Yeast spore germination: a requirement for Ras protein activity during re-entry into the cell cycle

Paul K.Herman¹ and Jasper Rine²

Department of Molecular and Cell Biology, 401 Barker Hall,
University of California, Berkeley, CA 94720, USA

¹Present address: Department of Molecular Genetics, The Ohio State
University, Columbus, OH 43210, USA

²Corresponding author

***Saccharomyces cerevisiae* spore germination is a process in which quiescent, non-dividing spores become competent for mitotic cell division. Using a novel assay for spore uncoating, we found that spore germination was a multi-step process whose nutritional requirements differed from those for mitotic division. Although both processes were controlled by nutrient availability, efficient spore germination occurred in conditions that did not support cell division. In addition, germination did not require many key regulators of cell cycle progression including the cyclin-dependent kinase, Cdc28p. However, two processes essential for cell growth, protein synthesis and signaling through the Ras protein pathway, were required for spore germination. Moreover, increasing Ras protein activity in spores resulted in an accelerated rate of germination and suggested that activation of the Ras pathway was rate-limiting for entry into the germination program. An early step in germination, commitment, was identified as the point at which spores became irreversibly destined to complete the uncoating process even if the original stimulus for germination was removed. Spore commitment to germination required protein synthesis and Ras protein activity; in contrast, post-commitment events did not require ongoing protein synthesis. Altogether, these data suggested a model for Ras function during transitions between periods of quiescence and cell cycle progression.**

Keywords: commitment/*G₀*/*Saccharomyces cerevisiae*/sporulation/spore germination

Introduction

Saccharomyces cerevisiae cells respond to specific nutrient deprivations by entering into either of two distinct resting states, stationary phase and the spore (Esposito and Klapholz, 1981; Werner-Washburne *et al.*, 1993). Entry into either of these states is reversible as both cell types will resume mitotic cell division when presented with the appropriate nutritional environment. Yeast spores are formed from vegetative diploid cells that are starved for nitrogen while in the presence of a non-fermentable carbon source. During sporulation, diploid cells proceed through a single round of meiotic division and the four resulting haploid progeny are packaged into individual spores

(reviewed in Esposito and Klapholz, 1981; Mitchell, 1994). Spores possess a number of unique structural elaborations that confer an elevated resistance to a variety of environmental stresses, including heat shock and starvation. The most notable spore-specific structure is the specialized, multi-layer coat that provides a physical barrier between the outside environment and the spore within (Briza *et al.*, 1986, 1988).

Saccharomyces cerevisiae spores regain the capacity for cell division during a process known as germination. A priori, it is likely that germination involves the recognition of germinant, the extracellular compound that triggers entry into the germination program; the production of an intracellular signal; the transduction of this signal into new transcription and translation; spore coat removal or uncoating; and re-entry into the cell cycle. During germination, the spore loses those attributes specific to this cell type and begins to take on the features associated with the vegetative cell. Therefore, spore germination will likely involve both processes unique to this transformation and others that are shared by similar transitions between quiescence and cell cycle progression, such as the exit from stationary phase in yeast and the *G₀*-to-*G₁* transition in mammalian cells.

Despite the importance of spore germination to the proliferative cycle of the yeast cell, there have been few recent studies of this process. No systematic genetic analysis of germination has ever been performed and hence the genes and precise environmental cues that control this process remain unknown. Early studies of spore germination indicate that this process is controlled by nutrient availability and identify a series of morphological changes that occur to the spore prior to the onset of the first cell cycle (Hashimoto *et al.*, 1958; Nagashima, 1959; Palleroni, 1961; Rousseau and Halvorson, 1973; Savarese, 1974; Kreger-Van Rij, 1978; Sando *et al.*, 1980). In these pioneering studies, germination assays based largely on morphological criteria were used to examine the requirements for spore germination. These assays revealed that germination does not require oxygen and is most efficient when a readily fermentable carbon source, such as glucose or fructose, is present (Palleroni, 1961; Seigel and Miller, 1971; Savarese, 1974; Tingle *et al.*, 1974). In addition, a role for protein synthesis was suggested by the ability of chemical inhibitors of translation to block the above morphological changes that occur during germination (Rousseau and Halvorson, 1973; Chohi *et al.*, 1977). However, most of these early studies of germination used assays that measured events occurring relatively late in germination. As a consequence, the identity and requirements of the critical early signaling events regulating spore germination are poorly understood.

We have initiated a study of spore germination to further the understanding of those mechanisms regulating the

resumption of growth from this resting state. This report describes a novel, quantitative assay for spore germination and an examination of the genetic and nutritional requirements for this process. We tested whether genes important for regulating the progression through the mitotic cell cycle were also required for spore germination. These experiments revealed a critical role for the Ras protein signaling pathway during the earliest events associated with spore germination. In addition, we identified and defined a commitment point in the germination process. These experiments indicated that the requirements for spore germination differed from those for mitotic division and represented a first step towards understanding yeast spore germination.

Results

Acquisition of Zymolyase sensitivity as an assay for spore germination

Spore germination transforms a non-dividing spore into a vegetative cell competent for mitotic division. During this process, the spore loses those attributes specific to this cell type and takes on the properties of a vegetative cell. In particular, because of a specialized coat structure, yeast spores are more resistant to the cell wall-degrading enzyme preparation, Zymolyase (Ballou *et al.*, 1977; Briza *et al.*, 1988, 1990). The relevant activity in Zymolyase is a β -1,3-glucanase that digests the glucan layer of the cell wall, thus disrupting its structural integrity and resulting in the production of wall-less cells known as spheroplasts (Kitamura *et al.*, 1974; Kitamura, 1982). The cell wall is the primary structure providing osmotic support to the vegetative cell and spheroplasts lyse when placed into a hypotonic environment. In general, spores exhibited a 10^5 -fold higher survival rate than vegetative cells when subjected to a Zymolyase-induced hypotonic lysis regimen (Figure 1A). We therefore evaluated the loss of Zymolyase resistance as a measure of spore germination.

Purified spores were incubated in a rich growth medium containing Zymolyase for different lengths of time and the number of survivors was determined as described in Materials and methods (Figure 1B). After 4 h, >90% of the spores were sensitive to Zymolyase-induced hypotonic lysis (hereafter referred to simply as Zymolyase sensitivity). The loss in viability did not occur if the spores were incubated in water or if Zymolyase was omitted from the reaction. To test whether this assay was measuring an early or late event in the germination program, the acquisition of Zymolyase sensitivity was timed relative to specific morphological changes that occur during germination, including spore swelling, elongation and bud emergence (Palleroni, 1961; Rousseau and Halvorson, 1973). Bud emergence occurred with much slower kinetics than the acquisition of Zymolyase sensitivity; only 50% of the spores possessed buds after 4–5 h of incubation at 30°C. Since bud emergence serves as an indicator of passage through the G_1/S boundary (Pringle and Hartwell, 1981), uncoating occurred several hours before the germinating spore encountered its first G_1/S transition. Moreover, Zymolyase sensitivity appeared prior to spore elongation and either before or concomitant with the initial swelling of the spore. Thus, the spore uncoating assay measured an early event in the germination program, and provided

Fig. 1. Acquisition of Zymolyase sensitivity as a measure of spore germination. (A) Spores exhibit an elevated resistance to Zymolyase-induced hypotonic lysis. Cultures of a sporulating diploid strain [JRY5216; Spo(+)] and a non-sporulating haploid strain [JRY2334; Spo(-)] were incubated for 3 days at 30°C in minimal spore medium. The ability of cells from both cultures to survive hypotonic lysis following treatment with the indicated concentrations of Zymolyase was measured as described in Materials and methods. The numbers for the sporulating culture were corrected for the percentage sporulation in each experiment. The average sporulation efficiency for JRY5216 was 40%. The plots shown represent the average of three independent trials. (B) Germinating spores rapidly acquire sensitivity to Zymolyase-induced hypotonic lysis. Purified wild-type spores, at a concentration of $\sim 1 \times 10^7$ per ml, were incubated in rich medium containing 3 mg/ml Zymolyase-20T at 30°C. At the indicated times, aliquots of this germination reaction were removed, diluted and then plated to solid rich growth medium to determine the number of survivors. The percentage of survivors relative to the original number of spores is plotted.

the basis of many of the following experiments. For this report, spore germination is operationally defined as the point at which spores acquire sensitivity to Zymolyase-induced hypotonic lysis.

