells that do not arrest in G1 in response to nutritional signals, whereas mutations that block cAMP formation produce cells that cannot enter the

mitotic cycle. As might be expected, some of these mutations also affect the shift in carbon sources. As an example, *BCY1* mutants producing a constitutively activated cAMP dependent protein kinase do not arrest growth in G1 in response to nutrient depletion and cannot be shifted to a nonfermentable carbon source (Toda *et al.*, 1987a). In view of these effects, changes in cAMP levels might be expected to play a role in the transient growth arrest at the diauxic shift.

In *Saccharomyces cerevisiae*, cAMP is produced by adenylate cyclase encoded by the *CYR1* gene (Matsumoto *et al.*, 1982). Adenylate cyclase is in turn stimulated by the GTP-binding products of the *RAS1* and *RAS2* genes (DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984; Toda *et al.*, 1985). Downstream in the pathway, cAMP binds to the regulatory subunit of the cAMP-dependent protein kinase (encoded by *BCY1*) and activates the kinases encoded by *TPK1*, 2, and 3 (Toda *et al.*, 1987b). Upstream in the pathway are both positive and negative regulators of Ras, *CDC25* and *IRA1* and 2, respectively. The *CDC25* gene product has Ras GDP/GTP exchange activity that serves to activate Ras proteins (Jones *et al.*, 1991). *Ira1* and *Ira2* proteins, homologues of mammalian GTPase

activating protein, stimulate the GTPase activity intrinsic to Ras proteins, converting active Ras-GTP to inactive Ras-GDP. In yeast, this decreases adenylate cyclase activity and ultimately reduces intracellular levels of cAMP (Tanaka *et al.*, 1989, 1990b). In addition to controls placed on cAMP production, it is likely that cAMP levels are also regulated at the level of degradation by cyclic nucleotide phosphodiesterase activity, as well as by a poorly understood process of feedback regulation that requires RAS2 (Nikawa *et al.*, 1987).

In this paper, we present experiments demonstrating that intracellular cAMP levels drop sharply as *S. cerevisiae* approach the diauxic shift. This drop is necessary for proper growth arrest at the diauxic shift; cells held at high cAMP do not properly arrest growth and are stranded, unable to grow on ethanol. The fall in cAMP levels is accompanied by a decrease in *CYR1* and *CDC25* expression and a marked increase in *IRA1* and *IRA2* expression. Mutant cells unable to express *IRA1* and *IRA2* cannot properly arrest growth and have a phenotype that is indistinguishable from the cells held at high cAMP. These results are consistent with a model in which growth is actively arrested at the diauxic shift by a decrease in cAMP. Additionally, the results indicate that along with the many other mechanisms known to regulate the Ras/adenylate cyclase system, changes in gene expression play a role in regulating cAMP production.

MATERIALS AND METHODS

All experiments were performed at least three times with similar results.

Materials

Adenylate cyclase grade [α - 32 P]ATP (40 cpm/pmol) was from Amersham (Arlington Heights, IL), [3 H] cAMP (10 000 cpm/assay) and [125 I] NaI were from New England Nuclear (Boston, MA).

Media

Yeast were grown on either rich medium containing 1% yeast extract, 2% bacto peptone, and either 2% glucose (YEPD) or 2% ethanol (YEPE) or on minimal medium containing 6.7 g/l yeast nitrogen base, 2% glucose, and the appropriate factors to support the growth of auxotrophic strains.

Disruption of the *IRA1* and *IRA2* Genes

IRA1 and *IRA2* were disrupted by one-step gene disruption (Rothstein, 1983). *IRA2* was disrupted in the strain HR125 by insertion of the *LEU2* gene into the *Bgl* II site at position 406 of the *IRA2* gene to produce the strain TJ106. The plasmid pNR1 (Tanaka *et al.*, 1989) provided by K. Tanaka (University of Tokyo) was used to disrupt the *IRA1* gene at position 8103 in the strain HR125 resulting in the mutant TM105 (Mitts *et al.*, 1991). The *URA3* gene was then inserted into the *Eco*RI site at position 328 of *IRA2* in TM105 to produce TD101. Disruptions were confirmed by Southern blot analysis.

