

# Efficient transition to growth on fermentable carbon sources in *Saccharomyces cerevisiae* requires signaling through the Ras pathway

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**Strains carrying *ras2*<sup>318S</sup> as their sole *RAS* gene fail to elicit a transient increase in cAMP levels following addition of glucose to starved cells but maintain normal steady-state levels of cAMP under a variety of growth conditions. Such strains show extended delays in resuming growth following transition from a quiescent state to glucose-containing growth media, either in emerging from stationary phase or following inoculation as spores onto fresh media. Otherwise, growth of such strains is indistinguishable from that of *RAS2*<sup>+</sup> strains. *ras2*<sup>318S</sup> strains also exhibit a delay in glucose-stimulated phosphorylation and turnover of fructose-1,6-bisphosphate, a substrate of the cAMP-dependent protein kinase A (PKA) and a key component of the gluconeogenic branch of the glycolytic pathway. Finally *Tpk*<sup>w</sup> strains, which fail to modulate PKA in response to fluctuations in cAMP levels, show the same growth delay phenotypes, as do *ras2*<sup>318S</sup> strains. These observations indicate that the glucose-induced cAMP spike results in a transient activation of PKA, which is required for efficient transition of yeast cells from a quiescent state to resumption of rapid growth. This represents the first demonstration that yeast cells use the Ras pathway to transmit a signal to effect a biological change in response to an upstream stimulus.**

**Keywords:** cAMP-dependent protein kinase A/ fermentable carbon/Ras signaling/*Saccharomyces cerevisiae*

## Introduction

Two Ras proteins, Ras1p and Ras2p, in the yeast *Saccharomyces* reside at the core of a signal transduction pathway whose components are well defined but whose function is less clear (Broach and Deschenes, 1990; Broach, 1991; Tatchell, 1993; Thevelein, 1994). As in other organisms, yeast Ras proteins can transmit a regulatory signal by shuttling between an inactive GDP-bound form and an active GTP-bound form. In yeast, the GTP-bound Ras proteins stimulate adenylyl cyclase, encoded by *CYR1*, to yield an increase in intracellular cAMP levels (Toda *et al.*, 1985; De Vendittis *et al.*, 1986; Field *et al.*, 1988). cAMP appears to function exclusively to liberate the yeast cAMP-

dependent protein kinase A (PKA) catalytic subunit, encoded redundantly by *TPK1*, *TPK2* and *TPK3*, from inhibition by the regulatory subunit encoded by *BCY1* (Matsumoto *et al.*, 1982; Toda *et al.*, 1987b). Active PKA can phosphorylate a number of proteins involved in transcription, energy metabolism, cell cycle progression, and accumulation of glycogen and trehalose (Broach and Deschenes, 1990; Tatchell, 1993; Thevelein, 1994; Smith *et al.*, 1998).

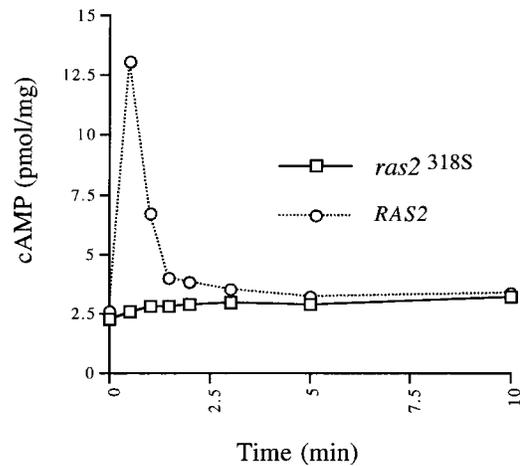
Rapid growth in rich media correlates with increased activity of the Ras pathway. The levels of cAMP in cells grown in rich media containing glucose are higher than those in cells grown in media lacking glucose (Russell *et al.*, 1993). This correlation appears to have functional significance, since cells with hyperactivated Ras/cAMP pathways, e.g. those carrying the activated *RAS2*<sup>19V</sup> allele or a deletion of *BCY1*, grow normally in rich media but fail to grow on non-fermentable carbon sources. Such strains also exhibit a number of phenotypes, including reduced glycogen and trehalose accumulation, heat shock sensitivity and nutrient-starvation sensitivity (Toda *et al.*, 1987a; Engelberg *et al.*, 1994), consistent with an inability to respond appropriately to conditions attenuating growth. In addition, mutationally activated forms of Ras facilitate transition from a yeast to a pseudohyphal growth pattern, but whether that results from increased adenylyl cyclase activity or from Ras activation of a MAP kinase pathway is not clear (Mosch *et al.*, 1996; Kubler *et al.*, 1997; Lorenz and Heitman, 1997). In contrast to activation of the pathway, loss of Ras activity results in diminished cAMP levels, which leads to growth arrest at the beginning of the G<sub>1</sub> stage of the cell cycle (Matsumoto *et al.*, 1985; Tatchell *et al.*, 1985; Toda *et al.*, 1985). This is the same stage at which cells arrest following nutrient deprivation. Growth arrest resulting from loss of Ras activity can be suppressed by downstream activation of the cAMP pathway, e.g. by mutational inactivation of *BCY1*, indicating that production of cAMP is the sole essential function of Ras in yeast. In sum, these observations indicate that the *RAS*/cAMP pathway performs one or more essential functions in the cell, and suggest a functional link between cAMP levels and growth rates.

While the phenotypes of activating and inactivating mutations of *RAS* suggest that Ras adjusts cAMP levels in response to nutrient availability and thereby controls the metabolic and cell cycle activities of the cell, other observations suggest that this simple model does not describe the role of Ras in yeast adequately. First, the differences in the cAMP levels in rich versus poor media or in the presence of activating versus wild-type alleles of Ras are quite modest (Nikawa *et al.*, 1987; Russell *et al.*, 1993). Secondly, high cAMP levels suppress, rather than enhance, the activity of G<sub>1</sub> cyclins and thus appear to control the size at which cells enter

the cell cycle but not the decision of whether to enter the cell cycle (Tokiwa *et al.*, 1994; Flick *et al.*, 1998). Finally, cells expressing constitutive, low-level PKA activity show essentially normal growth behavior, i.e. cells deleted for *BCY1*, *TPK2* and *TPK3* and carrying an attenuating mutation in *TPK1* exhibit low levels of PKA activity that do not fluctuate in response to changes in Ras activity or cAMP levels. Such strains, designated Tpk<sup>w</sup>, show wild-type resistance to heat shock, are not sensitive to nutrient starvation, execute a normal transition into stationary phase and, like wild-type cells, accumulate glycogen and trehalose as they enter stationary phase (Cameron *et al.*, 1988). Thus, the behavior of Tpk<sup>w</sup> strains suggests that, while cells require a basal level of Ras activity to survive, cells do not require signaling through the Ras pathway to adjust for growth under different conditions or to respond to growth arrest.

