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Genetic Assessment of Stationary Phase for Cells of the Yeast *Saccharomyces cerevisiae*

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Starvation of cells of the yeast *Saccharomyces cerevisiae* causes cessation of proliferation and acquisition of characteristic physiological properties. The stationary-phase state that results represents a unique developmental state, as shown by a novel conditional phenotype (M. A. Drebot, G. C. Johnston, and R. A. Singer, Proc. Natl. Acad. Sci. USA 84:7948–7952, 1987): mutant cells cannot proliferate at the restrictive temperature when stimulated to reenter the mitotic cell cycle from stationary phase but are unaffected and continue proliferation indefinitely if transferred to the restrictive temperature during exponential growth. We have exploited this reentry mutant phenotype to demonstrate that the same stationary-phase state is generated by nitrogen, sulfur, or carbon starvation and by the *cdc25-1* mutation, which conditionally impairs the cyclic AMP-mediated signal transduction pathway. We also show that heat shock, a treatment that elicits physiological perturbations associated with stationary phase, does not cause cells to enter stationary phase. The physiological properties associated with stationary phase therefore do not result from residence in stationary phase but from the stress conditions that bring about stationary phase.

Starvation of cells of the yeast *Saccharomyces cerevisiae* curbs cell proliferation and provokes physiological perturbations (45). These perturbations confer characteristic properties on starved cells that mark an altered physiological state known as stationary phase. Properties of stationary-phase cells include an unreplicated complement of DNA resulting from a regulated arrest of cell proliferation (33), increased thermotolerance (44), elevated levels of storage carbohydrates (22), and the induction of certain genes, including members of heat shock gene families (2, 5, 29). These distinctive stationary-phase properties are dissipated when cells reenter the mitotic cell cycle (10). For *S. cerevisiae*, restoration of satisfactory nutritional conditions triggers exit from stationary phase and resumption of cell proliferation.

The appropriate response of yeast cells to nutritional conditions depends, in part, on an adenosine 3′,5′-monophosphate (cAMP) effector pathway. Many components of this regulatory circuit have been identified genetically (11). An important component of this pathway is the *CDC25* gene product (6, 32, 35), which activates the *RAS2* G protein (31, 39) that in turn stimulates the *CYR1*-encoded adenylyl cyclase (3, 43). Mutant phenotypes reveal the role of cAMP-mediated signal transduction in nutrient sensing (21, 43). Conditional mutations in either *CDC25* or *CYR1* that decrease the activity of the cAMP-mediated pathway (6) bring about regulated arrest of cell proliferation, presumably because of specious signaling of nutrient status (17, 24, 25). Furthermore, cells blocked in proliferation by these *cdc25* or *cyr1* mutations exhibit properties of stationary-phase cells, even in nutrient-replete medium (17, 24, 25). Therefore the physiological changes characteristic of stationary phase can result from defined molecular changes in a nutrient signaling pathway.

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The display of stationary-phase properties is not confined to cells under starvation conditions; under certain circumstances it can be noted for growing cells. For example, some of these properties are seen upon the transfer of proliferating *S. cerevisiae* cells to an elevated but nonlethal growth temperature. This abrupt temperature shift, termed a heat shock, causes a transient cessation of proliferation in the prereplicative cell cycle interval (20) and is accompanied by changes in gene expression and cell physiology (26). Heat-shocked cells induce members of heat shock gene families (23), accumulate the storage carbohydrate trehalose (1, 15, 16), and acquire a thermotolerance that protects them from otherwise lethal effects of even higher temperatures (26). For heat-shocked cells these perturbations are only transient and have no long-term effects on cell proliferation. Nevertheless, a heat shock applied to proliferating cells leads to many alterations in cell physiology that are conventionally associated with stationary phase.

To evaluate the possibility that heat shock causes growing cells to enter, if only transiently, a stationary-phase state, we exploited a recently described mutant that is conditionally defective only for the resumption of proliferation from stationary phase (10). These mutant cells acquire typical stationary-phase properties when allowed to exhaust the growth medium but then upon incubation in fresh medium at the restrictive temperature cannot reenter the mitotic cell cycle to resume cell proliferation. This conditional defect is specific to the resumption of proliferation from stationary phase: if at a permissive temperature these reentry mutant cells are first allowed to resume proliferation, they become refractory to restrictive conditions and continue to proliferate at the restrictive temperature. Here we offer further evidence that the reentry mutant phenotype provides a reliable genetic criterion for stationary-phase status, and we use this phenotype to demonstrate that decreasing the activity of the cAMP pathway brings about an authentic stationary-phase state. We also show that the physiological perturbations elicited by heat shock do not signify the acquisition of stationary phase.