Preparation of Yeast Particulate Fractions

Yeast were grown to the indicated optical density. The cultures were centrifuged, and cells were disrupted by vortexing with glass beads

in YMB buffer (50 mM 2[N-morpholino] ethanesulfonic acid pH 6.0, 0.1 mM EDTA, 0.1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 50 μ g/ml leupeptin) as previously described (Caspersen *et al.*, 1983). The homogenate was centrifuged in a Beckman J6 (Fullerton, CA) for 5 min at 1000 rpm to remove nuclei and unbroken cells. The supernatant was then spun 30 min at 14 000 rpm to separate particulate and soluble fractions. Both particulate and soluble fractions were stored in YMB at -70°C until needed.

Adenylate Cyclase Assay

Adenylate cyclase was assayed by a modification of the method of Salomon *et al.* (1974) as described by Caspersen *et al.* (1985). Guanine nucleotides, where present, were at 10 μ M. Protein was assayed by the method of Lowry *et al.* (1951). Values are presented as the mean of triplicate determinations with SD < 15%.

Western Blots

Protein samples (yeast membranes) were separated on 5% polyacrylamide gels and transferred to nitrocellulose. The membranes were blocked with Blotto (Sigma, St. Louis, MO) (5% nonfat powdered milk in 50 mM Tris(hydroxymethyl)aminomethane [Tris] pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.01% sodium azide, 0.05% polyoxyethyl- enesorbitan monolaurate and developed with Ira1 serum (1:600 dilution) or anti Cyr1 serum (1:100) in Blotto. Immunoreactive proteins were visualized by the addition of [125 I]-labeled goat-anti-rabbit antibody (specific activity 2 000 000 cpm/ μ g) followed by autoradiography of the washed membrane.

Preparation of RNA and Northern Blots

Yeast cells (~ 20 OD units) were resuspended with 300 μ l lysis buffer (0.1 M Tris pH 7.5, 0.1 M LiCl, 0.01 M dithiothreitol) and added to tubes containing glass beads (500 μ l), 1% sodium dodecyl sulfate (SDS) (60 μ l), and phenol/chloroform (600 μ l). The cells were disrupted by six cycles of 30 sec bursts of vortexing and 30 sec on ice. Lysis was followed by multiple phenol chloroform extractions and ethanol precipitations as described (Ellwood and Craig, 1984). Fifteen microgram samples were electrophoresed and transferred onto a Gene Screen Plus membrane according to the suppliers directions (New England Nuclear). Blots were probed with two *Eco*RI fragments (1.3 and 1.0 kilobase [kb]) from *IRA1*, a 4.2-kb *Bgl* II fragment of *IRA2*, two *Hind*III fragments (2.0 and 3.3 kb) from *CYR1*, or a 1.6-kb *Pst* I fragment of *CDC25*. Probes were radiolabeled with 32 P by the random primer method (Promega Prime-A-Gene system, Madison, WI) to a specific activity of 1×10^9 cpm/ μ g. To confirm uniform loading and transfer, ribosomal RNA was visualized on the blot by staining with ethidium bromide. The stained blot was photographed, and the negative was scanned with a Molecular Dynamics Computing Densitometer (Sunnyvale, CA). Ribosomal RNA loading and transfer varied by less than 5% between lanes.

cAMP Measurements

Cells (~ 4 – 10 OD units/sample) were harvested, and extracts were prepared by resuspending pellets in 1 ml of 10% trichloroacetic acid. This extract was freeze thaw cycled three times and centrifuged in an Eppendorf (Hamburg, Germany) microcentrifuge (14 000 rpm) for 2 min. The supernatant was extracted with five washes of ether (2 ml each). The aqueous sample was then dried in a speed vac and resuspended in assay buffer. The samples were assayed for cAMP content with a Rianen cAMP [125 I] radioimmunoassay kit (New England Nuclear) according to the manufacturer's instructions. Values are presented as the mean of triplicate determinations, SD > 15%.

