JOURNAL OF BACTERIOLOGY, Feb. 1987, p. 779–784
0021-9193/87/020779-06$02.00/0

Vol. 169, No. 2

Effect of Cell Cycle Position on Thermotolerance in *Saccharomyces cerevisiae*

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Received 22 November 1985/Accepted 10 November 1986

We showed that the heat killing curve for exponentially growing *Saccharomyces cerevisiae* was biphasic. This suggests two populations of cells with different thermal killing characteristics. When exponentially growing cells separated into cell cycle-specific fractions via centrifugal elutriation were heat shocked, the fractions enriched in small unbudded cells showed greater resistance to heat killing than did other cell cycle fractions. Cells arrested as unbudded cells fell into two groups on the basis of thermotolerance. Sulfur-starved cells and the temperature-sensitive mutants *cdc25*, *cdc33*, and *cdc35* arrested as unbudded cells were in a thermotolerant state. Alpha-factor-treated cells arrested in a thermosensitive state, as did the temperature-sensitive mutant *cdc36* when grown at the restrictive temperature. *cdc7*, which arrested at the G1-S boundary, arrested in a thermosensitive state. Our results suggest that there is a subpopulation of unbudded cells in exponentially growing cultures that is in G0 and not in G1 and that some but not all methods which cause arrest as unbudded cells lead to arrest in G0 as opposed to G1. It has been shown previously that yeast cells acquire thermotolerance to a subsequent challenge at an otherwise lethal temperature during a preincubation at 36°C. We showed that this acquisition of thermotolerance was corrected temporally with a transient increase in the percentage of unbudded cells during the preincubation at 36°C. The results suggest a relationship between the heat shock phenomenon and the cell cycle in *S. cerevisiae* and relate thermotolerance to transient as well as to more prolonged residence in the G0 state.

The lethal effects of extreme temperature on *Saccharomyces cerevisiae* have been described. Exponentially growing cells are much more sensitive than are stationary-phase cells to a thermal shock (15). Thus, resting or quiescent cells are more thermotolerant than growing cells are. This heat resistance is acquired as the cells pass from the exponentially growing to the stationary state (12). Schenberg-Frascino and Moustacchi (15) isolated small unbudded cells from stationary cultures and observed their thermotolerance as they progressed through the first cell cycle into exponential growth. They observed that thermotolerance is decreased in budding cells. Yeast cells, when starved for an essential nutrient such as nitrogen, phosphorous, or sulfur, cease dividing and arrest their cell division as unbudded cells. The thermotolerance of cells starved for any of these nutrients resembles that of stationary-phase cells, and it has been suggested that this thermotolerance is a general characteristic of resting cells (11).

Our aim was to clarify the relationship between the cell cycle and thermotolerance. To this end we examined the effect of the cell cycle position on the thermotolerance of *S. cerevisiae* in exponentially growing cultures by using the elutriator rotor to separate cells by size and cell cycle position (9). We also examined the effects on the cell cycle of a mild heat shock, during which the cells acquire thermotolerance (10, 13). In addition, we used several methods to arrest cells in the unbudded state, and we determined the thermotolerance of the resulting populations. The methods included arrest via deprivation of essential nutrients, alpha-factor arrest, and the use of temperature-sensitive cell division cycle mutants.

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Our results demonstrate that a distinct population of unbudded cells was more thermotolerant than were cells in other morphological stages in the cell cycle. We suggest that this thermotolerance is associated with a defined state, G0, which has synthetic and physiological properties distinct from those of other unbudded cells.

MATERIALS AND METHODS

Strains. The following strains were used: SKQ2n (**a**/α *ade1*/+ +/*ade2* +/*his1*); A364A (**a** *lys2 tyr1 his7 gal1 ade1 ade2 ura1*); *cdc7-1* (*ts124*); *cdc25-1* (*ts321*); *cdc33-1* (E17a), derived from A364A (4; provided by L. Hartwell); *cdc35-1* (BR214-4a; **a** *ade1 ura1 his7 arg4 trp1*); and *cdc36-12* (ST21; **a** *met2 cyh2*) (provided by L. Hartwell).