MATERIALS AND METHODS

Strains and culture conditions. *S. cerevisiae* MD-G3-G02 (*MATa gcs1-1 sed1-1 ade2 his6 ura1*) is a backcrossed derivative of strain GR2 (*MATa his6 ura1*) (19) harboring the *gcs1-1 sed1-1* mutations (10). Strains MD25XG02 (*cdc25-1 gcs1-1 sed1-1 ade2 his6*) and MD28XG02 (*cdc28-4 gcs1-1 sed1-1 ura1*) are both products of repeated backcrosses, by standard yeast genetic procedures (28), of *cdc* mutant strains with strains GR2 and MD-G3-G02. Cells were grown at 29°C in YM1 complex medium (12) supplemented with adenine (20 μg/ml) and glucose (2%) or, for nitrogen and sulfur starvation, in YNB defined medium (12) supplemented with amino acids (40 μg/ml) and purines or pyrimidines (20 μg/ml) to satisfy auxotrophic requirements. Nitrogen starvation and sulfur starvation were imposed by incubation in nitrogen-free or sulfur-free YNB medium until cells were uniformly unbudded and proliferation had ceased (18, 21).

Assessment of cellular parameters. Cellular morphology was assessed by direct microscopic examination. Cell concentration was determined using a Coulter Counter (Coulter Electronics, Hialeah, Fla.). To quantify thermotolerance, 1-ml portions of a culture were incubated in a 52°C water bath for 5 min and then placed on ice for a minimum of 5 min. At the time of transfer to 52°C, an equivalent but untreated population of cells was placed on ice. Both samples were then diluted in phosphate-buffered saline and spread on solid medium (12). Thermotolerance was expressed as percent survival of heat-treated cells, as assessed by colony formation.

RESULTS

Stationary-phase status can be evaluated genetically. Physiological characteristics have been routinely used to identify a cell in stationary phase (33, 45). However, there is now a genetic indicator of stationary phase: a recently described mutant has been shown to be conditionally defective only for reentry into the mitotic cell cycle from stationary phase (10). Cells with this reentry mutant phenotype can not resume proliferation from stationary phase under restrictive conditions, but mutant cells that are already proliferating continue to proliferate when transferred to the restrictive temperature. This distinctive phenotype is not seen for cells of any other genotype.

The reentry mutant phenotype described above was characterized for cells growing in a rich, glucose-based medium (10), in which stationary phase most likely results from carbon deprivation (unpublished observations). Stationary phase, however, is thought to be a general state attained in response to starvation for many nutrients, including carbon (33). Therefore the generality of using this reentry mutant phenotype to assess stationary-phase status was evaluated. For this evaluation the resumption of proliferation was monitored for reentry mutant cells that had ceased proliferation due to various starvations or mutational blocks.

Reentry mutant cells were starved for nitrogen under permissive conditions. This starvation causes yeast cells to cease proliferation in a regulated fashion and acquire typical stationary-phase properties such as thermotolerance (4, 44). The starved cells then were transferred to fresh, nitrogen-replete complex medium and incubated either at the restrictive temperature of 14°C or at the permissive temperature of 29°C. The presence of fresh medium stimulated the nitrogen-starved reentry mutant cells to resume proliferation at 29°C (Fig. 1B), but identically starved mutant cells did not reenter