Results presented in this report suggest that optimal growth of yeast cells does require signaling through the Ras pathway—as distinct from maintenance of a basal cAMP level—but only during the transition to growth on a fermentable carbon source. Addition of glucose to starved cells or cells growing on a non-fermentable carbon source results in a large but transient increase in intracellular cAMP levels (Mazon *et al.*, 1982; Francois *et al.*, 1988). The rapid increase in cAMP levels results from stimulation by glucose of the Ras guanine nucleotide exchange factor, Cdc25p, and the attendant conversion of Ras to its active, GTP-bound form and stimulation of adenylyl cyclase (Broek *et al.*, 1987). The subsequent rapid decrease in cAMP levels results from PKA-dependent feedback inhibition of Cdc25p, Ras and adenylyl cyclase and perhaps activation of either one of the phosphodiesterases or a cAMP exporter (Nikawa *et al.*, 1987; Resnick and Racker, 1988; Mbonyi *et al.*, 1990; Gross *et al.*, 1992). These competing activities yield a 5- to 50-fold increase in cAMP levels within 1–2 min of glucose addition, followed by a decline to near basal levels within 20 min.

We previously constructed a mutant Ras2 protein that maintains normal basal levels of cAMP in the cell but fails to promote the transient increase in cAMP levels following glucose addition. The mutant carries a serine at position 318 instead of cysteine, which precludes the normal thioesterification of palmitate to that site and causes mislocalization of the mutant protein to the cytoplasm rather than the plasma membrane (Bhattacharya *et al.*, 1995). Here we show that strains carrying this mutant Ras2 protein grow normally under most conditions but show extended delays in transitions from poor to rich carbon sources. In addition, these strains show a delay in the phosphorylation and decay of fructose-1,6-bisphosphatase (FBPase), an enzyme that is inactivated by the PKA and required for gluconeogenesis. This suggests that the remodeling of the glycolytic pathway required for transition from gluconeogenic to glycolytic mode is impaired in *ras2*<sup>318S</sup> mutants. We take these results to indicate that efficient transition from poor to rich carbon sources requires signaling through the Ras pathway.



**Fig. 1.** *ras2*<sup>318S</sup> cells do not exhibit a transient increase in cAMP levels in response to glucose addition. Strains Y2446 (*RAS2*) and Y2455 (*ras2*<sup>318S</sup>) were grown at 30°C for 48 h in synthetic medium containing 3% glycerol. Glucose was then added to the cell cultures to 2%. At the indicated times after the addition of glucose, aliquots of cells were removed and the intracellular cAMP levels were determined as described in Materials and methods.

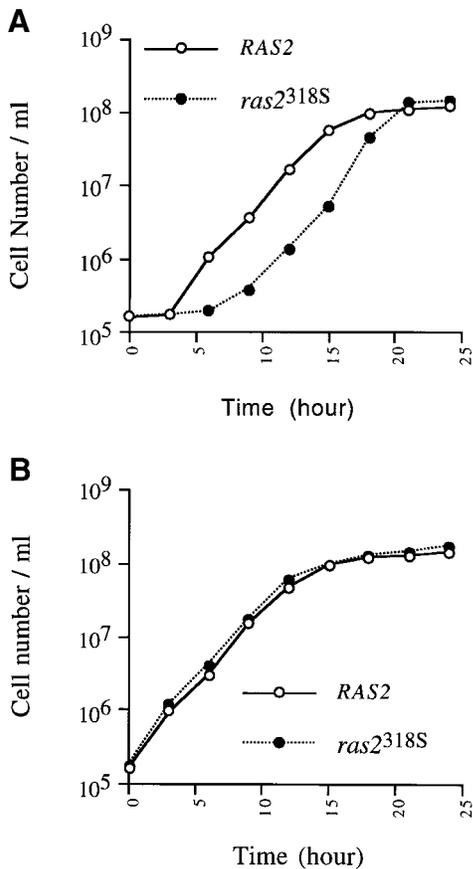
## Results

### *Ras2*<sup>318S</sup> protein does not support a glucose-stimulated transient increase in intracellular cAMP

As described in Materials and methods, we constructed closely related strains in which the only Ras protein expressed derived either from wild-type *RAS2* (strain Y2446) or from the *ras2*<sup>318S</sup> mutant allele (strain Y2455). As observed with other strains expressing wild-type Ras protein, addition of glucose to Y2446 cells grown in synthetic glycerol medium promoted a transient increase in cAMP levels, reaching a maximal value 5-fold that of the basal level within 2 min and then returning to near the initial basal level within 20 min. In contrast, as shown in Figure 1, addition of glucose to Y2455 cells grown in the synthetic glycerol medium did not elicit a transient increase in cAMP levels. We previously observed similar results for a similar strain grown in complex media (Bhattacharya *et al.*, 1995). Nonetheless, the basal cAMP levels of Y2455 cells grown in either glycerol or glucose, or in complex or synthetic media were indistinguishable from those of strain Y2446, or other related *RAS2* strains, grown under the same conditions (Figure 1; Bhattacharya *et al.*, 1995; data not shown). Thus, strains carrying the *Ras2*<sup>318S</sup> mutant protein maintain normal basal levels of cAMP but do not produce a transient increase in cAMP levels in response to glucose addition. The reason for this defect is not clear, but, as described below, the specific defect exhibited by this mutant strain provides a means of evaluating the role of the Ras-mediated transient increase in cAMP separate from the role of maintaining basal levels of cAMP in the cell.

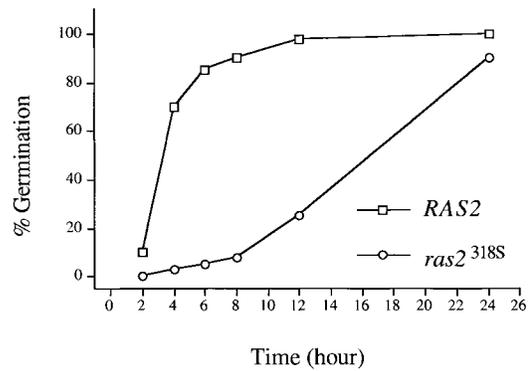
### *ras2*<sup>318S</sup> strains exhibit a delay in the transition from poor to rich carbon sources

Since strains dependent on *Ras2*<sup>318S</sup> as their sole Ras protein fail to exhibit a transient increase in cAMP levels following addition of glucose to starved cells, we asked whether these strains were defective in their ability to



**Fig. 2.** *ras2*<sup>318S</sup> strains show a delay in resumption of growth out of stationary phase. YEPD cultures (10 ml) were inoculated with 10<sup>6</sup> cells from cultures of strain Y2446 (*RAS2*) or Y2455 (*ras2*<sup>318S</sup>) that had been incubated for 48 h after the diauxic shift (A) or grown to 2×10<sup>7</sup> cells/ml (B). Growth of the cultures was monitored by cell count as a function of time after inoculation. Identical results were obtained with three different pairs of *RAS2* and *ras2*<sup>318S</sup> strains.