Media and cell growth. Cells were grown to mid-logarithmic phase at 23°C on a rotary shaker. Unless stated otherwise, growth was in YEDP medium (10 g each of yeast extract and Bacto-Peptone [Difco Laboratories, Detroit, Mich.] per liter and 2% glucose). For sulfur starvation experiments, cells were grown to mid-logarithmic phase in YNB medium without amino acids as described previously (9), supplemented with histidine, lysine, and tyrosine at 32 mg/liter each. The sulfur starvation medium itself was as follows (per liter): $NH_4H_2PO_4$, 6 g; KH_2PO_4, 0.5 g; NaH_2PO_4, 0.5 g; to pH 6.0 with K_2HPO_4; all 20 naturally occurring amino acids minus cysteine and methionine, 12.5 mg each; adenine and uracil, 10 mg each; $CaCl_2$ and $MgCl_2$, 100 mg each; H_3BO_3, KI, $CuCl_2 \cdot H_2O$, $ZnCl_3$, and $FeCl_2 \cdot 6H_2O$, 1 mg each; biotin, 0.02 mg; thiamine, 4 mg; pyridoxine, 4 mg; inositol, 20 mg; and pantothenate, 4 mg.

Sulfur starvation arrest. Cells were filtered onto sterile nitrocellulose, rapidly washed with starvation medium, and transferred on the filter to a fresh flask containing starvation medium.

Arrest of cell cycle mutants. Cultures were grown overnight at 23°C to mid-logarithmic phase. Culture (10 to 20 ml)

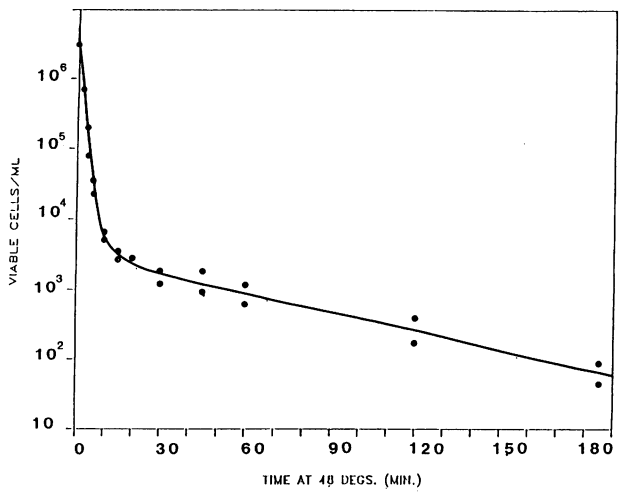

FIG. 1. Viability of SKQ2n after a shift from 23 to 48°C. Logarithmically growing cells were transferred from 23 to 48°C, and samples were taken at the times indicated. Viability was determined by colony counts as described in Materials and Methods.

was then transferred to a prewarmed (36°C) flask, and incubation was continued. Samples were removed at various times for a 10-min heat shock at 50°C.

Killing curves. Cells were grown to mid-log phase at 23°C before heat shock unless otherwise specified. For heat shock, cells (2.5 ml) were transferred to 2.5 ml of prewarmed media at 48°C for SKQ2n and 50°C for *cdc25*. Samples were removed into ice-cold sterile water at various times during the next 3 h, and the viabilities were determined as described below.

Heat shock of arrested cells. Cells (2 ml) were transferred to prewarmed 50-ml flasks containing 2 ml of warmed medium in a rotary shaker water bath. At the time of the heat shock, a control sample was mixed with ice-cold sterile water to stop growth. After the 10-min heat shock at 50°C, 2 ml of the heat-shocked culture was rapidly cooled in sterile ice water. Both control and heat-shocked samples were washed once with cold water, and viability was determined as described below.

Viability determinations. Cells in an ice water bath were sonicated by using a Branson Sonifier (CD 200; Branson Sonic Power Co., Danbury, Conn.) at a setting of 2 for 20 s to disrupt cell aggregates. After appropriate dilutions into sterile, ice-cold water, cells were plated onto YEPD agar and incubated at 23°C for 3 to 5 days. All platings were done in duplicate, and colonies were counted.

Cell cycle fractionation and heat shock. The diploid strain SKQ2n was used in all cell cycle fractionation experiments. The fractionation and morphology determination procedures have been described previously (3, 9). The following modifications were made for this study: cell separation and washing were performed at 23°C, not at 4°C; and the cells were fractionated in YEPD media, not water. The cells were heat shocked at a constant interval after each rotor fraction was collected (25 min). Each fraction was suspended in medium, and control samples were taken for dilution, sonication, and plating. For heat shock, 0.5 ml of cells was added to a 50-ml flask prewarmed to 48°C and containing 1.5 ml of prewarmed medium. After the 10-min heat shock, the samples were diluted in ice-cold sterile water, sonicated, and plated for viability determinations.

Alpha-factor arrest. Alpha factor was obtained from Sigma Chemical Co., St. Louis, Mo., and suspended in methanol. Alpha-factor arrest was monitored by counting cell types in a hemacytometer.