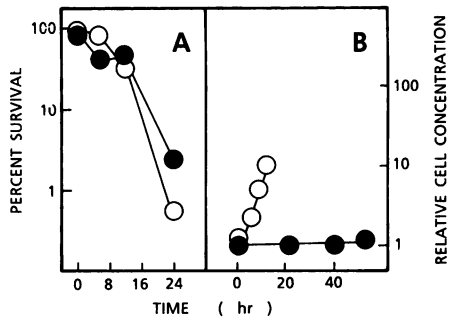

FIG. 1. Nitrogen starvation elicits the reentry mutant defect that blocks the resumption of cell proliferation. Nitrogen-starved cells were transferred to complete medium and incubated at 14°C and at intervals were assessed for thermotolerance (A) and for cell concentration (B). (A) Symbols: ○, wild-type cells at 14°C; ●, reentry mutant cells at 14°C. (B) Symbols: ○, reentry mutant cells at 29°C; ●, reentry mutant cells at 14°C.

the mitotic cell cycle or even bud when similarly stimulated at the restrictive temperature of 14°C (Fig. 1B). Wild-type cells resumed proliferation within 14 h at this temperature (data not shown). Despite the inability of nitrogen-starved mutant cells to resume proliferation at the restrictive temperature, the starved cells did respond metabolically to the stimulation conditions. Stimulated mutant cells, like wild-type cells, lost the characteristic thermotolerance of stationary phase (Fig. 1A).

Mutant cells starved for sulfur or starved by growth in only 0.1% glucose instead of the usual 2% glucose were similarly unable to resume proliferation at the restrictive temperature (data not shown). Therefore, with respect to the mutational block to the resumption of proliferation in the reentry mutant cells, starvation for nitrogen, sulfur, or carbon all blocked cells in a similar stationary-phase state.

Certain temperature-sensitive mutations in the cAMP-mediated signal transduction pathway also bring about changes characteristic of a stationary-phase arrest, even under conditions of satisfactory nutrient supply. For example, cells harboring a temperature-sensitive *cdc25* mutation promptly cease cell proliferation in a regulated fashion and acquire stationary-phase properties at the restrictive temperature of 36°C (17, 24, 25). The restrictive temperature for this cAMP pathway mutation is different than the restrictive temperature (14°C) that elicits the reentry mutant phenotype. Therefore each type of mutational block can be imposed separately and independently. This feature allowed the blockage caused by a *cdc25* mutation to be imposed by incubation at a high temperature, where the reentry mutant phenotype is not manifested; then the effects of the reentry mutant defect on the resumption of proliferation from that blocked state were studied by transfer of arrested mutant cells to the low restrictive temperature, where the *cdc25* blockage is no longer manifested.

Mutant cells bearing the *cdc25-1* mutation in combination with the reentry mutations, *gcs1-1* and *sed1-1* (10; see Materials and Methods), were first arrested at the *cdc25* block by incubation at 36°C. Portions of the arrested cell population were then incubated at two different temperatures permissive for the *cdc* mutation: 29 and 14°C, the temperature at which the reentry mutant phenotype is manifested. The mutant cells recovered fully from the blockage induced by the *cdc25-1* mutation when incubated at 29°C but did not resume proliferation when incubated at 14°C (Fig.

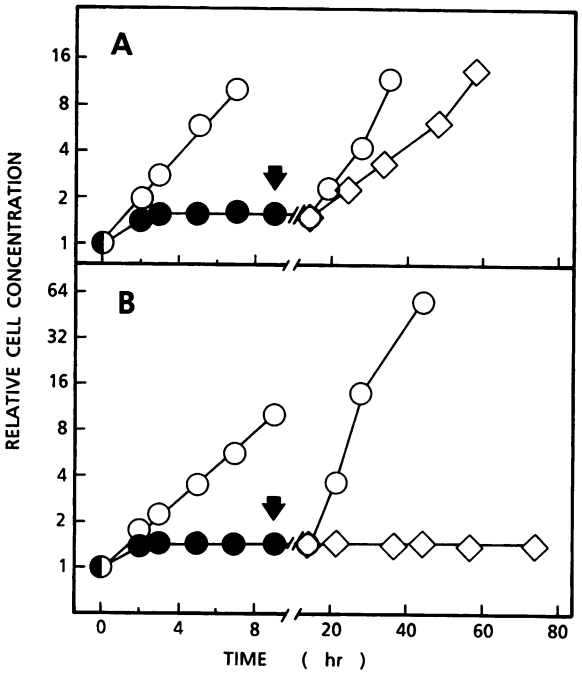

FIG. 2. Some *cdc*-mediated start blockages are accompanied by stationary-phase arrest. Reentry-mutant cells bearing either the *cdc28-4* mutation (A) or the *cdc25-1* mutation (B) were transferred from the permissive temperature of 29°C to the restrictive temperature of 37°C (●) and incubated for 9 h to impose the *cdc*-mediated cell cycle block. After complete cessation of proliferation, portions of each population of arrested cells were incubated at either 29°C (○) or 14°C (◇) to assess resumption of proliferation.