make the transition from starvation to growth in the presence of glucose. Accordingly, we grew strains Y2446 (*RAS2*) and Y2455 (*ras2*<sup>318S</sup>) into stationary phase, diluted the cultures into fresh media containing glucose and then monitored the growth of the strains. As shown in Figure 2, strain Y2446 resumed exponential growth ~4 h after transfer to fresh medium. In contrast, strain Y2455 exhibited a delay of >10 h before resuming exponential growth. This differential effect was not due simply to dilution into fresh media, since neither strain exhibited a delay in exponential growth when diluted from exponentially growing cultures (Figure 2B). Nor was the delay in resumption of growth by the Y2455 culture due to loss of viability of cells during stationary phase; both Y2446 and Y2455 cultures retained >90% viability during extended incubation in stationary phase (data not shown). In a related experiment, we found that *ras2*<sup>318S</sup> cells exhibited a 5 h delay in assuming a faster growth rate following addition of glucose to cells growing exponentially in glycerol media (data not shown). In contrast, *RAS2* cells assumed the faster growth rate almost immediately. Finally, as noted in Figure 2, while strain Y2455 exhibited a delay in resumption of growth out of stationary phase, the growth rate of the strain once it resumed growth was indistinguishable from that of the *RAS2*<sup>+</sup> strain. Thus,

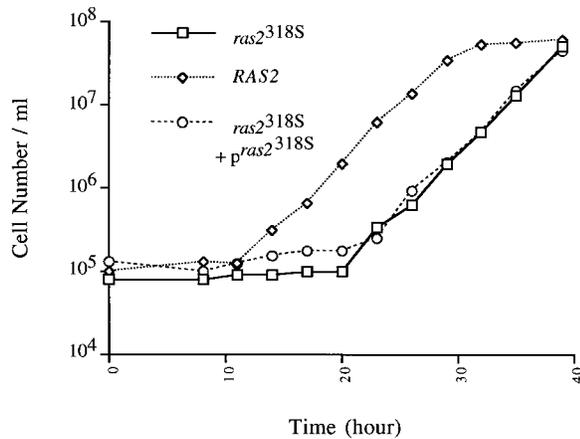


**Fig. 3.** *ras2*<sup>318S</sup> spores germinate more slowly than *RAS2* spores. Diploid strain Y1810 (*ras1/ras1.RAS2-URA3/ras2*<sup>318S</sup>) was sporulated and dissected on YEPD plates. Germination of spores at 30°C from 30 tetrads was monitored by microscopic visualization using initial appearance of the first bud as the metric that germination had occurred. The *RAS2* allele in each spore was determined upon colony maturation by following the *URA3* marker, which was linked to *RAS2*<sup>+</sup>. The cumulative germination of spores as a percentage of the total number of spores examined is shown for both sets of spores at the indicated time following dissection.

the *ras2*<sup>318S</sup> strain grows normally under steady-state conditions but fails to make an efficient transition to growth in rich media.

Spore germination represents a second condition under which cells make a transition from quiescence to growth in the presence of glucose. In addition, spore germination depends on Ras function (Herman and Rine, 1997). Accordingly, we examined whether cells carrying the *ras2*<sup>318S</sup> allele exhibited a delay in germination relative to the time required for germination of *RAS2*<sup>+</sup> cells. We sporulated a *ras1/ras1 RAS2-URA3/ras2*<sup>318S</sup> strain and then determined the time of germination of each spore by monitoring microscopically the time of appearance of the first bud. The allele present in each spore was subsequently determined by scoring the resultant spore colonies for the presence of a *URA3* marker linked to the *RAS2*<sup>+</sup> allele. As evident from the results shown in Figure 3, >80% of the *RAS2*<sup>+</sup> spores had germinated by 8 h, while <10% of the *ras2*<sup>318S</sup> spores germinated within the same time. However, by 24 h, >95% of both *RAS2*<sup>+</sup> and *ras2*<sup>318S</sup> spores had germinated, i.e. spores dependent on the *Ras2*<sup>318S</sup> protein germinate but do so more slowly than those containing wild-type Ras protein. Thus, the *ras2*<sup>318S</sup> allele prevents efficient resumption of growth of spores.

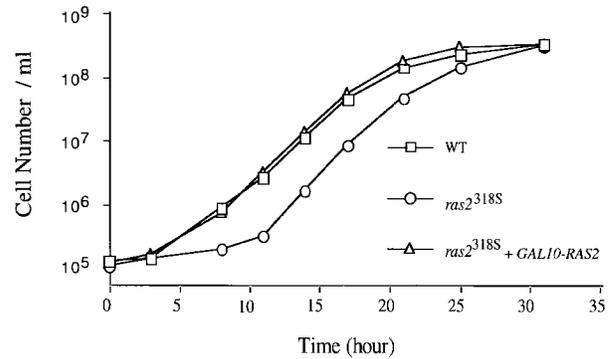
As noted above, cells carrying the *ras2*<sup>318S</sup> allele contain the same steady-state levels of cAMP as do cells carrying the *RAS2* allele but fail to generate the transient increase in cAMP following addition of glucose. Accordingly, we surmise that the delay in resumption of growth of spores or of stationary phase cells results from the inability of these cells to generate the transient increase in cAMP. However, to rule out the possibility that the salient defect in *ras2*<sup>318S</sup> cells is a subtle difference in basal Ras activity, we examined the behavior of cells carrying multiple copies of *ras2*<sup>318S</sup> by creating strains carrying the *ras2*<sup>318S</sup> gene on a 2 μ-based plasmid. Such strains produce 10- to 20-fold more Ras2 protein than do cells carrying a single copy of the gene (Deschenes and Broach, 1987; Bhattacharya *et al.*, 1995). Thus, if the phenotypic effects of the *ras2*<sup>318S</sup> allele were due simply to reduced activity



**Fig. 4.** Overexpression of the *RAS2*<sup>318S</sup> allele does not alleviate its phenotypic defects. Strain Y2455 was transformed with either plasmid pRS426 (a 2  $\mu$  circle-based *URA3* vector) (Sikorski and Hieter, 1989) or pRS426 containing the *ras2*<sup>318S</sup> gene. Transformants were grown at 30°C in synthetic media lacking uracil for 5 days and then inoculated in fresh medium to a cell density of  $1 \times 10^5$  cells/ml. Growth of the culture at 30°C was monitored by cell count as a function of the time after inoculation.

of the Ras2 protein in these cells, then overproducing the protein should alleviate the defect. As shown in Figure 4, cells carrying multiple copies of the *ras2*<sup>318S</sup> allele show the same delay in resumption of growth out of stationary phase as do cells carrying a single copy of the gene. Thus, we conclude that the phenotypic effects of the *ras2*<sup>318S</sup> allele derive from its inability to elicit a transient increase in cAMP rather than a simple reduction in the overall activity of the protein.