Determination of unbudded cells in arrested cell cultures. Samples were taken at various times for control and heat shock survival determinations. The control cells that had been sonicated and kept on ice were used for morphology determinations, which were done with a light microscope and hemacytometer.

Note on terminology. A number of physical, nutritional, and genetic effects can lead to the enrichment of a population for nonproliferating unbudded cells. These cells have variously been referred to as being in a G1-arrest phase, G0, or stationary phase. We believe that there are problems associated with the use of such terms to describe all nonproliferating unbudded cells in a population. Our data as well as the data of a number of other groups suggest that although each of the terms may accurately describe some nonproliferating unbudded cells, the terms are not interchangeable, and therefore their use prejudges the physiological characteristics of a particular population of cells. To avoid this semantic bias, we avoid these terms in presenting our data and use them only in the analysis of the data.

RESULTS

Thermotolerance of cells at different positions in the cell cycle in exponentially growing cultures. We determined the kinetics of killing for exponentially growing SKQ2n cells. Cells were transferred from a growth temperature of 23°C to 48°C, aliquots were taken, and viable cell numbers were determined by colony counting after 3 to 5 days at 23°C. The results are shown in Fig. 1. The curve is biphasic. There was an initial rapid decline in viability during the first 10 min; after this period, the number of viable cells fell off much more slowly with continued incubation at 48°C. The biphasic curve suggests the presence of two populations of cells with very different thermal killing characteristics. We estimated the relative size of the two populations by extrapolating the second linear portion back to time zero, giving a value of approximately 3×10^3 cells per ml, which was about 0.1% of the total population at time zero (3×6^{10} cells per ml).

As this was a very small fraction of the total population, we wished to know whether this population was in any way related to cell cycle position. Is there any position in the cell cycle that is inherently more thermotolerant? To determine this, we separated exponentially growing cells in cell cycle-specific fractions via centrifugal elutriation. Aliquots of cells from each resulting fraction were plated both before and after a 10-min heat shock at 48°C. The viable cells in each were determined by colony counts after 3 to 5 days of growth at 23°C.

The relative survival found in each cell cycle fraction is shown in Fig. 2A, and the cytology of cells in each rotor fraction is shown in Fig. 2B. Morphology was determined as described previously by using the fluorescent stain DAPI (9). Because the fractions did not contain equal numbers of cells, we expressed survival relative to the average survival of the sum of all the cells in all the fractions. The total survival of all cells recovered from the rotor after heat shock was 0.17%. Rotor fractions 2 and 3 showed an almost 20-fold-higher survival after a heat shock than other fractions did (Fig. 2A). The predominant cell type of these two fractions was small unbudded cells (9; Fig. 2B).

It is known that stationary-phase cells are more resistant to heat killing than logarithmically growing cells are (11).

Because of our heat shock regimen, the total number of survivors we found was low (fractions 2 and 3 were small). We wished to eliminate the possibility that the survivors were the result of some small population of cells present in the original inoculum persisting throughout the growth period. To determine whether this interpretation could be eliminated, using an inoculum of similar size, we grew cultures to mid-log phase and continued to keep them growing logarithmically by diluting with fresh medium for 3 days. At various dilutions of the original inoculum, but always at mid-log-phase cell densities, samples were heat shocked at 48°C for 10 min and the percent survival was determined.

If the few survivors we saw at the earliest time point were due to cells present in the original inoculum, then survival should have declined as the original inoculum was further diluted. This was not the case. Survival did not decrease as the culture was maintained via dilution in the log phase. We diluted the original inoculum of $1:1.3 \times 10^{10}$ through growth (survival was 0.029% after 10 min at 48°C) and saw no decrease in survival over that found in the original overnight culture (dilution from inoculum of $1:1.7 \times 10^3$ with 0.029% survival after 10 min at 48°C). We conclude that the unbudded cell fraction that we observed surviving the heat shock was not a function of the original inoculum. The cells express a property of the exponentially growing cells, which we showed (Fig. 1) to contain two distinct populations with respect to thermotolerance.