Nutritional requirements for spore germination

The nutritional requirements for spore germination were assessed using the Zymolyase sensitivity assay. For these measurements, spores were incubated in the relevant medium at 30°C in the presence or absence of Zymolyase. At the indicated times, dilutions of the germination reactions were plated to determine the number of survivors. The sample lacking Zymolyase tested the viability of the strains in each of the media used. The rich growth medium, YPD, served as the best germinant tested (Table II). A defined synthetic complete medium containing yeast

Table I. Yeast strains used in this study

Strain		Source
JRY5215	<i>MATα his3-11 leu2-3,112 lys2Δ::hisG trp1-1 ura3-1 can1-100</i>	
JRY2334	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100</i>	
JRY3009	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100</i>	
JRY5216	diploid JRY2334/JRY3009	
JRY5217	diploid JRY2334/JRY5215	
STX461-3A	<i>MATα prt1-1 ade1 ade2 gal1 tyr1</i>	YGSC ^a
E17	<i>MATα cdc33-1 ade1 ade2 gal1 his7 lys2 tyr1 ura1</i>	YGSC ^a
JRY0891	<i>MATα cdc35-1 ade1 arg4 his2 trp1 ura1</i>	
JRY0890	<i>MATα cdc25-1 ade1 ade2 gal1 his7 lys2 tyr1 ura1</i>	
JT249	<i>MATα cdc28-4 met tyr ura1</i>	J.Thorner
SR672-1	<i>MATα cdc37-1 cyh2 gal2 ura1</i>	A.Mitchell
JRY5220	<i>JRY2334 ras2-23 ras1Δ::HIS3</i>	
JRY5221	<i>JRY2334 ras2-23 ras1Δ::HIS3</i>	
JRY5222	<i>JRY5215 ras2-23 ras1Δ::HIS3</i>	
JRY5223	diploid JRY5220/JRY5222	
JRY5224	diploid JRY5221/JRY5222	
JRY5225	<i>JRY5217 gal1Δ::HIS3/gal1Δ::HIS3</i>	
HM56-1A	<i>MATα ras2-47 ras1::HIS3 ade8 his3 leu2 trp1 ura3</i>	H.Mitsuzawa
HM55-3A	<i>MATα ras2-23 ras1::HIS3 his3 leu2 met4 trp1 ura3</i>	H.Mitsuzawa

^aYeast Genetics Stock Center, University of California, Berkeley.

nitrogen base and 2% glucose was also an excellent germinant. The rate of germination was slightly slower in this synthetic medium than in the rich medium: 50% of the spores acquired Zymolyase sensitivity after 3 h in the complete minimal medium as opposed to ~2.2 h for the rich medium.

To determine which components of the media were required for germination, different constituents of the defined synthetic medium were omitted systematically from the germination reactions. Neither the nitrogen source, amino acid nor nitrogenous base (uracil and adenine) supplements were essential for germination as spore uncoating occurred with near wild-type kinetics in media lacking these ingredients. In each case, >95% of the spores were Zymolyase-sensitive after 12 h in the medium (Table II). However, if glucose was omitted from the medium, <5% of the spores germinated, indicating that glucose was necessary for germination. Moreover, a solution of 2% glucose was an efficient germinant allowing for ~95% germination after 12 h. Therefore, the presence of glucose alone was sufficient to trigger spore germination. Although other carbon sources could substitute for glucose, efficient germination was seen only with readily fermentable sugars, such as glucose and fructose (Table II; see also Palleroni, 1961; Seigel and Miller, 1971; Donnini *et al.*, 1986; Xu and West, 1992). Therefore, the primary requirement for efficient germination was the presence of a fermentable carbon source.

Two experiments indicated that the metabolism of the carbon source, and not simply its presence, was required for germination. First, two different non-metabolizable analogs of glucose, 6-deoxyglucose and 2-deoxyglucose, were unable to trigger spore germination. Less than 5% of the spores in yeast minimal medium (SC) with either analog substituting for glucose became sensitive to Zymolyase after 16 h at 30°C (Table III). The presence of these glucose analogs was not inhibitory to germination, as spores germinated with normal kinetics in medium containing both glucose and either non-metabolizable glucose analog. For the second experiment, a yeast *gal1* mutant

Table II. Nutritional requirements for spore germination

Medium	% Germination
YPD	>95
Water	<5
SC	>95
SC no amino acids	>95
SC no bases (uracil/adenine)	>95
SC no nitrogen source	>95
SC no glucose	<5
YNB alone	<5
2% glucose	95
YP-fructose	91
YP-galactose	51

Table III. Germination required a metabolizable carbon source

Genotype	Carbon source	% Germination
Wild-type	glucose	>99
Wild-type	galactose	91
Wild-type	none	<5
<i>gal1</i>	glucose	>99
<i>gal1</i>	galactose	<5
<i>gal1</i>	none	<5
Wild-type	6-deoxyglucose	<5
Wild-type	2-deoxyglucose	<5
Wild-type	glucose + 6-deoxyglucose	>99

was used to evaluate spore germination in media with either glucose or galactose as the sole carbon source. The *GAL1* gene encodes a galactokinase which is required for the first step in the conversion of galactose into glucose prior to the entry into glycolysis (Johnston and Carlson, 1992). *gal1* mutant spores were unable to germinate on galactose-containing medium; <5% of the spores became sensitive to Zymolyase after 16 h of incubation (Table III). In contrast, >90% of wild-type spores had germinated in galactose medium after this time. The *gal1* spores were not generally defective for germination as they exhibited wild-type germination kinetics in medium containing

Fig. 2. Protein synthesis requirements during spore germination. (A) Protein synthesis was required for the acquisition of Zymolyase sensitivity by germinating spores. Left columns: wild-type spores were incubated in rich medium containing 3 mg/ml Zymolyase-20T for 6 h at 30°C in the absence (column 1, WT) or presence (column 2, CHX) of 40 µg/ml of cycloheximide. Center columns: either wild-type, *prt1-1* or *cdc33-1* spores were incubated for 6 h at 37°C in rich medium containing 3 mg/ml Zymolyase-20T. Right columns: same strains incubated for 14 h at 23°C. ' % Germination' indicates the percentage of spores that became sensitive to the Zymolyase treatment. Values shown represent the average of three independent trials. In addition, three different diploid strains were examined for each genotype shown. (B) Protein synthesis was required for early steps of the germination program. Wild-type spores were incubated in rich medium containing 3 mg/ml Zymolyase-20T at 30°C. At the indicated time, cycloheximide was added to a final concentration of 40 µg/ml and the 30°C incubation was continued. After 8 h of total incubation, dilutions of the reactions were plated on solid rich medium. ' % Germination' represents the percentage of the original spores that became sensitive to Zymolyase-induced lysis after the total 8 h incubation.

glucose as the carbon source. Therefore, a metabolizable carbon source was both necessary and sufficient to trigger *S.cerevisiae* spore germination.