We tested a second prediction of our hypothesis that the delay in resumption of growth of stationary *ras2*<sup>318S</sup> cells following transfer to glucose results from the absence of the glucose-induced transient increase in cAMP. If the hypothesis were correct, then we would expect that providing wild-type Ras2 protein only at the time of transition should be sufficient to restore wild-type behavior. Accordingly, we constructed strain Y2453, which was essentially identical to the *ras2*<sup>318S</sup> strain Y2455 except that it also carried a *GAL10p*-*RAS2* construct consisting of wild-type *RAS2* under control of the galactose-inducible *GAL10* promoter. We grew the strain into stationary phase, harvested cells, and incubated one sample in synthetic media containing 2% galactose for 15 min and one sample in synthetic media lacking any carbon source. Cells were then washed and diluted into YEPD medium. As shown in Figure 5, cells that had been exposed to galactose resumed exponential growth with the same kinetics as that exhibited by the *RAS2* strain. In contrast, *ras2*<sup>318S</sup> cells lacking the *GAL10p*-*RAS2* construct but treated similarly showed the same delay in resumption of exponential growth as reported above (Figure 5). Mock treatment of Y2453 cells with synthetic media lacking galactose did not diminish its delay in resumption of growth (data not shown). From these results, we conclude that efficient transition to growth in rich media does not require Ras2 protein to be present in the cell prior to or during stationary phase. For efficient restoration of growth, cells require Ras2 protein only during the transition phase itself.

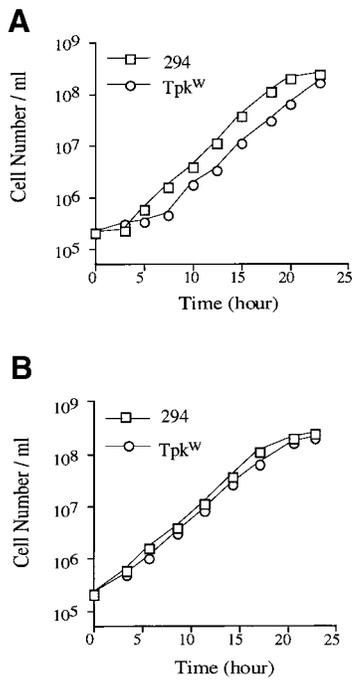


**Fig. 5.** Transient expression of *RAS2* in *ras2*<sup>318S</sup> cells restores rapid resumption of growth out of stationary phase. Cultures of Y2466 (wild-type), Y2455 (*ras2*<sup>318S</sup>) and Y2453 (*ras2*<sup>318S</sup> + *GAL10*-*RAS2*) were grown to saturation (72 h) in YEPD. Cells from 1 ml of each culture were pelleted, resuspended in 1 ml of synthetic media containing 2% galactose, and incubated at 30°C for 15 min. Cells were then washed with YEPD and inoculated into a fresh 10 ml YEPD culture to a cell density of  $10^5$ /ml. Growth of the culture was monitored by cell count as a function of the time after the inoculation.

### *Tpk*<sup>W</sup> strains exhibit a delay in resumption of growth in rich media

Our results suggest that quiescent cells require a transient increase in cAMP levels in order to resume growth rapidly. Essential functions of cAMP in the cell are mediated exclusively by PKA. To determine whether PKA mediates this signaling function of cAMP, we examined the ability of *Tpk*<sup>W</sup> strains to resume growth in glucose from quiescent states. As noted in the Introduction, *Tpk*<sup>W</sup> strains maintain a constitutive, low-level PKA activity. Thus, while cAMP levels increase in response to glucose addition to starved *Tpk*<sup>W</sup> cells, PKA activity does not change under these conditions. We examined a *Tpk*<sup>W</sup> strain (Y1278) and a related wild-type strain (Y294) for their ability to resume growth in glucose media following extended incubation in stationary phase. As evident from the results presented in Figure 6, stationary phase Y294 cells resumed growth within 4 h of dilution into fresh media. In contrast, stationary phase Y1278 cells required almost twice as long to resume growth in fresh media. Dilution of exponentially growing cells of either strain into fresh media did not result in a lag in resumption of growth, nor was the viability of either strain diminished during incubation in stationary phase. Finally, the growth rates of both strains during exponential growth were indistinguishable. Thus, the inability to modulate PKA activity has the same effect on resumption of growth as does the lack of a transient cAMP signal.

We also examined the ability of *Tpk*<sup>W</sup> cells to germinate. As shown in Figure 7, *Tpk*<sup>W</sup> spores germinate ~2 h more slowly than do wild-type cells, as judged by the time of emergence of the first bud. However, the growth resumption of *Tpk*<sup>W</sup> spores may be even more severe than indicated by the time of appearance of the first bud. Figure 7B shows the spore clone size 36 h following dissection of the homozygous *Tpk*<sup>W</sup> diploid. These clones are substantially smaller than those from the related wild-type strain, even though the sizes of the wild-type and *Tpk*<sup>W</sup> colonies obtained by restreaking growing cells are essentially indistinguishable. Thus, the reduced sizes of the spore colonies reflect a significant lag in the resumption of



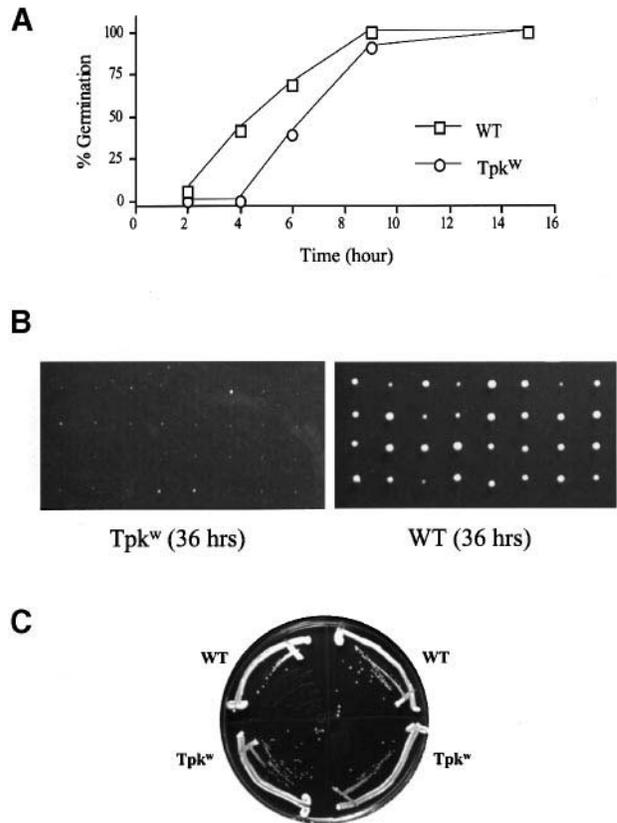
**Fig. 6.** Tpk<sup>W</sup> cells show a delay in resumption of growth out of stationary phase. YEPD cultures (10 ml) were inoculated to 10<sup>5</sup> cells/ml from cultures of strain Y294 (wild-type) and Y1278 (Tpk<sup>W</sup>) grown in YEPD to stationary phase (>2×10<sup>8</sup> cells/ml) (A) or to exponential phase (2×10<sup>7</sup> cells/ml) (B). The cell concentration at various times after the inoculation was determined by counting cells on a hemacytometer.

growth of Tpk<sup>W</sup> cells following germination. These observations, in conjunction with the results from the dilution from stationary phase, suggest that efficient restoration of growth following quiescence requires a transient burst in PKA activity, which is normally achieved by the transient increase in cAMP levels at the time of transition into rich media.