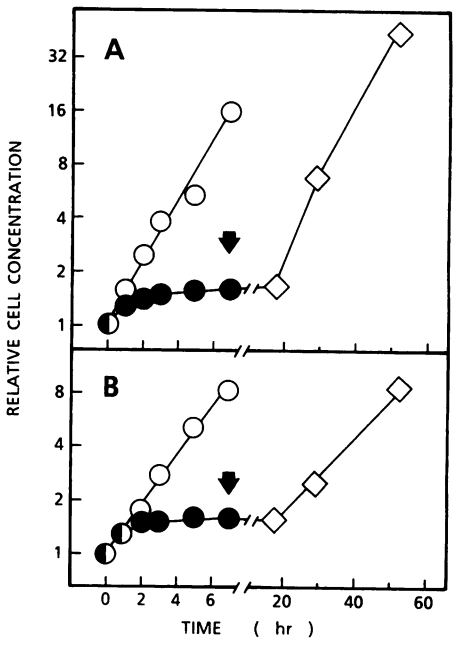

FIG. 3. Resumption of proliferation after arrest by α factor. Proliferating wild-type (A) and reentry mutant (B) cells were incubated with the mating pheromone α factor for 7 h to arrest proliferation at start. Arrested cells were then incubated at 14°C in the absence of α factor and assessed for proliferation. Symbols: ○, 29°C without α factor; ●, 29°C with α factor; ◇, 14°C without α factor.

2B). A control experiment showed that a mutant strain bearing the *cdc25-1* mutation but without the *gcs1-1* and *sed1-1* reentry phenotype mutations resumed proliferation normally at 14°C after arrest by incubation at 36°C (data not shown). Therefore the effects of the *cdc25-1* mutation cause mutant cells to enter a state that, by the criterion of the reentry mutant phenotype, resembles that imposed by carbon, nitrogen, or sulfur starvation. These findings are consistent with previous studies on the effects of this mutation in particular (17, 25) and the function of the cAMP-mediated signal-transduction pathway in nutrient signaling in general (21, 40).

Stationary-phase cells contain an unreplicated complement of DNA, as do cells in the prereplicative portion of the cell cycle (33). With respect to landmarks of the mitotic cell cycle, stationary-phase cells can be shown by a partial order-of-function analysis (33) to be at or before the cell-cycle regulatory step termed start that occurs in G_1 (13, 14). Blockage in G_1, demonstrably at start, can be imposed by certain other mutations that affect the cell cycle (45) and also by the yeast mating pheromones (7, 46); many of these start-blocking situations, unlike those imposed by starvation or the *cdc25-1* mutation, do not cause cells to acquire the properties of stationary-phase cells. For example, cells arrested at start by the actions of a yeast mating pheromone or by a conditional *cdc28* mutation (13) show none of the physiological changes characteristic of stationary phase (45). It might therefore be expected that the resumption of proliferation from these sorts of G_1 blockages would not be affected by the reentry mutant defect; this expectation was tested.

In the first way used to impose G_1 arrest, reentry mutant haploid cells (of *MAT***a** mating type) were arrested in the G_1 interval at start by treatment with the mating pheromone α factor and then transferred to fresh medium devoid of α factor and incubated at either 29 or 14°C. In the second method, triple-mutant *cdc28-4 gcs1-1 sed1-1* reentry mutant cells were incubated at 36°C to bring about arrest at start through the effects of the thermosensitive *cdc28-4* start mutation (34) and then transferred to either 29 or 14°C for further incubation. For both arrest situations start-arrested reentry-mutant cells resumed proliferation both at 29 and at 14°C (Fig. 2A and 3). These results verify that neither α-factor treatment nor a *cdc28* start mutation causes cells to enter stationary phase.