Protein synthesis was required only for early events in the germination program

A requirement for protein synthesis during spore germination was tested by using both genetic and pharmaceutical blocks to translation. The addition of the protein synthesis inhibitor cycloheximide to a spore germination reaction resulted in the complete inhibition of germination (Figure 2A). This requirement for protein synthesis was confirmed by analyzing the effects of two temperature-sensitive mutations, *prt1-1* and *cdc33-1*, that block protein transla-

tion. The yeast *PRT1* gene encodes a component of the translation initiation factor eIF-3 (Naranda *et al.*, 1994; Danaie *et al.*, 1995) and *CDC33* encodes the cap-binding protein, eIF-4E (Brenner *et al.*, 1988). Both of these proteins are essential for translation. Neither *prt1-1* nor *cdc33-1* spores were able to germinate at the non-permissive temperature; in both cases, <5% of the spores became sensitive to Zymolyase. However, both *prt1-1* and *cdc33-1* mutant spores were able to germinate at the permissive temperature of 23°C (Figure 2A). These data established that protein synthesis was required for yeast spore germination.

Broadly, protein synthesis might have one of several roles during spore germination. It might be required only to initiate this transformation, or alternatively, ongoing translation might be necessary for the completion of the germination process. To test whether protein synthesis was required for all, or only a portion of the germination program, cycloheximide was added to a germination reaction at different times after the addition of growth medium to spores (Figure 2B). For these reactions, Zymolyase sensitivity of the spores was assessed at a common point, 8 h following the addition of growth medium. The effects of cycloheximide varied depending upon when this inhibitor was added to the germination reaction. If the drug was added during the first 30 min of incubation, essentially no spores were Zymolyase-sensitive after the 8 h incubation (Figure 2B). However, if the cycloheximide addition took place at 60 min, ~50% of the spores were Zymolyase-sensitive 7 h later. In contrast, if cycloheximide was added 3 h or later after the addition of medium, there was only a modest effect upon the final level of germination. The inhibitory effects of cycloheximide upon translation at all points of addition were confirmed by removing aliquots of the culture after drug addition and assessing protein synthesis rates by measuring the incorporation of [³⁵S]methionine and [³⁵S]cysteine into protein; in all cases, protein synthesis was decreased to <10% of the uninhibited rate. Taken together, these data indicated that ongoing protein synthesis was required early during the germination process but was not essential for later events.

Spore commitment to germination

Many biological processes consist of multiple distinct steps, any of whose execution requires the completion of a previous step in the pathway. In some cases, the completion of an early event predisposes the cell to complete later steps, even in the absence of the stimulus that originally started the process. To test for the presence of such a commitment step in the germination pathway, spores were incubated for a variable length of time in rich medium and then transferred to water that contained Zymolyase, as described in Materials and methods. The difference between the original number of spores and the final number of survivors represents the number that became committed to germination (Figure 3). Approximately 60% of the spores became committed to germination after 1 h in the rich growth medium. At this time, <5% of the spores had germinated. In general, spore commitment preceded the acquisition of Zymolyase sensitivity by ~1.5 h. Therefore, a brief exposure to germinant

Fig. 3. Spores became committed to the germination pathway after a brief exposure to germinant. The percentage of spores committed to the completion of the uncoating reaction (% Commitment) was compared with the percentage already sensitive to Zymolyase-induced lysis (% Germination) at the indicated times. Wild-type spores were incubated at 30°C in rich medium containing 3 mg/ml Zymolyase-20T. At the indicated times, two aliquots of the cultures were removed and processed as follows. To determine the percent germination, one aliquot was plated on solid media and the number of survivors of this Zymolyase treatment was determined. The fraction of spores that had germinated was equal to the fraction of spores killed during the incubation with Zymolyase in rich medium. To determine the percent of the spores committed to the germination program, the second aliquot was pelleted and resuspended in water containing 3 mg/ml Zymolyase-20T. This incubation in water was continued such that the total incubation in rich medium and water was 9 h. After 9 h, the final cultures were plated on solid rich medium and the number of survivors of this commitment regimen was determined. The percentage of spores committed to germination after the indicated time in rich medium was equivalent to the percentage found to be sensitive to Zymolyase after the total 9 h incubation period. Note that spores became committed to the germination pathway ~1.5 h before they germinated.

triggered a series of events that allowed spore uncoating to proceed, even in the absence of the original stimulant.

The commitment period for spore germination was roughly coincident with the interval of essential protein synthesis defined by the cycloheximide experiments described above. This observation suggested that protein synthesis was required for the commitment process, but not for post-commitment events. To test this hypothesis, the effects of adding cycloheximide to either the pre- or post-commitment phases of the uncoating reaction were analyzed. To assess the protein synthetic requirements for commitment, spores were exposed to rich medium containing cycloheximide for 1, 2 or 3 h at 30°C. The spores then were transferred to water that contained Zymolyase, and the incubation was continued such that the total reaction time was 9 h. With protein synthesis inhibited, <5% of the spores committed to the germination program after 3 h of exposure to rich medium (Figure 4). Therefore, protein synthesis was required for spore commitment.

To evaluate the translational requirements for post-commitment events, we performed the reciprocal experiment and included cycloheximide in the incubation with water and Zymolyase after exposure to rich medium for 1 h. The presence of cycloheximide during the post-commitment period had no effect on the fraction of spores

Fig. 4. Protein synthesis was required for spore commitment. Spores were incubated in rich medium for 1, 2 or 3 h at 30°C in the presence or absence of 2 µg/ml cycloheximide (CHX). Following the rich medium incubation, the cultures were treated as described in Materials and methods to determine the percentage of spores that had committed to the germination process.

that acquired Zymolyase sensitivity. When cycloheximide was present in the post-commitment reaction, 59% of the spores were found to be Zymolyase-sensitive, as compared with 58% in the control reaction. Similar results were obtained with the *prt1-1* mutant; *PRT1* gene function was required only during the pre-commitment period and not for later events (data not shown). Taken together, these results indicated that translation was required only for early events in the germination program, including spore commitment. The later post-commitment steps of the germination program, including spore uncoating, proceeded in the absence of ongoing protein synthesis.

Many regulators of the mitotic cycle were dispensable for spore germination

Spore germination likely involves some events that are unique to this process and others that are shared with a continuing mitotic cycle. Although a large number of genes are known to be essential for yeast cell cycle progression (Pringle and Hartwell, 1981; Norbury and Nurse, 1992; Murray and Hunt, 1993), there has not been a systematic analysis performed to test whether any of these genes also play a role in regulating spore germination. Therefore, we tested whether known regulators of the mitotic cell cycle influenced either the absolute levels or the rate of spore germination. These experiments used conditional mutants that were defective in passage through various phases of the cell cycle.