Viability Measurements

Cells were diluted 1:100 in YEPD and plated onto YEPD plates containing 1 mM cAMP. The plates were incubated overnight and ex-

aminated under a microscope to determine the percentage of cells able to form colonies. Each determination contained ≥300 cells.

RESULTS

Changes in cAMP Levels During Growth

The rapid increase in cAMP observed as starved cells resume growth in fresh glucose medium is well documented (Eraso and Gancedo, 1985; Nakafuku *et al.*, 1988; Tanaka *et al.*, 1989). Much less attention has been devoted to changes in cAMP levels as cells deplete the glucose supply and stop growing. We have found that the correlation between cAMP levels and growth extends to conditions where cells exit the mitotic cycle. Cells grown in rich medium steadily accumulate ethanol as they deplete the glucose in the medium, reaching the highest ethanol concentration at the point where glucose is exhausted and the cells show a characteristic plateau in growth (Kappeli, 1986). This plateau in the growth curve corresponds to the diauxic shift, where the cells switch to oxidative growth on ethanol. We measured cAMP levels in samples of yeast taken throughout growth in YEPD, concentrating on cAMP levels in cells at the growth plateau of the diauxic shift, where cells exit and re-enter the mitotic cycle. As shown in Figure 1, cAMP levels dropped during the early fermentative phase of growth, reaching a sharp minimum as cells stopped dividing at the diauxic shift (~4–5 OD). As the cells resumed growth, cAMP levels rose again; however, neither cAMP nor growth rate returned to the levels observed during the fermentative phase.

Role of cAMP in the Diauxic Shift

To more directly examine the effects of cAMP at the diauxic shift, we used a strain in which we could manipulate cAMP levels. This strain (TC41-1, Table 1) cannot make its own cAMP because it carries a deletion in the structural gene for adenylate cyclase, *CYR1*. These cells are able to take up cAMP from the growth medium by virtue of *cam* mutations (Matsumoto *et al.*, 1982) and can be propagated by addition of exogenous cAMP

Figure 1. cAMP levels drop at the diauxic shift. Wild-type cells (HR125) were grown in YEPD medium, and samples were harvested at the indicated densities and frozen on dry ice/ethanol in aliquots for later measurement of cAMP content. cAMP assays were performed as indicated in MATERIALS AND METHODS. □, culture density at 660 nm; ▲, cAMP levels.

(Figure 2). Thus, cAMP levels in these cells can be manipulated by changing the cAMP concentration of the growth medium. When TC41-1 cells were held at constant cAMP in rich medium, cultures at 2 or 5 mM cAMP grew well during the fermentative phase of growth but failed to grow at all in the oxidative phase of the growth curve. When we examined the cells held at the higher cAMP concentrations, we found that they had stopped growth at all stages of the cell cycle, indicating a failure to arrest growth in G1. Cultures held at 0.5 and 1.0 mM cAMP grew more slowly during the fermentative phase but were able to enter the oxidative phase and continue growth. We found no fixed concentration of cAMP that would allow the TC41-1 cells to achieve the overall rate of growth seen in the wild-type culture.

These results are consistent with the idea that yeast require a decrease in cAMP level to utilize a nonfermentable carbon source. We tested this by varying the cAMP concentration as cells growing in glucose were

Table 1. Yeast strains used in this study

Strain	Genotype
HR125 ^a (wild-type)	<i>MAT a leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4</i>
TC41-1 ^b (<i>cyr1</i> deletion)	<i>MAT a leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1::URA3 cam</i>
TM105 ^c (<i>IRA1</i> disruption at codon 2520)	<i>MAT a leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ira1::LEU2</i>
TJ106 (<i>IRA2</i> disruption at codon 59)	<i>MAT a leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ira2::LEU2</i>
TD101 (<i>IRA1</i> disruption at codon 2520, <i>IRA2</i> disruption at codon 32)	<i>MAT a leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ira1::LEU2 ira2::URA3</i>

^a Caspersen *et al.*, 1983.

^b Heideman *et al.*, 1990.

^c Mitts *et al.*, 1991.