Of the experimental manipulations tested here, the only ones that blocked cell proliferation in ways that made the subsequent resumption of proliferation sensitive to the reentry-mutant defect were those of starvation or altered nutrient sensing; these treatments are also the ones that confer stationary-phase properties on cells.

Heat shock does not elicit stationary phase. Proliferating cells subjected to an abrupt increase in growth temperature display increased thermotolerance (26, 30) and become transiently arrested in the G_1 phase of the cell cycle (20). G_1 arrest and thermotolerance are also features of stationary phase. To assess whether a heat shock causes cells to enter stationary phase, proliferating reentry mutant cells were transferred from 29 to 37°C and incubated at that elevated growth temperature for 1 h. During this incubation the mutant cells acquired the usual thermotolerance, as defined by the maintenance of viability during a subsequent 52°C test incubation (data not shown). After the 1-h incubation at 37°C, at the time of maximum accumulation of G_1-phase cells (20), a portion of the heat-shocked reentry mutant population was transferred to 14°C for further incubation to

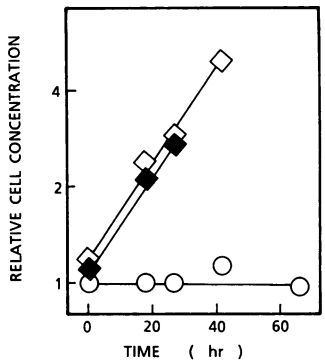

FIG. 4. Heat shock does not bring about stationary phase. One portion of a culture of proliferating reentry mutant cells was transferred to 14°C for further incubation (◇), whereas another portion was first heat shocked by incubation at 37°C for 1 h before further incubation at 14°C (◆). For comparison, another population of stationary-phase reentry mutant cells was incubated in fresh medium at 14°C (○).

assess stationary-phase status. At this restrictive temperature the heat-shocked mutant cells resumed proliferation and grew at the same rate as proliferating reentry mutant cells transferred directly to 14°C without an intervening incubation at 37°C (Fig. 4). These results show that the reentry mutant defect, which is specific for the resumption of proliferation from stationary phase, does not come into play upon heat shock.

Because cells subjected to a heat shock only display a transient response, including a transient arrest of proliferation (20, 38), it could be argued that the 1-h heat treatment, which causes only a subpopulation of cells to arrest in G_1, similarly allows only a subpopulation of cells at the appropriate point in the cell cycle to undergo a stationary-phase arrest; cells in this subpopulation could be responsible for acquired properties such as thermotolerance, whereas the other subpopulation that does not enter stationary phase could account for the observed resumption of proliferation at the restrictive temperature. This hypothesis was refuted by the following two experiments.

To determine whether cell-cycle position at start influences the response of heat-shocked cells with respect to stationary phase, reentry mutant cells were arrested uniformly in G_1 at start by inhibition with α factor (7) or, as a control, uniformly blocked in S phase by inhibition with hydroxyurea (39). The arrested mutant cells were then heat shocked at 37°C for 1 h, transferred to fresh medium, and incubated at either 14 or 29°C. Heat-shocked reentry mutant cells arrested at start by α-factor treatment or in S phase by hydroxyurea treatment resumed proliferation under restrictive conditions (Fig. 5). Therefore cell-cycle position, or even cell-cycle blockage, does not bring about a stationary-phase response upon heat shock.

To examine the response of individual unperturbed cells to heat shock, the abilities of individual reentry mutant cells to proliferate at 14°C after a heat shock were assessed by using time-lapse photomicrography. Of 140 heat-shocked cells examined, 137 (98%) resumed proliferation at the restrictive temperature, suggesting that few, if any, cells in the population were forced into stationary phase by the heat shock.

These findings reinforce the conclusion that heat shock does not cause cells to enter stationary phase.