None of the tested genes essential for progress through either the S, G₂ or M phases of the mitotic cycle was required for spore germination; the representative *cdc* mutants defective in these genes exhibited no significant quantitative or kinetic defects in the acquisition of Zymolyase sensitivity (data not shown). These assays included an analysis of two genes whose products act at the G₁/S boundary (*CDC4* and *CDC34*), one that acts early in S phase (*CDC7*) and others (*CDC24* and *CDC43*) that are required for bud emergence (see Pringle and Hartwell, 1981; Murray and Hunt, 1993, and references therein). The other genes tested included *CDC2*, *CDC8*, *CDC14*, *CDC15* and *CDC46*. Our results were consistent with

Fig. 5. The Ras protein signaling pathway was required for spore germination. Wild-type and the indicated mutant spores were incubated in rich medium containing 3 mg/ml Zymolyase-20T for 6 h at 37°C. The fraction of spores that exhibited sensitivity to Zymolyase-induced lysis after this incubation represented those spores that had undergone the germination process. The results shown represent the average of three experiments with at least two different diploid strains for each mutant analyzed.

previous analyses of these mutants that had failed to uncover a role for the respective wild-type genes in spore germination using indirect measures of germination.

The role of genes that regulate G_1 functions was analyzed by measuring germination in yeast mutants defective in the control of 'Start'. Start identifies a major point of cell cycle regulation that occurs in late G_1 ; once a cell passes this point it is committed to completing the current cell cycle (Pringle and Hartwell, 1981). A key regulator of Start is the cyclin-dependent kinase encoded by the *CDC28* gene (Pringle and Hartwell, 1981; Reed, 1992). The role of this kinase in germination was tested by assessing the acquisition of Zymolyase sensitivity by *cdc28* mutant spores at the non-permissive temperature of 37°C. *cdc28* mutants exhibited germination kinetics identical to that of wild-type spores (Figure 5). After 6 h at 37°C, 95% of the *cdc28* spores had germinated. In the 37°C control culture lacking Zymolyase, we observed that >95% of the *cdc28* cells were arrested as unbudded cells, indicating that the *cdc* block had been imposed. This latter result also indicated that, upon germination, spores were in G_1 or they would not have arrested at the characteristic *cdc28* block. *CDC37* is another yeast gene required for the execution of Start and *cdc37* and *cdc28* mutants exhibit a similar terminal phenotype (Pringle and Hartwell, 1981). *CDC37* gene function also was dispensable for spore germination as >95% of the *cdc37* spores germinated after 6 h at the non-permissive temperature (Figure 5). Both *cdc28* and *cdc37* mutant spores underwent the characteristic set of morphological changes associated with germinating spores, such as swelling and elongation. Therefore, several steps of the germination program, including commitment, uncoating and spore shape changes, occurred in the absence of *CDC28* and *CDC37* gene activity. These results indicated that two primary regulators of exit from the G_1 phase of the yeast cell

cycle, Cdc28p and Cdc37p, were not required for spore germination.

We also examined the role of two genes, *UBC1* and *CMK1*, reported to play a role in spore germination or mitotic growth following germination. Previous studies suggest that *ubc1* mutants exhibit a slow growth phenotype for several rounds of mitotic division immediately following spore germination (Seufert *et al.*, 1990). A separate study suggests a role for the *CMK1* gene product in spore outgrowth as the colonies formed from *cmk1* spores are smaller than those formed from wild-type spores (Pausch *et al.*, 1991). Spore germination in *ubc1* and *cmk1* spores was analyzed with the Zymolyase sensitivity assay and by determining the kinetics with which the first bud appeared from the spore. Both *ubc1* and *cmk1* spores germinated with wild-type kinetics (data not shown). Therefore, the growth defect in these mutants is likely associated with the first few rounds of mitotic division immediately following germination and not with germination *per se*.

The Ras signaling pathway was required for spore germination and commitment

The components of the Ras protein signaling pathway constitute the second primary regulator of passage through Start and the G_1 phase of the cell cycle (Pringle and Hartwell, 1981; Broach, 1991). In *S.cerevisiae*, the Ras proteins, encoded by the *RAS1* and *RAS2* genes, control the level of cellular cAMP and hence the activity of the cAMP-dependent protein kinase (Toda *et al.*, 1985; Gibbs and Marshall, 1989; Broach, 1991). We found that the Ras signaling pathway was required for spore germination. Temperature-sensitive mutant spores defective in the genes encoding components of this signaling pathway, including *CDC25* (the guanine nucleotide exchange factor for Ras proteins; Broek *et al.*, 1987), *RAS2* and *CDC35/CYR1* (adenylyl cyclase; Matsumoto *et al.*, 1982), were all unable to germinate at the non-permissive temperature (Figure 5). The *cdc25-1*, *ras2-23* (*ras1Δ*) and *cdc35-1* mutant spores all exhibited <10% germination after a 6 h incubation at 37°C. Therefore, some regulators of the G_1 phase of mitotic division, including the Ras pathway, were required for spore germination, whereas the Cdc28p cyclin-dependent kinase was not.

Spore germination was relatively slow in media containing galactose instead of glucose as the sole carbon source. Because glucose is known to stimulate the Ras pathway (Thevelein, 1994) and we have shown that the Ras pathway is required for germination, this delay in germination might reflect a poor activation of the Ras pathway by galactose. To test this hypothesis, we asked whether overproducing Ras2p in spores would overcome this delay. A yeast plasmid containing a hybrid gene with the *GAL1* promoter upstream of the coding region of the *RAS2* gene was introduced into a wild-type diploid (JRY5216). The yeast *GAL1* promoter is regulated by galactose and the level of Ras2p was increased by more than 10-fold when galactose replaced glucose in medium. After 8 h in a minimal galactose-containing medium, only 20–30% of the wild-type spores with normal Ras2p levels had germinated (Figure 6). By contrast, spores that contained the *GAL1-RAS2* hybrid gene exhibited ~85% germination at this time. Both of the spore populations

Fig. 6. Overproduction of the Ras2 protein accelerated spore germination. Wild-type spores containing either pGAL1-RAS2 or a control vector, were incubated in a glucose- or galactose-containing minimal medium for 8 h at 30°C. Both media contained Zymolyase-20T at a concentration of 3 mg/ml. Following this incubation, the cultures were plated to glucose-containing minimal medium (lacking uracil to maintain a selection for the plasmids) and the percentage of spores that became sensitive to Zymolyase was calculated as previously. The germination of *gal1* spores containing either pGAL-RAS2 or the control vector also was analyzed after an 8 h incubation at 30°C in the galactose-containing medium.

displayed >95% germination after 8 h at 30°C in a glucose-containing medium. Therefore, the presence of an elevated level of Ras2p increased the rate of germination in galactose-grown cells several-fold, indicating that activation of the Ras pathway may be rate-limiting for spore germination. The levels of overall protein synthesis in spores expressing wild-type and elevated levels of Ras2p were compared by determining the rate at which [³⁵S]methionine was incorporated into acid-precipitable material. Both spore populations exhibited similar rates of protein synthetic activity (data not shown). It is important to note that the overproduction of Ras2p did not bypass the requirement for a metabolizable carbon source as *gal1* mutant spores carrying the GAL1-RAS2 chimera were unable to germinate on galactose-containing medium (Figure 6).

We further tested the role of the Ras signaling pathway in spore germination by examining the commitment of *ras2* spores at the non-permissive temperature for these mutants. Mutant and wild-type spores were purified and the fraction of spores capable of undergoing commitment was determined at 37°C. Approximately 90% of the wild-type spores underwent commitment after 3 h in rich medium at 37°C. However, the *ras2* spores were defective for the commitment step of germination; <10% of the mutant spores became committed after this 3 h incubation period at 37°C. Therefore, the yeast RAS pathway was required for a very early event in the spore germination process.