Figure 2. Growth of *cyr1⁻* cells with exogenous cAMP. A *cyr1⁻* strain (TC41-1) was diluted from a stationary culture into YEPD medium containing varying concentrations of cAMP. Culture densities were measured at the indicated times. Concentrations of cAMP: 0 mM, ○; 0.1 mM, ▲; 0.2 mM, □; 0.5 mM, ◆; 1 mM, △; 5 mM, ●. For comparison, a culture of wild-type cells (HR125) at 0 mM cAMP is also shown, -■-.

shifted into ethanol medium. Yeast were grown in glucose medium at 5 mM cAMP, centrifuged, and then transferred to ethanol medium at varying cAMP concentrations (Figure 3). As observed in the previous experiment, cells maintained at high cAMP failed to grow in the ethanol medium, but if the cAMP level in the ethanol medium was reduced to 1.5 mM or lower, the yeast were able to proliferate. This demonstrates that a drop in cAMP is necessary for successful progression

Figure 3. Effect of cAMP on shift to ethanol metabolism. TC41-1 cells were grown in YEPD with 5 mM cAMP to a density of 0.2 OD, centrifuged 5 min in a Beckman J6 at 3000 rpm, and resuspended in YEP medium with 2% ethanol in place of glucose. The ethanol medium contained final cAMP concentrations of 0.5 mM, △; 1.5 mM cAMP, □; or 5 mM cAMP, ●. Culture densities were measured at 660 nm at the indicated times.

Figure 4. Reduction of cAMP rescues ethanol growth only in early cultures. Cells were grown in YEPD medium at 5 mM cAMP as indicated in the growth curve shown in A. At the densities indicated in A, aliquots were centrifuged 5 min in a Beckman J6 at 3000 rpm and resuspended in YEPE at either 1 mM cAMP (open symbols, ---) or 5 mM cAMP (closed symbols, —). The densities of the ethanol cultures were then monitored at 660 nm (B). Densities at the transfer: A, 0.2 OD; B, 0.46 OD; C, 1.0 OD; D, 2.56 OD.

to oxidative growth. The effects of high cAMP could not be attributed to toxicity but appeared to be specific to the carbon source. Grown on glucose, cells cultured at 2 or 5 mM cAMP invariably grew more rapidly than those cells maintained at lower cAMP.

Further experiments showed that this decrease in cAMP levels must occur at the right time to allow growth on ethanol medium (Figure 4). As in the previous experiment, cells were grown in glucose at high cAMP (5 mM). Cells were spun down and then transferred to ethanol medium at either the same high cAMP concentration or at reduced cAMP (1 mM). However, in this experiment, we transferred cells from the glucose medium at varying points in the glucose growth curve as indicated in Figure 4A. The growth of these samples after transfer to ethanol medium is plotted in Figure 4B. As was the case in the experiment shown in Figure 3, if cAMP concentration was lowered early in the growth curve (below 0.5 OD), the cells were able to grow in

ethanol medium. However, if the cultures were allowed to proceed farther in the glucose growth curve (1–3 OD), reducing cAMP no longer allowed growth on ethanol medium. The critical density appeared to correlate with the density at which cAMP falls in the wild-type cells.

Although the cells at 3 OD could no longer be shifted to ethanol, they were still viable if transferred to fresh glucose medium. After growth had ceased, a sample of the high cAMP cells were plated onto YEPD with 1 mM cAMP; 90% of these cells were able to form colonies. However the TC41-1 cells cultured at high cAMP steadily lost viability in a manner similar to that observed with cells carrying either *ras2^{val19}* or *bcy1* mutations (Toda *et al.*, 1985, 1987a). They lost viability during storage on plates, and they steadily lost viability after depletion of nutrients in liquid culture. Four days after inoculation, only 12% of the cells at 5 mM cAMP remained viable, whereas 47% of the cells held at 1 mM cAMP remained viable, and 90% of the wild-type cells were able to form colonies.