**Phosphorylation and inactivation of fructose-1,6-bisphosphatase are delayed in RAS2<sup>318S</sup> strains**

Cells require FBPAse during growth on non-fermentable carbon sources for gluconeogenesis. However, the reciprocal reaction is catalyzed by phosphofructokinase (PFK) during growth on a fermentable carbon source, and FBPAse expression is repressed under these conditions. In the transition from non-fermentative to fermentative growth, phosphorylation of FBPAse by PKA inactivates it and targets it for Pep4-mediated degradation in the vacuole (Pohligh and Holzer, 1985; Rose *et al.*, 1988; Chiang and Schekman, 1991; Huang and Chiang, 1997). To address whether the lack of a transient increase in cAMP in *ras2<sup>318S</sup>* cells affects the activity of PKA, we examined the phosphorylation and decay of FBPAse in cells following addition of glucose to glycerol-grown cells.

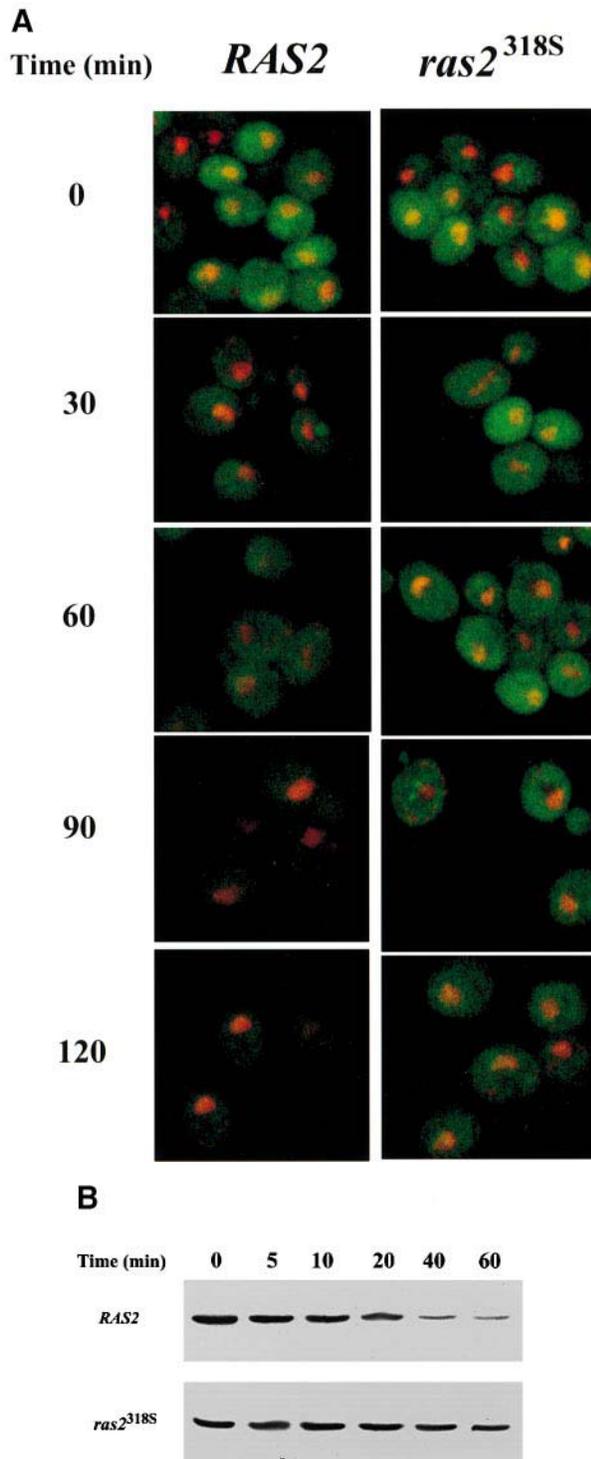
We examined the glucose-induced turnover of FBPAse in *RAS2<sup>+</sup>* and *ras2<sup>318S</sup>* cells by immunofluorescence and immunoprecipitation. Cells were grown in synthetic media containing 3% glycerol to exponential phase and then glucose was added to the media to 2%. As monitored by immunofluorescence microscopy using anti-FBPAse antibodies, decay of FBPAse following addition of glucose was delayed in the *ras2<sup>318S</sup>* strain compared with that in



**Fig. 7.** Tpk<sup>W</sup> cells germinate more slowly than wild-type cells. A homozygous Tpk<sup>W</sup> diploid strain and a wild-type diploid strain were sporulated and dissected on YEPD plates. The germination of spores for each strain was monitored microscopically. (A) The percentage of germinated spores of each allele is plotted as a function of time after dissection. (B) A photograph of the dissection plates after 36 h at 30°C. (C) Two representative Tpk<sup>W</sup> spores and two wild-type spores were struck out for single colonies 48 h after germination and the plate photographed after 36 h growth at 30°C.

the *RAS2* strain (Figure 8A). This result was confirmed by Western blot analysis of extracts of these strains, using anti-FBPAse antibody. As shown in Figure 8B, the steady-state levels of FBPAse were significantly reduced within 40 min following transfer of the *RAS2* strain to glucose media, while the *ras2<sup>318S</sup>* strain retained most of the FBPAse protein during the same time frame. In agreement with previous results (Chiang and Schekman, 1991), this decay in FBPAse protein in the *RAS2* strain was dependent on Pep4p (Figure 9).

We also examined directly the *in vivo* phosphorylation of FBPAse following glucose addition. Since in a *PEP4* background the absolute level of accumulation of label in FBPAse would reflect the competing activities of phosphorylation and protein decay, we examined *in vivo* phosphorylation of FBPAse in a *pep4* background. As is evident from the results in Figure 9, phosphorylation of FBPAse in the *RAS2* strain reached a maximum within 10 min following addition of glucose and remained at that level for the duration of the experiment. Incorporation of label into FBPAse in the *ras2<sup>318S</sup>* strain occurred significantly more slowly and to a substantially reduced level than that observed in the *RAS2* strain. Incorporation of label into FBPAse in *RAS2 PEP4* and *ras2<sup>318S</sup> PEP4* cells showed initial rates identical to those seen in the



**Fig. 8.** *ras2*<sup>318S</sup> cells exhibit a delay in the decay of fructose-1,6-bisphosphatase, following the addition of glucose. Glucose was added to cultures of strains Y2446 (*RAS2*) and Y2455 (*ras2*<sup>318S</sup>) grown for 48 h in YEP media containing 3% glycerol. Aliquots of cells were removed at the indicated times following addition of glucose, and the amount of FBPase in cells was visualized by immunofluorescence (A) and by Western analysis (B) using affinity-purified anti-FBPase antibody. In (A), DAPI staining is shown in red and FBPase indirect immunofluorescence is shown in green.