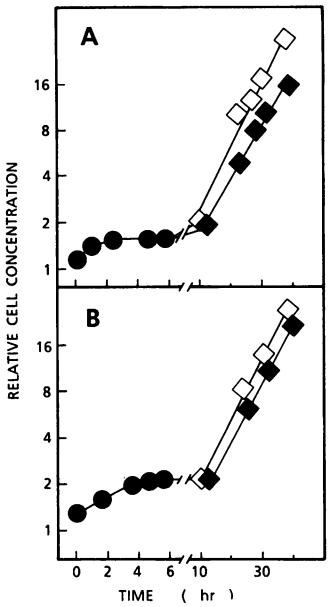

FIG. 5. The state of cells after heat snock is unaffected by prior cell cycle arrest. Proliferating reentry mutant cells were incubated for 7 h with α factor (A) to arrest cells at start or with hydroxyurea (B) to arrest cells in S phase. Portions of each arrested population were then subjected to a 1-h heat shock at 37°C before transfer to inhibitor-free medium and incubation at 14°C. Symbols: ●, 29°C with inhibitor; ◆, 14°C after heat shock; ◇, 14°C without heat shock.

DISCUSSION

The observations that proliferating yeast cells can acquire many stationary-phase properties upon incubation at 37°C (27, 30) led us to investigate whether the 37°C incubation and resultant biosynthetic stress (26, 27) cause cells to enter stationary phase. The criterion used here to define residence in stationary phase was a functional test that relies on the novel phenotype of mutant cells harboring both the *gcs1-1* and *sed1-1* mutations. These double-mutant cells under restrictive conditions are specifically impaired only in the resumption of proliferation from stationary phase (10) and thus can be used to distinguish stationary-phase cells from other types of nonproliferating cells. We demonstrate here that these double-mutant cells exhibit the same distinctive response when stationary phase is imposed in different ways. These findings expand the scope of the reentry mutant phenotype and provide additional evidence that sensing a nitrogen source involves cAMP-mediated signal transduction (37, 43). The reentry mutant phenotype thus provides a reliable tool for evaluating stationary-phase status of cells. We also show that the stress imposed by heat shock does not cause cells to enter stationary phase.

The stationary phase that is a consequence of nutrient deprivation is characterized by a constellation of physiological properties (33, 45). Similar properties are also adopted at a restrictive temperature, without starvation, by cells mutant in the *CDC25* gene (17, 24, 25, 30), which encodes a component of the cAMP-mediated signal-transduction pathway. Mutations in this gene decrease cAMP levels (3, 9) and would thereby be expected to decrease the activity of cAMP-dependent protein kinases (42). These observations have suggested that this cAMP effector pathway and the responsive cAMP-dependent protein kinase activities play key roles in nutrient sensing and consequent responses such

as entry into stationary phase (4). Here we show that a *cdc25* cAMP pathway mutation causes cells with the reentry mutant phenotype to become defective for resumption of proliferation upon the restoration of permissive conditions. With respect to this functional test, therefore, the same stationary phase is produced by carbon, nitrogen, or sulfur deprivation and by perturbation of the cAMP-mediated signal transduction pathway. These findings also support the conclusion that the effects of starvation are mediated through altered cAMP levels (45).

The biosynthetic limitation that leads to stationary phase imposes a stress situation on a cell that elicits characteristic physiological perturbations. Our observations suggest that many of these physiological changes that characterize stationary phase may be related only to the stress imposed by starvation rather than to the stationary-phase status of the cell itself. This conclusion derives from observations concerning the situation instigated by heat shock, which produces a stress related to the production of denatured or abnormal proteins (8, 36) rather than to starvation. Nevertheless, the responses to heat shock include many physiological perturbations, including an arrest of proliferation and increased thermotolerance, that are similar to those that accompany starvation (30). Moreover, the responsiveness of cells to heat shock, like the response to the stress of nutrient depletion, requires an intact cAMP-dependent signal transduction pathway: cells with unregulated protein kinase activity or with elevated cAMP levels do not arrest proliferation or become thermotolerant upon heat shock (38, 41, 42). Thus the cAMP effector pathway mediates cellular responses to a variety of environmental stresses. However, we found that heat-shocked reentry mutant cells, with or without prior arrest in G_1 or S phase, did not become impaired in the resumption of proliferation under restrictive conditions. These findings demonstrate that heat shock does not cause cells to enter stationary phase, even though the accompanying physiological perturbations are associated with stationary-phase cells. These physiological perturbations exhibited by heat-shocked or nutrient-deprived cells are therefore more reasonably understood as responses to the physiological stress imposed, rather than as characteristics of stationary phase itself.

ACKNOWLEDGMENTS

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