Discussion

Saccharomyces cerevisiae spore germination is a multi-step process in which a non-dividing, quiescent spore becomes competent for mitotic cell division. In this report, a novel, quantitative assay for spore germination was developed and used to characterize the nutritional and genetic requirements for this process. This assay exploited

Fig. 7. A temporal map of landmark events occurring during the spore germination program. This flow-diagram depicts a schematic representation of the sequence of events currently known to occur during spore germination. See the text for further discussion.

structural differences that exist between the yeast spore and mitotic cell. In particular, the yeast spore is encapsulated within a specialized coat structure that must be removed, or disrupted, prior to the re-initiation of cell division (Hashimoto, 1958; Kreger-Van Rij, 1978; Sando *et al.*, 1980; Briza *et al.*, 1986, 1988). This process of spore uncoating was operationally defined as the point at which the spore became sensitive to digestion by the cell wall-degrading enzyme preparation, Zymolyase. The acquisition of Zymolyase sensitivity by the germinating spore provided a robust and direct measure of spore germination. Moreover, spore uncoating was an early event in the germination program (Figure 7), and therefore this assay was able to distinguish between the requirements for germination *per se*, and those for the ensuing round of mitotic division. This assay allowed us to determine the critical parameters necessary for re-entry of quiescent spores back into the cell cycle.

Our analysis of different germination media indicated that the only component essential for spore germination was the carbon source. The role of this medium component was further clarified by establishing that the metabolism, and not simply the presence, of the carbon source was both necessary and sufficient for the initiation of germination. Altogether, these results suggested that the trigger for germination was an intracellular by-product of carbon source metabolism. Since yeast germination can be triggered by both fermentable and non-fermentable carbon sources (Donnini *et al.*, 1986; Xu and West, 1992; but also see Palleroni, 1961; Seigel and Miller, 1971), and can occur under aerobic or anaerobic conditions (Palleroni, 1961; Tingle *et al.*, 1974), the yeast spore is likely responding to changes in cellular energy levels and not to intermediates in the fermentation pathway. This hypothesis is consistent with the rate and absolute efficiency of germination being much lower in media containing only a non-fermentable carbon source. The energy charge of yeast is much higher during growth by fermentation than

during growth by oxidative phosphorylation (Fraenkel, 1982).

Although both spore germination and mitotic cell division are regulated by nutrient availability, significant differences in the nutritional requirements for the two processes were observed. Specifically, spore germination proceeded efficiently in media that did not support mitotic cell division. For example, yeast spores germinated efficiently in solutions of glucose that contained no additional medium components (see also Palleroni, 1961; Savarese, 1974); in contrast, mitotic division was not supported by this limited medium. The more stringent requirements for mitotic division were further demonstrated by the fate of spores following germination in pure glucose. After the completion of uncoating, spores arrested early in the mitotic cycle, prior to the formation of the first bud and the point of Cdc28p action. Therefore, media that could not sustain even a single round of cell division could trigger efficient spore germination.

The ability of yeast spores to become competent for mitotic division in media that cannot support further cell division is not unique to this quiescent cell type. Yeast stationary phase cells also respond to glucose solutions by exiting quiescence and re-entering the mitotic cycle (Granot and Snyder, 1991, 1993). Following the exit from these quiescent states, the yeast cell is in the G₁ phase of the cell cycle and has a decreased ability to survive without division relative to the quiescent cell (Granot and Snyder, 1991, 1993). This response by yeast resting cells to a growth medium that contains glucose, but is deficient in other essential nutrients, would seem to be maladaptive due to the dire consequences for the ultimate survival of the cell. The response of yeast spores and stationary cells to such growth media may occur because they lack a nutritional checkpoint responsible for evaluating the level of other nutrients besides the carbon source. Such a checkpoint would presumably be functional in mitotic cells as similar growth media can induce mitotically dividing yeast cells to cease division and exit from the cell cycle. Without this checkpoint, spores and stationary phase cells would respond simply to the presence or absence of a metabolizable carbon source in the growth medium. The fact that yeast spores do not possess a mechanism to prevent germination under these medium conditions that cannot sustain mitotic division, suggests that these growth conditions are not frequently encountered in the wild.

Although a metabolizable carbon source was required to trigger germination, the completion of spore uncoating did not require the presence of this carbon source for the entire germination period. Instead, a brief exposure to this germinant resulted in the completion of some early steps in the pathway that committed the spore to the subsequent completion of later steps, including spore uncoating (Figure 7). These later steps of germination could be completed even in the absence of the original germinant. Upon completion of these early steps of the pathway, the spores were committed to the germination program. Formally, the commitment point of germination was defined as the time at which spores were irrevocably destined to complete the uncoating process, even in the absence of the original germinant that triggered germination. An analogous commitment step is present in many

multi-step biological pathways, including the mitotic cell cycle, yeast sporulation and DNA recombination (Esposito and Klapholz, 1981; Pardee, 1989). Such a commitment step often serves as a focal point for cellular control of the entire multi-step process; by regulating the completion of one event, the cell can effectively modulate the execution of multiple steps in a pathway.

An analysis of mutants defective in specific stages of cell cycle progression indicated that the Ras signal transduction pathway was a key regulator of spore germination. If the Ras pathway was rendered non-functional, yeast spores remained quiescent even when presented with the appropriate nutritional cues necessary for germination. In contrast, the other key regulators of the mitotic cell cycle tested, including the Cdc28p cyclin-dependent kinase, were dispensable for germination. Moreover, increasing the levels of Ras protein, and hence Ras signaling activity, accelerated the rate at which spores initiated germination. Together, these data suggested that activation of the Ras pathway was the rate-limiting step of spore germination. Consistent with this hypothesis, Ras activity was required for a very early step in germination; mutants defective in Ras signaling were unable to complete commitment, the first measurable event of the germination program. Further experimentation is necessary to determine whether there is a single threshold level of Ras activity required to trigger germination or if increasing Ras levels result in a corresponding increase in the rate of spore germination.

A role for protein synthesis at some stage of germination was suggested by previous studies of this process (Rousseau and Halvorson, 1973; Choih *et al.*, 1977). Our experiments with chemical inhibitors of protein synthesis and mutants defective in the initiation of translation confirmed and extended these previous observations. We found that ongoing protein synthesis was not required for all stages of the germination program. Instead, protein synthesis was necessary only for those events leading up to and including the commitment step of the pathway. Post-commitment steps, including spore uncoating, occurred in the absence of ongoing protein synthesis. Therefore, protein synthesis and Ras protein activity are required for similar steps of the germination pathway. Since mutants defective in Ras pathway activity exhibit decreased rates of protein synthesis (see Broach, 1991), the requirement for Ras protein activity during germination might simply reflect the demand for ongoing protein translation during this process. However, our data indicated that Ras protein signaling during germination regulated more than just the protein synthetic activity of the spore, as increased Ras2p levels resulted in an elevated rate of germination without significantly affecting the rate of overall protein synthesis. These data therefore suggested that Ras signaling was controlling other key events necessary for the initiation of spore germination.

Passage through the G₁ phase of the eukaryotic cell cycle is controlled primarily by the Ras pathway and the cyclin-mediated activation of cdk protein kinases, such as Cdc28p. However, the precise order of action of these two pathways has not been resolved (Matsumoto *et al.*, 1983; Hubler *et al.*, 1993). Our experiments with *cdc28* and Ras pathway mutants indicated that the yeast Ras proteins acted before Cdc28p during spore germination.