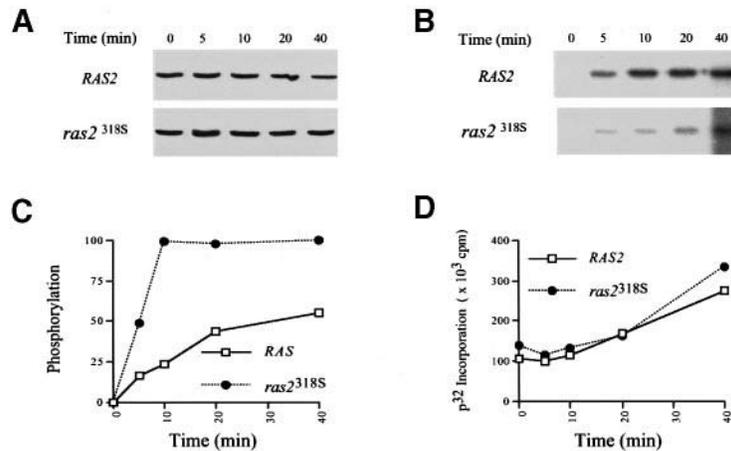
corresponding *pep4* cells, although absolute levels of label in the *RAS2* strain declined at the later time points, coincident with loss of FBPase from the cell. Thus, phosphorylation and decay of FBPase is significantly retarded in the *ras2*<sup>318S</sup> cells relative to that in *RAS2* cells. From these results, we conclude that activation of PKA occurs at a significantly slower rate in a *RAS2*<sup>318S</sup> strain following glucose addition than it does in a *RAS2* strain.

## Discussion

We have shown that strains carrying *ras2*<sup>318S</sup> as their sole *RAS* gene fail to elicit a transient increase in cAMP levels following addition of glucose to starved cells and also show extended delays in resuming growth following transition from a quiescent state to glucose-containing growth media. Otherwise, both growth of such strains and steady-state cAMP levels in such strains are indistinguishable from those of *RAS2*<sup>+</sup> strains. In addition, the viability of *ras2*<sup>318S</sup> strains is identical to that of wild-type strains in stationary phase or following sporulation. This latter observation, plus the fact that the delay in resumption of growth in the mutant can be eliminated by expression of the wild-type *RAS2* gene solely during the transition phase, excludes the possibility that the delay results from some loss of the integrity of mutant cells during incubation in stationary phase. Finally, increased expression of the mutant gene fails to rescue the mutant phenotype. Thus, the transition phenotype is not due simply to reduced basal activity of the mutant Ras2 protein; rather, the mutant phenotype is a consequence of a qualitative rather than a quantitative defect in the Ras protein. From these results, we conclude that the failure of *ras2*<sup>318S</sup> strains to resume growth following transition to rich media is a direct consequence of the failure to produce a transient increase in cAMP at the onset of the transition.

The phenotypic effects of the transient increase in cAMP levels are probably mediated by PKA. First, we showed that the rate of *in vivo* phosphorylation of FBPase, a PKA substrate, is substantially reduced in a *ras2*<sup>318S</sup> strain. Thus, the absence of the transient cAMP increase translates into an attenuated activation of PKA. Furthermore, we find that cells that are unable to modulate PKA activity in response to cAMP (*Tpk*<sup>w</sup> strains) exhibit the same phenotypes as do *ras2*<sup>318S</sup> cells, i.e. *Tpk*<sup>w</sup> strains exhibit a delay in resuming growth when transferred from stationary phase to rich media or following inoculation of spores onto fresh media. Thus, Ras-mediated signaling through PKA is required for efficient resumption of growth following starvation.

Why should the absence of a transient increase in PKA activity cause a delay in resumption of growth in rich media? Growth on a fermentable versus a non-fermentable carbon source requires a substantially different configuration of the glycolytic pathway and citric acid cycle. Cells achieve long-term reconfiguration by altering the level of expression of enzymes within the pathways (DeRisi *et al.*, 1997; Goncalves *et al.*, 1997; Lashkari *et al.*, 1997). However, cells achieve short-term reconfiguration by allosteric regulation of enzymes lying at thermodynamically sensitive points in the pathway. For instance, PFK catalyzes conversion of fructose-6-phosphate to fructose-1,6-bisphosphate during glycolysis, while FBPase catalyzes



**Fig. 9.** *ras2*<sup>318S</sup> cells exhibit a delay in phosphorylation of fructose-1,6-bisphosphatase following the addition of glucose. Cultures of strain Y2460 (*RAS2 pep4*) and Y2461 (*ras2*<sup>318S</sup> *pep4*) were grown in low phosphate synthetic media with 3% glycerol as carbon source and the cultures divided into two at a density of  $5 \times 10^6$  cells/ml as described in Materials and methods.  $^{32}\text{PO}_4$  was added to one of each pair of cultures and  $\text{PO}_4$  added to the other. Cells were harvested and disrupted, and the amount of FBPase in unlabeled cells was determined, using affinity-purified anti-FBPase antibodies, by Western analysis of extracts fractionated by SDS-PAGE (A). FBPase was immunoprecipitated from extracts of the labeled cultures, fractionated by SDS-PAGE and the incorporation of  $^{32}\text{P}$  into the protein was visualized by autoradiography (B) and quantitated using a PhosphorImager (C). Values are arbitrary intensity units from integration over appropriate sections of the PhosphorImager data (Molecular Dynamics). The incorporation of  $^{32}\text{P}$  label in each strain at the indicated times was determined by measuring the radioactivity in 10  $\mu\text{l}$  of diluted cell extracts (D) (see Materials and methods).

the reverse reaction during gluconeogenesis. The activities of these two enzymes are reciprocally regulated by cAMP through PKA (Broach and Deschenes, 1990). PKA stimulates PFK indirectly by stimulating production of fructose-2,6-bisphosphate (F-2,6-BiP), an allosteric activator of PFK and inhibitor of FBPase. In addition, direct phosphorylation of FBPase by PKA inhibits its activity, increases its sensitivity to F-2,6-BiP and targets the protein for degradation by stimulating its vesicle-mediated transport to the vacuole where it is proteolyzed in a Pep4-dependent manner (Pohlig and Holzer, 1985; Rose *et al.*, 1988; Chiang and Schekman, 1991; Huang and Chiang, 1997). Thus, PKA can play a direct role in reconfiguring the primary carbon metabolic pathways in the cell.