Expression of Components of the Ras/Adenylate Cyclase System

A number of factors might be expected to contribute to the decrease in cAMP levels observed at the diauxic shift. These might include a decrease in the CDC25-mediated guanine nucleotide exchange activity that initiates Ras activation; an increase in the rate of termination of the Ras signal, mediated by the products of the *IRA1* and 2 genes; or an increase in the degradation of cAMP by phosphodiesterase activity. In addition to changes in the specific activities of these enzymes, an unexplored means to account for the observed changes in cAMP might be changes in the levels of the gene products involved in the pathway. To test this idea, triplicate samples of cells were harvested at 1-h intervals during culture, RNA was isolated, and triplicate Northern blots were prepared and probed with *CDC25*, *CYR1*, *IRA1*, and *IRA2* (Figure 5). Each probe identified unique products (at ~5 kb for *CDC25*, 9.5 kb for *IRA1* and *IRA2*, and 6.5 kb for *CYR1*). The bands were quantitated with a scanning densitometer, and the averages from the blots are plotted in the top panel in Figure 5. Representative blots are shown in the bottom panel. None of the messages examined remained constant during the course of the culture. As shown in Figure 5 at the point in the growth curve at which cAMP levels were lowest, mRNA for *IRA1* and *IRA2* were increasing dramatically, whereas the messages for *CDC25* and *CYR1* were falling. This corresponds to the point just before the plateau in growth in which cells are halting proliferation.

The changes in *CYR1* and *IRA1* expression were confirmed at the protein level by Western blotting using antibodies against Cyr1 and Ira1 (Heideman *et al.*, 1990; Mitts *et al.*, 1991). As measured by densitometer scan-

Figure 5. Changes in the levels of mRNA for *IRA1*, *IRA2*, *CDC25*, and *CYR1* at the diauxic shift. Wild-type cells (HR125) were grown in YEPD medium, and triplicate samples were harvested at the indicated densities for Northern blotting as described in MATERIALS AND METHODS. Samples of total RNA were extracted, and 15 μ g samples were loaded onto each lane. Uniform loading and transfer was confirmed as described in MATERIALS AND METHODS. Northern blots from three separate preparations of RNA were quantitated by scanning with a Molecular Dynamics Computing Densitometer, and the averages are plotted along with culture density at 660 nm. *IRA1* message, $\blacklozenge$; *IRA2* message, $\circ$; *CYR1* message, $\blacksquare$; *CDC25* message, $\triangle$; culture density, —. Error bars indicate SD of the triplicate samples.

ning of the Western blots, levels of Cyr1 and Ira1 protein varied at least fivefold over the course of culture growth (Figure 6). Cyr1 protein levels reached a peak in mid log phase growth, where cAMP levels were high and the cells were dividing most rapidly. However as the cells approached the diauxic shift, Cyr1 protein levels were dropping rapidly. As Cyr1 levels were falling, Ira1 levels peaked. Ira1 levels then diminished as the culture resumed proliferation in the oxidative phase of growth. The drop in Ira1 protein at the point where cells resumed growth (the 6.9 OD point in this experiment) was consistently observed.

All of the changes in expression that we observed as the cells stopped dividing at the diauxic shift would be expected to decrease the production of cAMP. The drop in *CDC25* expression should decrease adenylate cyclase

Figure 6. Changes in Cyr1 and Ira1 protein at the diauxic shift. Wild-type cells (HR125) were grown in YEPD medium, and samples were harvested at the indicated densities for Western blotting with Cyr1 or Ira1 antibodies as described in MATERIALS AND METHODS. Membrane samples (30 μ g) were run on a 5% SDS polyacrylamide gel and transferred to nitrocellulose for Western blotting. Uniform loading and transfer was confirmed by staining the blots with Ponceau-S. The bands were quantitated by scanning with a Molecular Dynamics Computing Densitometer, and the relative density of each band is plotted along with culture density at 660 nm. Solid symbols, culture density; Δ , IRA1 protein; $\square$, CYR1 protein.