Although Ras pathway mutants could not germinate, *cdc28* mutants germinated with wild-type kinetics and then arrested at Start, in the G₁ phase of the cell cycle. The order of function determined by our experiments conflicts with results from a study with mitotically dividing cells, suggesting that Ras and Cdc28p act at the same point in the mitotic cycle (Hubler *et al.*, 1993). These differences in the order of function can be explained by a model proposing that the Ras proteins act at Start to control the entry into or out of a quiescent state. At Start, cells would assess the activity of both the Ras and Cdc28p pathways and determine whether a new round of mitotic division should be initiated. If Ras activity was low, the cell would exit from the cell cycle and enter into the appropriate quiescent state. Alternatively, if Cdc28p activity was low, the cell would arrest division, but not growth, and remain in G₁. According to this model, during mitotic growth, the Ras proteins and Cdc28p would execute their respective functions at the same time. During spore germination, however, the Ras proteins would act first to initiate the re-entry into the cell cycle. The germinated spore would then be at Start in G₁ and dependent upon the *CDC28*-encoded protein kinase for further progression into the cell cycle.

This study examined the genetic requirements for spore germination by testing whether particular genes essential for cell cycle transit had any role in the regulation of germination. These analyses identified the Ras protein signaling pathway as a key regulator of yeast spore germination and excluded an involvement of *CDC28* and *CDC37*, two key regulators of exit from G₁, and a collection of other *CDC* genes. The next step in this analysis will involve testing whether there are genes required for spore germination but not for mitotic division. The isolation of such germination-specific genes would clearly establish the spore as a state distinct from G₁ and the rest of the mitotic cycle and would provide insight into the genes regulating the resumption of growth from a period of quiescence.

Materials and methods

Yeast media

Yeast rich medium (YPD), minimal medium (SC) and 5-FOA medium were as described previously (Kaiser *et al.*, 1994). The pre-spore growth medium, PSP, was 50 mM potassium phosphate, pH 5.0, 0.1% yeast extract, 0.67% yeast nitrogen base (DIFCO), and 1% potassium acetate. The minimal spore medium was 1% potassium acetate and 2.5 µg/ml zinc acetate. PSP, SC and minimal spore media were supplemented with the appropriate amino acids and nitrogenous bases at the concentrations described previously (Kaiser *et al.*, 1994).

Yeast methods

Standard yeast genetic methods were as described previously (Kaiser *et al.*, 1994). For sporulation, yeast strains were grown in PSP medium to mid-log phase (OD₆₀₀ ~0.7) and then transferred to minimal spore medium for 3–5 days. For wild-type strains, the incubations were performed at 30°C; for *ts* strains, all incubations were performed at 23°C. The percentage of cells sporulated in each culture was determined by microscopic examination. Yeast transformations were performed as described previously (Ito *et al.*, 1983; Schiestl and Gietz, 1989).

Plasmid constructions

Standard recombinant DNA methods were as described previously (Sambrook *et al.*, 1989). The plasmid pPHY329 was used to construct a null allele of the yeast *LYS2* gene. The *LYS2* gene was cloned into pUC8 vector as a 4.8 kb *EcoRI*–*HindIII* fragment from YIp600 (Barnes

and Thorner, 1986). The 2.9 kb *BglIII*–*BamHI* fragment of the *LYS2* gene then was replaced with the 3.8 kb *BglIII*–*BamHI* fragment of pNKY51 (Alani *et al.*, 1987) that contains the *hisG*–*URA3*–*hisG* gene disruption cassette to form pPHY329. The p*GAL1*–*RAS2* plasmid (a gift from Jennifer Whistler) contained a hybrid gene where the *GAL1* promoter is fused to the *RAS2* coding sequence and 3' transcriptional terminator.

Strain constructions

The *S.cerevisiae* strains used in this study are listed in Table I. Unless otherwise designated, the strains were either constructed during the course of this study or obtained from the Rine laboratory collection. The strain JRY5215 was constructed from JRY3009, a *MATα* derivative of JRY2334 (an isolate of W303-1A). The *ade2-1* allele of JRY3009 was gene converted to *ADE2* by transforming with the 2.2 kb *BglIII* fragment of pASZ11 carrying the *ADE2* gene (Stotz and Linden, 1990) and selecting for adenine prototrophy. The *LYS2* gene of the resulting strain, JRY5258, was then disrupted in two steps, as follows. In the first step, JRY5258 (*LYS2 ura3-1*) was transformed with the 5.8 kb *HindIII*–*BamHI* fragment, containing the *lys2Δ::hisG-URA3-hisG* allele, of plasmid pPHY329 and uracil prototrophs were selected. These Ura⁺ strains then were plated onto 5-FOA medium, and revertants that had lost the *URA3* gene were selected. The presence of the *lys2Δ::hisG* allele was confirmed by Southern blotting and the lysine auxotrophy associated with the resulting strain. The final *MATαADE2 lys2Δ::hisG* strain was JRY5215 (see Table I for full genotype).

Diploids homozygous for mutations to be tested in a germination assay were constructed by the following procedure. A haploid strain containing the mutation of interest was crossed with either the *MATa* (JRY2334) or *MATα* (JRY5215) wild-type strain. The heterozygous diploids were sporulated and two haploid progeny of opposite mating type containing the mutation of interest were then mated to form homozygous diploids. In general, three homozygous mutant diploids were constructed for each mutation analyzed. To control for strain background differences, two homozygous wild-type diploids also were constructed for each examined mutation from segregants of the same initial cross that lacked the relevant mutation. The haploid starting strains for these diploid strain constructions are listed in Table I. The strain numbers for each of the final homozygous mutant diploids are: *cdc28-4/cdc28-4*, JRY5227, JRY5228 and JRY5229; *cdc37-1/cdc37-1*, JRY5232, JRY5233 and JRY5234; *cdc25-1/cdc25-1*, JRY5237, JRY5238 and JRY5239; *ras2-47/ras2-47*, JRY5242, JRY5243 and JRY5244; *cdc35-1/cdc35-1*, JRY5247 and JRY5248; *cdc33-1/cdc33-1*, JRY5254 and JRY5255; and *prt1-1/prt1-1*, JRY5249, JRY5250 and JRY5251.

Spore purification

Sporulated cultures of the appropriate yeast diploid were pelleted by a 5 min centrifugation at 1000 g at room temperature. The cells and spores were resuspended in softening buffer (10 mM dithiothreitol, 100 mM Tris–SO₄, pH 9.4) at a cell density of 5 OD₆₀₀ U/ml and incubated for 10 min at 30°C. The cells and spores were pelleted, as above, and resuspended in spheroplasting buffer (2.1 M sorbitol, 10 mM potassium phosphate, pH 7.2) at 25 OD₆₀₀ U/ml. Zymolyase-20T (Seikagaku America Inc.) was added to a concentration of 0.5 mg/OD₆₀₀ unit and the spheroplasting reaction was carried out for 30 min at 30°C. This suspension was spun at 1000 g for 10 min at room temperature. The spore pellet was washed once with 0.5% Triton X-100 and then resuspended in the same solution; the amount of 0.5% Triton X-100 added for this final resuspension was one-fourth the volume of spheroplasting buffer used in the previous step. The final resuspension was sonicated briefly to disperse the spores and was stored at 4°C. The spheroplasts formed from yeast cells by the Zymolyase treatment were effectively lysed by the resuspension in 0.5% Triton X-100 and subsequent sonication. The spore preparation could be stored for at least several weeks without a significant loss in viability. This spore purification protocol had several advantages over previously described schemes (Rousseau and Halvorson, 1969; Dawes and Hardie, 1974) including its relative simplicity and that the procedure did not alter the germination properties of the spores.