As shown in this report, the absence of the cAMP transient increase causes a delay in the elimination of FBPase from the cell. Thus, the reconfiguration of the metabolic machinery that is a prerequisite for transition to growth on glucose does not occur as quickly in the mutant cell as it does in a wild-type cell. We note, however, that loss of FBPase is not the rate-limiting step in resumption of growth, since the time required for resumption of growth is substantially longer than the time required for elimination of FBPase, both in the mutant and wild-type cells. Rather, we assume that a variety of metabolic, structural and transcriptional components, among which FBPase serves as a single illustrative example, undergo remodeling during this transition. In wild-type cells, the burst of PKA activity probably coordinates and accelerates this extensive transcriptional, metabolic and structural reconfiguration to minimize the delay in resuming growth. In the absence of this signal, the reconfiguration occurs more slowly and in a less coordinated fashion, the result of which is a significant delay in initiating growth under the new conditions.

A separate, but not necessarily different way of viewing the role of the transient increase in cAMP is to consider the different configurations of metabolic enzymes and

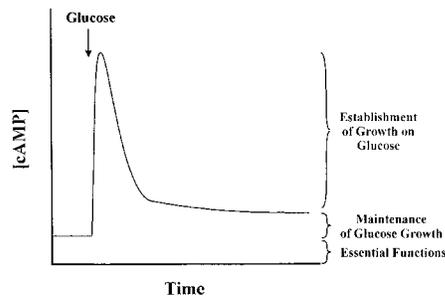
cytoskeletal components during growth on fermentable carbon sources, during growth on non-fermentable carbon sources or during quiescence (in stationary phase or as spores) as distinct quasi-stable states. Once the appropriate expression patterns and configuration of metabolic enzymes and cellular structure appropriate for a particular growth state are established, then that state becomes essentially self-sustaining. In this scenario, the burst of PKA activity would serve as a state switch to convert the cell from one quasi-stable metabolic state to a different state, i.e. from a non-fermentative mode to a fermentative mode. From this perspective, we distinguish a role for signaling through the RAS/cAMP pathway as a means of establishing a particular metabolic state of the cell as distinct from the role of basal levels of cAMP in maintaining those cellular states.

Studies of metabolic oscillation and feedback regulatory circuits provide a precedent for the concept of a state switch in biological systems. For instance, oscillations in metabolic flux through the glycolytic pathway in yeast can exhibit two distinct but stable patterns (Markus and Hess, 1984). Introduction of a burst of substrate can induce interconversion between these quasi-stable metabolic oscillatory states (Moran and Goldbeter, 1984; Goldbeter, 1996). Thus, in this model system, the burst of substrate functions as a state switch. Similarly, distinct but heritable states of *lac* operon expression can be obtained as a result of an autoregulatory loop for inducer transport (Novick and Weiner, 1957). Transiently inactivating the *lac* repressor can convert *lac* expression from one heritable level to another. Thus, the inducer functions as a state switch in this setting. These examples document the feasibility of using a single pulse of a signaling molecular to convert one biological state into another.

In this model of cAMP function, we can distinguish different roles for different levels of the intracellular cAMP. As suggested in Figure 10, the basal, or pre-stimulated, level of cAMP supports a number of essential

functions, including entry into the cell cycle (Matsumoto *et al.*, 1985; Tatchell *et al.*, 1985; Toda *et al.*, 1985) and completion of mitosis (Morishita *et al.*, 1995). Secondly, the level of cAMP in cells grown in glucose versus that in cells grown in glycerol maintains the fermentative metabolic state. This could explain why strains carrying activated *RAS* alleles, which have somewhat higher levels of cAMP than wild-type strains, fail to grow on non-fermentable carbon sources. Finally, the large transient spike in cAMP concentration observed following glucose stimulation would be the signal necessary to promote efficient transition from quiescence or non-fermentative growth to fermentative growth. In the absence of this signal, cells could convert to fermentative growth but would do so only slowly, perhaps in a stochastic fashion.

The distinction between signaling functions and basal maintenance function of the *RAS* pathway in yeast mimics the situation in other eukaryotes. In *Caenorhabditis elegans*, Ras acts in a signaling cascade in several developmental pathways to promote cell-cell communication required for cell fate determination (Kayne and Sternberg, 1995). Aside from this role in development, Ras also performs one or more undefined essential functions, which may or may not involve Ras-mediated signaling *per se*. As we find in yeast, the signaling functions of Ras, in this case required for cell fate determination, can be genetically separated from its essential functions.



**Fig. 10.** The various roles of cAMP in the cell. Our observations in conjunction with prior results suggest that pre-stimulated levels of cAMP support essential cellular functions; the slightly higher basal levels of cAMP present in glucose-grown cells are required for maintenance of fermentative metabolic state and the large transient spike promotes efficient transition from non-fermentative growth to fermentative growth.

Similarly, Ras serves as part of a signaling cascade required for cell-cell communication underlying development of photoreceptor in the *Drosophila* eye, and Ras performs essential functions in the organism as well (Wassarman *et al.*, 1995). These signaling functions in eye development are also genetically separable from the essential functions. Thus, the multifaceted role of Ras in yeast mirrors the complexity of its functions in metazoans. The facility of manipulating the yeast system may allow us to tease apart these various functions of Ras.

## Materials and methods

### Strains and growth assays

All yeast strains used in this study are listed in Table I and derive from an S288C background through strains SP-1 (Kataoka *et al.*, 1984) or Y294. Isogenic strains Y1426 and Y1857 were obtained by transforming strain Y1420 to 5-fluoro-orotic acid resistance using linear DNA fragments spanning the *RAS2* or *ras2*<sup>318S</sup> gene, respectively, and then isolating subclones that had lost the YEp13-TPK1 plasmid. Strain Y335 [designated JR33 in Kataoka *et al.* (1985)] was dissected on galactose media to obtain an  $\alpha$  *ras1::HIS3 ras2::URA3 leu2::GAL10p-RAS2* segregant, which was then mated to strain Y1857 to yield strain Y2452. Strains Y2446, Y2453 and Y2455 are haploid segregants from strain Y2452.

Growth curves for various strains were obtained by diluting cells into YEPD medium (2% yeast extract, 1% bacto-peptone, 2% glucose) to  $1-2 \times 10^5$  cells/ml, either from exponentially growing cultures ( $\sim 2 \times 10^7$  cells/ml) in YEPD or from stationary phase cultures in YEPD in which cells had been incubated for at least 48 h following the diauxic shift. Growth was monitored by counting cells in a hemacytometer. The viability of cells in each inoculum was determined by plating appropriate dilutions onto YEPD plates and scoring the number of colonies after 3 days incubation at 30°C.