activity by decreasing the rate of GTP activation of Ras. Similarly, the increase in IRA1 and IRA2 expression would be expected to accelerate the inactivation of GTP $\cdot$ RAS, decreasing the GTP stimulation of adenylate cyclase. Because CYR1 encodes the catalytic subunit of adenylate cyclase, the drop in CYR1 expression would also decrease production of cAMP. We were able to confirm that the changes in expression were associated with the expected changes in adenylate cyclase activity by measuring GTP stimulation of adenylate cyclase *in vitro*. Membranes were prepared from cells sampled at various densities along the growth curve and assayed for adenylate cyclase activity with either Mg^{2+} alone, to measure basal activity, or with GTP, to measure Ras-stimulated activity (Figure 7). GTP (Ras)-stimulated adenylate cyclase activity was lowest in membranes prepared from the cells at the plateau of the growth curve, a point at which CDC25 expression had fallen, and IRA1 and IRA2 expression was at its highest. At this density, GTP-stimulated activity fell almost to basal levels as cells arrested growth. This effect was very reproducible;

in four separate experiments, stimulation by GTP was consistently lowest in cells at the growth plateau of the diauxic shift. Although GTP stimulation of adenylate cyclase matched the expression of CDC25, IRA1, and IRA2 very well, adenylate cyclase activity in membranes harvested from cells at the higher densities was at odds with both CYR1 expression and cAMP measurements. The increase in adenylate cyclase activity at the higher cell densities may be because of activation of the enzyme during membrane preparation. It is known that protease activity markedly increases after the diauxic shift (Jones, 1991), and adenylate cyclase in *S. cerevisiae* is very sensitive to protease activation (Heideman *et al.*, 1987).

Effect of IRA1 and IRA2 Disruption on Growth at the Diauxic Shift

The increase in IRA1 and IRA2 expression at the diauxic shift coupled with the decreased GTP stimulation of adenylate cyclase suggested that IRA gene expression might play a role in lowering cAMP levels and halting proliferation. To test this, we constructed a strain carrying disruptions in both IRA genes (TD101). As observed previously, these cells were viable, indicating that the IRA genes are not essential for normal growth (Tanaka *et al.*, 1990). On rich medium the mutants grew normally in the early phase of culture (where IRA expression is normally low), but they were unable to progress through the diauxic shift (Figure 8). Indeed, the phenotype of these cells was identical to that of TC41-1 cells grown at 5 mM cAMP (Figure 2). The mutant cells did not arrest growth as they approached the diauxic shift but rather became stranded in all stages of the cell cycle. Cells carrying a single IRA mutation had

Figure 7. GTP-stimulated adenylate cyclase activity is decreased at the diauxic shift. Samples from the growth curve in Figure 1 were collected, and membranes were prepared as described in MATERIALS AND METHODS. Membranes were assayed for adenylate cyclase activity in the presence of Mg^{2+} alone, $\blacksquare$, or Mg^{2+} with GTP (10 μ M), $\square$.

Figure 8. Disruption of *IRA1* and *IRA2* prevents cells from progressing past the diauxic shift. Cultures of a wild-type strain (HR125) and an otherwise isogenic mutant were grown at 30°C in YEPD. Culture densities were monitored at 660 nm at the indicated times. (A) HR125 (■) and TD101, an isogenic double *IRA1*, *IRA2* disruptant (Δ). (B) HR125 (■) and TM105, an isogenic *IRA1* disruptant (□). (C) HR125 (■) and TJ106, an isogenic *IRA2* disruptant (□).

an intermediate phenotype; they were able to progress through the diauxic shift, but oxidative growth was slow compared to the isogenic wild-type (Figure 8, B and C).

DISCUSSION

Our results support two conclusions. The first is that a drop in cAMP plays an important role in the processes occurring at the diauxic shift. The second conclusion is that the expression of genes involved in the Ras/cAMP pathway is not constant during growth in culture. In proposing models for the regulation of the adenylate cyclase by Ras, we will need to make allowances for these changes.

Drop in cAMP at the Diauxic Shift

The drop in cAMP levels in cells approaching the diauxic shift suggested a possible role for cAMP in the G1 arrest at the diauxic shift. This correlative evidence was reinforced by experiments in which we manipulated cAMP levels in cells grown in culture. In our experiments, the drop in cAMP was necessary for cells to successfully enter the oxidative phase of growth. Elevated cAMP levels did not appear to affect cells in the fermentative phase of growth. Indeed, cells in high cAMP medium (5 mM) grew more rapidly than cells held at lower cAMP (0.5 mM) during the early phase of growth. The same response was observed in the cells carrying the double disruption in *IRA1* and *IRA2*. These cells grew normally during the fermentative growth but were unable to proceed to the oxidative phase. Like the cells at high cAMP, the *ira1,2⁻* cells failed to arrest in G1 when they reached the growth plateau.