To determine whether the survivors in the sporulated diploid culture were the haploid spores as expected and not Zymolyase-resistant diploids, the ploidy of the survivors was determined by a mating assay. Greater than 99% of the surviving colonies from the sporulated diploid culture were mating-proficient, indicating that only the haploid spores survived this lysis regimen. In addition, log-phase cultures of either haploid or diploid strains exhibited an even greater sensitivity to this Zymolyase-induced hypotonic lysis than did spores or stationary phase cells; to

produce the same drop in viability, 10-fold lower concentrations of Zymolyase were required for log-phase cultures relative to stationary-phase cultures. Upon germination, spores more resembled log-phase cells than stationary-phase cells with respect to Zymolyase resistance.

Acquisition of Zymolyase sensitivity assays

Purified spores were added to the appropriate medium at a concentration of $\sim 1 \times 10^7$ per ml and incubated at 30°C. Following this incubation, the spores were pelleted by a 5 min centrifugation at 1000 g at room temperature and treated with softening buffer and Zymolyase-20T, as described above. An aliquot then was removed from the spheroplasting reaction and diluted into 0.5% Triton X-100. Serial dilutions of this suspension were plated to solid rich medium and the plates were incubated for 2–4 days, usually at 30°C. At this time, the number of colonies on the plates were counted and the fraction of spores sensitive to Zymolyase digestion (the definition of germination) was calculated. The percent germination was calculated with the following formula: % germinated = (No. of input spores – No. of survivors of Zymolyase treatment) $\times 100$ / No. of input spores.

For most experiments, we used a modified form of this acquisition of Zymolyase sensitivity assay. Instead of assessing Zymolyase sensitivity of the spore population after incubation in the relevant medium, Zymolyase-20T was added directly into the germination medium with the spores. The germination reaction was allowed to proceed and the number of survivors over time was determined by the number of colonies formed following the plating of dilutions of the reaction to solid rich medium. The final concentration of Zymolyase-20T in the reactions was 3 mg/ml. The germination kinetics observed with this modified protocol was identical to that seen with the previous assay for all temperatures from 23° to 38°C. In addition, similar kinetics for the acquisition of Zymolyase sensitivity was observed for spores in the original sporulated cultures, indicating that the purification process itself did not alter the germination properties of the spores. The digestion by Zymolyase occurred rapidly, because the same efficiency of cell lysis was obtained when Zymolyase was added just 15 min prior to plating for survivors as when the enzyme was present for the entire germination period. For each assay, a control reaction omitting the Zymolyase was performed to test for any Zymolyase-independent loss of viability during the incubation period. Spore survival was >95% for most media conditions used. In addition, a Zymolyase reaction in water was performed to test for any germination-independent loss of viability that may have occurred due to the high Zymolyase concentrations used in these assays. Less than 5% of the input spores were killed by such an incubation in water and Zymolyase. Therefore, this chronic Zymolyase-exposure assay simplified the measurement of germination kinetics by minimizing the number of manipulations required prior to plating for survivors.

For germination assays of *ts* mutants, the mutant spores were pre-incubated at the appropriate non-permissive temperature, usually 37°C, for 30 min prior to the addition of germination medium. For each mutant analyzed, the pre-incubation conditions were established by ensuring that such an incubation would produce the relevant cell cycle block in mitotic cells. A control reaction lacking Zymolyase also was performed to test whether any loss of viability was due to spore germination or to the possible irreversibility of the temperature-sensitive step. For germination assays with cycloheximide (Sigma), the spores were pre-incubated with 40 µg/ml cycloheximide for 15 min at 23°C prior to the addition of germination medium. Cycloheximide concentrations of 1–40 µg/ml were found to be equally effective in inhibiting germination.

Commitment assays

Purified spores were incubated at a concentration of $\sim 1 \times 10^7$ per ml in rich growth medium for varying lengths of time at 30°C. Depending on the experiment, this incubation was performed in either the presence or absence of 3 mg/ml Zymolyase-20T; identical results for the subsequent commitment assays were obtained with either method. Following this incubation, the spores were pelleted by centrifugation at 1000 g for 5 min, washed once with water and then resuspended in water containing 3 mg/ml Zymolyase-20T. This resulting spore suspension then was incubated at 30°C for a period of time such that the total combined incubation period in rich growth medium and water was 9 h. At 9 h, dilutions of the spore suspension were plated to solid rich growth medium to determine the number of survivors. The percentage of spores committed to germination was equal to the percentage of spores killed by the subsequent Zymolyase treatment in water. As an example, to test the fraction of the spores that were committed after 1 h, spores were incubated in rich medium for 1 h and then transferred to water containing 3 mg/ml Zymolyase for an additional 8 h prior to plating to a rich

medium. The difference between the original number of spores and the final number of survivors represented the number of spores committed after 1 h in rich medium at 30°C.

Acknowledgements

We thank Aaron Mitchell, Hiroshi Mitsuzawa, Robert Sclafani and Jeremy Thorner for providing yeast strains and plasmids. We are especially grateful to Jeffrey Stack for critical reading of this manuscript. This work was supported by a Leukemia Society of America Special Fellow Award (to P.K.H.), Damon Runyon–Walter Winchell Cancer Research Fund fellowship DRG-113 (to P.K.H.), National Institutes of Health grant GM35827 (to J.R.) and the California Tobacco-Related Disease Research Program IRT26 (to J.R.). Additional core support was provided by a National Institute of Environmental Health Sciences Mutagenesis Center grant (P30 ESO 1896).