### Immunofluorescence microscopy

Yeast cells were grown for 48 h in SC medium containing 3% glycerol. Glucose was then added to the cell culture to a final concentration of 2%. At various times after the addition of glucose, an aliquot of cells was removed and fixed directly in the medium with 3.7% formaldehyde (pH 6.5). After incubation at room temperature for 30 min, cells were transferred to 100 mM potassium phosphate buffer, pH 7.5, containing 3.7% formaldehyde and incubated for another 30 min. Fixed cells were then prepared for immunofluorescence staining as previously described (Pringle *et al.*, 1991). Affinity-purified rabbit anti-FBPase antibody (1:100 dilution) (kindly provided by Dr H.Chiang) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (1:100 dilution) (Jackson Laboratory) were used as the primary and secondary antibodies, respectively. Cells were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) prior to mounting. Immunofluorescence images were obtained with a Bio-Rad MRC600 confocal imaging unit.

**Table I.** Strains used in this study

Strain	Genotype	Source
Y294	$\alpha$ <i>leu2-3,112 trp1-289 ura3-52 his3<math>\Delta</math>1</i>	laboratory stock
Y335	$\alpha/\alpha$ <i>leu2-3,112/leu2-3,112::LEU2-GAL10-RAS2 ura3/ura3 trp1/trp1 his3/his3 RAS1/ras1::HIS3 ras2::URA3/RAS2 can1/can1</i>	Kataoka <i>et al.</i> (1985)
Y1278	$\alpha$ <i>his3 leu2 ura3 trp1 ade8 tpk1<sup>W1</sup> tpk2::HIS3 tpk3::TRP1 bcy1::URA3</i>	laboratory stock
Y1420	$\alpha$ <i>leu2-3,112 ura3-52 his3<math>\Delta</math>1 ade trp1-289 cyh2 ras1::HIS3 ras2::URA3 [YEp13-TPK1]</i>	laboratory stock
Y1426	$\alpha$ <i>leu2-3,112 ura3-52 his3<math>\Delta</math>1 ade trp1-289 cyh2 ras1::HIS3</i>	laboratory stock
Y1810	$\alpha/\alpha$ <i>leu2-3,112/leu2-3,112 his3<math>\Delta</math>1/his3<math>\Delta</math>1 ura3-52/ura3-52 trp1/trp1 ras1::HIS3/ras1::HIS3 ras2<sup>318S</sup>/RAS2-URA3</i>	Bhattacharya <i>et al.</i> (1995)
Y1857	$\alpha$ <i>leu2-3,112 ura3-52 his3<math>\Delta</math>1 ade trp1-289 cyh2 ras1::HIS3 ras2<sup>318S</sup></i>	this study
Y2452	$\alpha/\alpha$ <i>leu2-3,112/leu2-3,112::LEU2-GAL10-RAS2 ura3/ura3 trp1/trp1 his3/his3 RAS1/ras1::HIS3 ras2::URA3/ras2<sup>318S</sup> ade/+ cyh2/+ +/can1</i>	this study
Y2446	$\alpha$ <i>ura3 leu2 trp1 his3 ras1::HIS3 can1</i>	this study
Y2453	$\alpha$ <i>ura3 leu2::LEU2-GAL10-RAS2 trp1 his3 ras1::HIS3 ras2<sup>318S</sup></i>	this study
Y2455	$\alpha$ <i>ura3 leu2 trp1 his3 ras1::HIS3 ras2<sup>318S</sup></i>	this study
Y2460	$\alpha$ <i>ura3 leu2 trp1 his3 ras1::HIS3 pep4::URA3</i>	this study
Y2461	$\alpha$ <i>ura3 leu2 trp1 his3 ras1::HIS3 ras2<sup>318S</sup> pep4::URA3</i>	this study

**Immunoprecipitation and immunoblot analysis**

Yeast cells were grown in synthetic complete (SC) medium (Kaiser *et al.*, 1994) containing 3% glycerol for 48 h, after which the cells were shifted to low phosphate medium (SC medium modified to consist of a final potassium phosphate concentration of 0.2 mM) containing 3% glycerol for an additional 12 h. Cells were then collected and resuspended in 6 ml ( $\sim 10^8$  cells/ml) of low phosphate medium plus 3% glycerol.  $^{32}\text{PO}_4$  (3 mCi) (Amersham) was added to the cell suspension and incubated for 15 min prior to the addition of glucose (final concentration 2%). Following the addition of glucose, cells (1 ml) were removed at the indicated times and fixed by addition of trichloroacetic acid to 5%. Cells were washed twice with ice-cold acetone and dried in a Speed Vac. Cell pellets subsequently were resuspended in 100  $\mu\text{l}$  of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) and lysed by vortexing with glass beads. Cell lysates were heated 95°C for 5 min and then diluted with 1 ml of IP buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1 mM EDTA and 0.5% Tween-20). Insoluble cell debris was removed by centrifugation for 15 min. Supernatants were transferred into a new tube and incubated with affinity-purified anti-FBPase antibody (2  $\mu\text{g}$ /tube) at 4°C for 2 h. The protein-antibody complex was precipitated by adding to the supernatant 100  $\mu\text{l}$  of a 50% slurry of protein A beads (Zymed), followed by incubation at 4°C for 90 min. Beads were then washed once with IP buffer, twice with IP buffer plus 2 M urea and twice with 1%  $\beta$ -mercaptoethanol. Proteins were released from the beads by boiling in 70  $\mu\text{l}$  of 2 $\times$  SDS sample buffer for 5 min. An aliquot (20  $\mu\text{l}$ ) of each sample was fractionated by electrophoresis on an SDS-polyacrylamide gel.  $^{32}\text{P}$ -Labeled proteins were visualized by autoradiography. Incorporated  $^{32}\text{P}$  was quantitated on a PhosphorImager (Molecular Dynamics).

Cell samples for Western blot analysis were prepared exactly as described above except that  $^{32}\text{PO}_4$  was replaced with 100  $\mu\text{l}$  of 10 mM  $\text{KH}_2\text{PO}_4$  buffer. After the cells were lysed with glass beads, 300  $\mu\text{l}$  of 1 $\times$  SDS sample buffer was added to the cell lysate and the samples were boiled for 5 min. An aliquot (20  $\mu\text{l}$ ) of each sample was fractionated by electrophoresis. Western blots were performed with affinity-purified anti-FBPase antibody (1:500 dilution).

**cAMP assay**

Cells were grown in SC medium (100 ml) containing 3% glycerol for 48 h to a density of  $1\text{--}2 \times 10^7$  cells/ml, at which point glucose was added to a final concentration of 2%. At the indicated times, cells were collected from a sample (4 ml) of the cell culture by filtration through a nitrocellulose filter and the filter immediately immersed in 1 ml of *n*-butanol-saturated 1 M formic acid. Cell debris was removed by centrifugation and an aliquot of the supernatant was lyophilized in a Speed Vac. The cAMP content in the lysates was measured using a cAMP scintillation proximity assay kit (Amersham). Immediately prior to addition of glucose, a sample of cells was removed for determination of total protein, and cAMP levels were normalized to total protein content in an equivalent volume of cells.

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