The diauxic shift involves at least two processes, including a halt in cell division and a shift in metabolic machinery (Wills, 1990). The drop in cAMP appears to be involved in both of these processes. In addition to failing to arrest growth after nutrient depletion, the TC41-1 cells at high cAMP appeared unable to convert to oxidative metabolism, as illustrated by the finding that (although not arrested at any particular stage of the cell cycle) these cells failed to grow on the nonfermentable carbon source.

Changes in Gene Expression at the Diauxic Shift

We found that none of the components of the Ras/adenylate cyclase system that we examined were expressed at constant levels during growth in culture. As cells approached the growth plateau of the diauxic shift, the expression of both *CDC25* and *CYR1* decreased, whereas *IRA1* and *IRA2* expression increased. All of these changes were consistent with the fall in cAMP levels that we observed at the diauxic shift. In addition to the changes that we observed, *RAS1* message levels are also known to decrease as cells change from glucose to nonfermentable carbon sources (Breviario *et al.*,

1986). In light of these results, we will not be able to assume a constant stoichiometry between the components of the Ras/adenylate cyclase pathway when constructing models for the regulation of cAMP production by Ras proteins.

In addition to the changes in expression of *CDC25*, *RAS1*, *CYR1*, *IRA1*, and *IRA2* mentioned above, there are several other mechanisms that might potentially cause the drop in cAMP as cells prepare for growth arrest at the diauxic shift. For example, addition of glucose to starved cells produces a very rapid *CDC25*-mediated increase in cAMP. There is no reason to believe that this mechanism cannot be reversed to reduce cAMP production as glucose is depleted. Additionally, regulation of phosphodiesterase activity might play a role in the decrease in cAMP levels. Our results indicate that in addition to these potential posttranslational regulatory mechanisms, regulation at the level of gene expression must be considered.

Models for Ras function have centered around regulation of the GTPase cycle that is central to both the Ras and G proteins. Why do yeast need to add regulation of gene expression to this system? In a G protein type of model, cAMP levels would change in direct proportion to nutrient levels. Although this produces a useful working model, such a system may be too simple to meet the needs of growing yeast. Yeast are under strong selective pressure to proliferate rapidly. Over several generations, a slight increase in doubling rate will provide a large selective advantage. On the other hand, it is perilous for a yeast cell to traverse the mitotic cycle with inadequate nutrient supplies. Thus for yeast, growth on glucose is like a race to the edge of a cliff; the cells must grow and divide at the fastest possible pace and stop at the last moment. A system that directly couples growth rate to nutrient levels would have growth gradually slow as the nutrient supply becomes depleted. Such a system is much better suited to maintaining a homeostatic balance than it is to controlling the decision to halt growth. The evidence presented above indicates that the Ras/adenylate cyclase system is not static but rather changes with growth conditions. This demonstrates an active process for halting growth. It also demonstrates yet another signal affecting growth—that which regulates expression of the components of the Ras/adenylate cyclase system. We will need to identify this signal before we completely understand growth regulation in *S. cerevisiae*.

ACKNOWLEDGMENTS

The authors thank Sin Fai Cheung for assistance in the laboratory and Kazuma Tanaka and Fuyuhiko Tamanoi for providing the plasmid pNR1 and the *IRA2* probe. This work was supported by Public Health Service grant GM42406 from the National Institutes of Health and by Institutional Research Grant IN-35-31 from the American Cancer Society.