References

- Alani, E., Cao, L. and Kleckner, N. (1987) A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics*, **116**, 541–545.
- Ballou, C.E., Maitra, S.K., Walker, J.W. and Whelan, W.L. (1977) Developmental defects associated with glucosamine auxotrophy in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **74**, 4351–4355.
- Barnes, D.A. and Thorner, J. (1986) Genetic manipulation of *Saccharomyces cerevisiae* by use of the *LYS2* gene. *Mol. Cell. Biol.*, **6**, 2828–2838.
- Brenner, C., Nakayama, N., Goebel, M., Tanaka, K., Toh, E.A. and Matsumoto, K. (1988) *CDC33* encodes mRNA cap-binding protein eIF-4E of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **8**, 3556–3559.
- Briza, P., Winkler, G., Kalchauer, H. and Breitenbach, M. (1986) Dityrosine is a prominent component of the yeast ascospore wall. *J. Biol. Chem.*, **261**, 4288–4294.
- Briza, P., Ellinger, A., Winkler, G. and Breitenbach, M. (1988) Chemical composition of the yeast ascospore wall: the second outer layer consists of chitosan. *J. Biol. Chem.*, **263**, 11569–11574.
- Briza, P., Ellinger, A., Winkler, G. and Breitenbach, M. (1990) Characterization of a DL-dityrosine-containing macromolecule from yeast ascospore walls. *J. Biol. Chem.*, **265**, 15118–15123.
- Broach, J.R. (1991) *Ras* genes in *Saccharomyces cerevisiae*: signal transduction in search of a pathway. *Trends Genet.*, **7**, 28–32.
- Broek, D., Toda, T., Michaeli, T., Levin, D., Birchmeier, C., Zoller, M., Powers, S. and Wigler, M. (1987) The *S. cerevisiae* *CD25* gene product regulates the RAS/adenylyl cyclase pathway. *Cell*, **48**, 789–799.
- Choi, S., Ferro, A.J. and Shapiro, S.K. (1977) Function of S-adenosylmethionine in germinating yeast ascospores. *J. Bacteriol.*, **131**, 63–68.
- Dawes, I.W. and Hardie, I.D. (1974) Selective killing of vegetative cells in sporulated yeast cultures by exposure to diethyl ether. *Mol. Gen. Genet.*, **131**, 281–289.
- Danaie, P., Wittmer, B., Altmann, M. and Trachsel, H. (1995) Isolation of a protein complex containing translation initiation factor Ptl1 from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **270**, 4288–4292.
- Donnini, C., Artoni, N. and Marmiroli, N. (1986) Germination conditions that require mitochondrial function in *Saccharomyces cerevisiae*: utilization of acetate and galactose. *J. Bacteriol.*, **168**, 1250–1253.
- Esposti, R.E. and Klapholz, S. (1981) Meiosis and ascospore development. In Strathern, J.N., Jones, E.W. and Broach, J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 211–287.
- Fraenkel, D.G. (1982) Carbohydrate metabolism. In Strathern, J.N., Jones, E.W. and Broach, J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–37.
- Gibbs, J.B. and Marshall, M.S. (1989) The *ras* oncogene – an important regulatory element in lower eucaryotic organisms. *Microbiol. Rev.*, **53**, 171–185.
- Granot, D. and Snyder, M. (1991) Glucose induces cAMP-independent growth-related changes in stationary-phase cells of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **88**, 5724–5728.
- Granot, D. and Snyder, M. (1993) Carbon source induces growth of stationary phase yeast cells, independent of carbon source metabolism. *Yeast*, **9**, 465–479.

- Hashimoto,T., Conti,S.F. and Naylor,H.B. (1958) Fine structure of microorganisms: III electron microscopy of resting and germinating ascospores of *Saccharomyces cerevisiae*. *J. Bacteriol.*, **76**, 406–416.
- Hubler,L., Bradshaw-Rouse,J. and Heideman,W. (1993) Connections between the Ras-cyclic AMP pathway and G₁ cyclin expression in the budding yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **13**, 6274–6282.
- Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.
- Johnston,M. and Carlson,M. (1992) Regulation of carbon and phosphate metabolism. In Jones,E.W., Pringle,J.R. and Broach,J.R. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 193–281.
- Kaiser,C., Michaelis,S. and Mitchell,A. (1994) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Kitamura,K. (1982) Re-examination of Zymolyase purification. *Agric. Biol. Chem.*, **46**, 963–969.
- Kitamura,K., Kaneko,T. and Yamamoto,Y. (1974) Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. II. Purification and properties of an enzyme, Zymolyase, which lyses viable yeast cells. *J. Gen. Microbiol.*, **20**, 323–344.
- Kreger-Van Rij,N.J.W. (1978) Electron microscopy of germinating ascospores of *Saccharomyces cerevisiae*. *Arch. Microbiol.*, **117**, 73–77.
- Matsumoto,K., Uno,I., Oshima,Y. and Ishikawa,T. (1982) Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. *Proc. Natl Acad. Sci. USA*, **79**, 2355–2359.
- Matsumoto,K., Uno,I. and Ishikawa,T. (1983) Control of cell division in *Saccharomyces cerevisiae*. mutants defective in adenylate cyclase and cAMP-dependent protein kinase. *Exp. Cell Res.*, **146**, 151–161.
- Mitchell,A.P. (1994) Control of meiotic gene expression in *Saccharomyces cerevisiae*. *Microbiol. Rev.*, **58**, 56–70.
- Murray,A. and Hunt,T. (1993) *The Cell Cycle: An Introduction*. W.H.Freeman and Company, New York.
- Nagashima,T. (1959) On the germination of yeast spores. *Ecol. Rev.*, **15**, 75–78.
- Naranda,T., MacMillan,S.E. and Hershey,J.W. (1994) Purified yeast translational initiation factor eIF-3 is an RNA-binding protein complex that contains the PRT1 protein. *J. Biol. Chem.*, **269**, 32286–32292.
- Norbury,C. and Nurse,P. (1992) Animal cell cycles and their control. *Annu. Rev. Biochem.*, **61**, 441–470.
- Palleroni,N.J. (1961) The nutritional requirements for the germination of yeast spores. *Phyton*, **16**, 117–128.
- Pardee,A.B. (1989) G₁ events and regulation of cell proliferation. *Science*, **246**, 603–608.
- Pausch,M.H., Kaim,D., Kunisawa,R., Admon,A. and Thorner,J. (1991) Multiple Ca²⁺/calmodulin-dependent protein kinase genes in a unicellular eukaryote. *EMBO J.*, **10**, 1511–1522.
- Pringle,J.R. and Hartwell,L.H. (1981) The *Saccharomyces cerevisiae* cell cycle. In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 97–142.
- Reed,S.I. (1992) The role of p34 kinases in the G₁ to S-phase transition. *Annu. Rev. Cell Biol.*, **8**, 529–561.
- Rousseau,P. and Halvorson,H.O. (1969) Preparation and storage of single spores of *Saccharomyces cerevisiae*. *J. Bacteriol.*, **100**, 1426–1427.
- Rousseau,P. and Halvorson,H.O. (1973) Effect of metabolic inhibitors on germination and outgrowth of *Saccharomyces cerevisiae* ascospores. *Can. J. Microbiol.*, **19**, 1311–1318.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sando,N., Oguchi,T., Nagano,M. and Osumi,M. (1980) Morphological changes in ascospores of *Saccharomyces cerevisiae* during aerobic and anaerobic germination. *J. Gen. Appl. Microbiol.*, **26**, 403–412.
- Savarese,J.J. (1974) Germination studies on pure yeast ascospores. *Can. J. Microbiol.*, **20**, 1517–1522.
- Schiestl,R.H. and Gietz,R.D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.*, **16**, 339–346.
- Seigel,J.L. and Miller,J.J. (1971) Observations on acid-fastness and respiration of germinating yeast ascospores. *Can. J. Microbiol.*, **17**, 837–845.
- Seufert,W., McGrath,J.P. and Jentsch,S. (1990) *UBC1* encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation. *EMBO J.*, **9**, 4535–4541.
- Stotz,A. and Linder,P. (1990) The *ADE2* gene from *Saccharomyces cerevisiae*: sequence and new vectors. *Gene*, **95**, 91–98.
- Thevelein,J.M. (1994) Signal transduction in yeast. *Yeast*, **10**, 1753–1790.
- Tingle,M.A., Kuenzi,M.T. and Halvorson,H.O. (1974) Germination of yeast spores lacking mitochondrial deoxyribonucleic acid. *J. Bacteriol.*, **117**, 89–93.
- Toda,T. *et al.* (1985) In yeast, RAS proteins are controlling elements of adenylate cyclase. *Cell*, **40**, 27–36.
- Werner-Washburne,M., Braun,E., Johnston,G.C. and Singer,R.A. (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.*, **57**, 383–401.
- Xu,G. and West,T.P. (1992) Nutritional and physiological factors affecting germination of heterothallic *Saccharomyces cerevisiae* ascospores. *Microbios*, **72**, 27–34.

Received on June 5, 1997; revised on July 4, 1997