REFERENCES

- Breviario, D., Henebusch, A., Cannon, J., Tatchell, J., and Dhar, R. (1986). Carbon source regulation of *RAS1* expression in *Saccharomyces cerevisiae* and the phenotypes of *ras2⁻* cells. *Proc. Natl. Acad. Sci. USA* 83, 4152–4156.
- Casperson, J.F., Walker, N., and Bourne, H.R. (1985). Isolation of the gene encoding adenylate cyclase in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 82, 5060–5063.
- Casperson, J.F., Walker, N., Brasier, A.R., and Bourne, H.R. (1983). A guanine nucleotide sensitive adenylate cyclase in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 258, 7911–7914.
- DeFeo-Jones, D., Scolnick, E.M., Koller, R., and Dhar, R. (1983). *ras*-related gene sequences identified and isolated from *Saccharomyces cerevisiae*. *Nature* 306, 707–709.
- Ellwood, M.S., and Craig, E.A. (1984). Differential regulation of the 70K heat shock gene and related genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4, 1454–1459.
- Eraso, P., and Gancedo, J.P. (1985). Use of glucose analogues to study the mechanism of glucose-mediated cAMP increase in yeast. *FEBS Lett.* 19, 51–54.
- Heideman, W., Caspersen, G.F., and Bourne, H.R. (1987). Adenylyl cyclase in yeast: hydrodynamic properties and activation by trypsin. *J. Biol. Chem.* 262, 7087–7091.
- Heideman, W., Caspersen, G.F., and Bourne, H.R. (1990). Adenylyl cyclase in yeast: antibodies and mutations identify a regulatory domain. *J. Cell. Biochem.* 42, 229–242.
- Jones, E.W. (1991). Tackling the protease problem in yeast. *Methods Enzymol.* 194, 428–452.
- Jones, S., Vignais, M.-L., and Broach, J.R. (1991). The *CDC25* protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotides bound to Ras. *Mol. Cell. Biol.* 11, 2641–2646.
- Kappeli, O. (1986). Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Adv. Microb. Physiol.* 28, 181–209.
- Lowry, O.H., Rosebrough, N.S., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Matsumoto, K., Uno, I., and Ishikawa, T. (1985). Genetic analysis of the role of cAMP in yeast. *Yeast* 1, 15–24.
- Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982). Isolation and characterization of yeast mutants defective in adenylate cyclase and cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* 79, 2355–2359.
- Mitts, M.R., Bradshaw-Rouse, J., and Heideman, W. (1991). Interactions between adenylate cyclase and the yeast GTPase-activating protein, *IRA1*. *Mol. Cell. Biol.* 11, 4591–4598, 1991.
- Nakafuku, M., Obara, T., Kaibuchi, K., Miyajima, I., Miyajima, A., Itoh, H., Nakamura, S., Arai, K., Matsumoto, K., and Kaziro, Y. (1988). Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: studies on structure and possible functions. *Proc. Natl. Acad. Sci. USA* 85, 1374–1378.
- Nikawa, J., Cameron, S., Toda, T., Ferguson, K.M., and Wigler, M. (1987). Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. *Genes & Dev.* 1, 931–937.
- Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian *ras* genes. *Cell* 36, 607–612.
- Pringle, J.R., and Hartwell, L.H. (1981). The *Saccharomyces cerevisiae* cell cycle. In: *The Molecular Biology of the Yeast Saccharomyces cerevisiae: Life Cycle and Inheritance*, ed. E.W. Jones and J.R. Broach,

- Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 97–142.
- Rothstein, R.J. (1983). One-step gene disruption in yeast. *Methods Enzymol.* 101, 202–211.
- Salomon, Y., Londos, C., and Rodbell, M. (1974). A highly sensitive adenylate cyclase assay. *Anal. Biochem.* 58, 541–548.
- Tanaka, K., Matsumoto, K., and Toh-e, A. (1989). *IRA1*, an inhibitory regulator of the RAS-cyclic AMP pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 9, 757–768.
- Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M.S., Gibbs, J., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990a). *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian *ras* GTPase activating protein. *Cell* 60, 803–807.
- Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990b). *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian GTPase-activating protein. *Mol. Cell. Biol.* 10, 4303–4313.
- Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987a). Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cAMP-dependent protein kinase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7, 1371–1377.
- Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987b). Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* 50, 277–287.
- Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, *RAS* proteins are controlling elements of adenylate cyclase. *Cell* 40, 27–36.
- Wills, C. (1990). Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. *Crit. Rev. Biochem. Mol. Biol.* 25, 245–280.