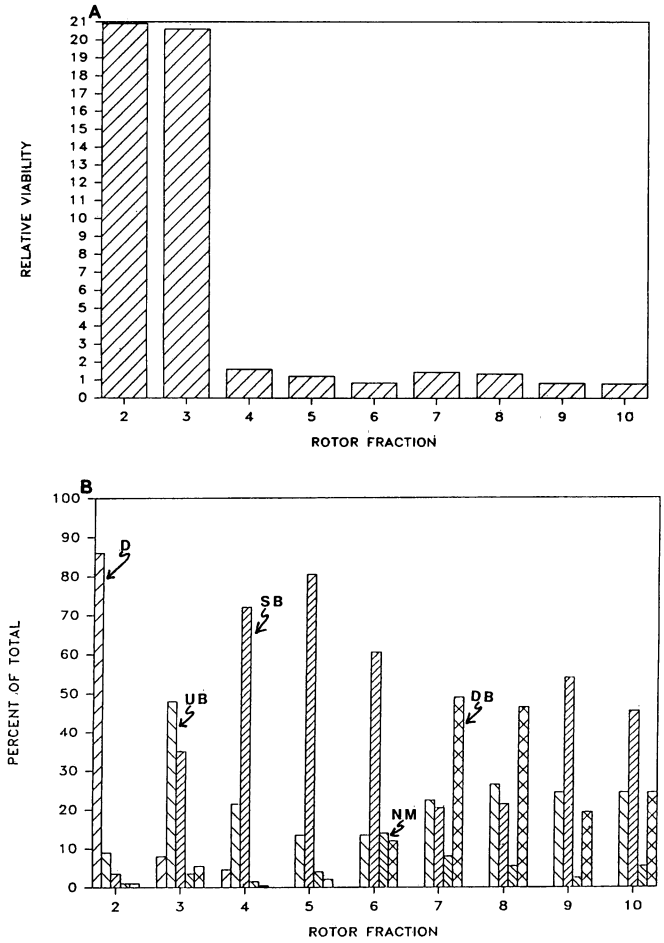

FIG. 2. Thermotolerance as a function of cell cycle position. SKQ2n cells were fractionated into cell cycle-specific fractions via centrifugal elutriation (see Materials and Methods). (A) Cells from each fraction were heat shocked at 48°C for 10 min, after which viability was determined. Survival is expressed relative to the average survival of the sum of all the cells in all the fractions. Samples were also taken from each fraction for morphology determinations. (B) Percentages of cells found as dead (D), unbudded (UB), small budded (SB), nuclear migrants (NM), and doublets (DB).

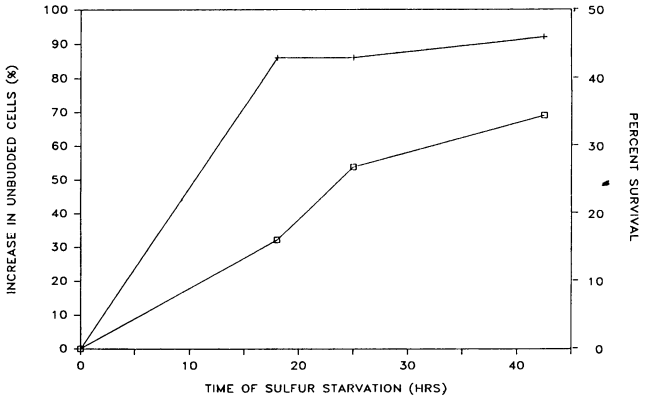

FIG. 3. Thermotolerance of A364a cells starved for sulfur. Cells were grown for 48 h in logarithmic phase in low-sulfur medium and transferred via filtration to minimal medium minus sulfur. Cells were maintained in the starvation medium for the times indicated, and aliquots were then heat shocked at 50°C for 10 min. Survival was determined by plating (□). Samples were taken at the times indicated, and the percent unbudded cells was determined microscopically (+).

Thermotolerance of wild-type cells arrested in the unbudded state. The results established an interrelationship between the unbudded phase of the cell cycle and thermotolerance. To examine this relationship in more detail, we arrested cells with nutritional stress (sulfur starvation), with alpha factor, and by the use of cell cycle division mutants and determined their thermotolerance.

A364A cells were shifted to sulfur starvation medium (see Materials and Methods) at time zero. At the times indicated in Fig. 3, samples were taken and examined cytologically; the cells were plated for viability both before and after a 10-min heat shock at 50°C. By 42.5 h the percent unbudded cells increased from 50 to 96% (Fig. 3), indicating that the culture had arrested. At the time of the sulfur starvation, less than 0.1% (0.03%) of the population survived the heat shock. Thermotolerance gradually increased, reaching 34.5% survival by 42.5 h after the shift to sulfur starvation medium (Fig. 3). There was a rapid acquisition of thermotolerance for the first 18 h of starvation as cells arrested (Fig. 3). There was an increase in thermotolerance that occurred as the cells remained in the arrested state during the next 20 h, whereas there was little apparent change in the cytology. The percent unbudded cells remained constant (at 93 to 96%) from 18 to 42.5 h, whereas thermotolerance increased from 15 to 35% during this period. The unbudded cells continued to acquire thermotolerance as they stayed for longer periods of time in the sulfur-starved state.

The behavior of cells arrested by the mating hormone alpha factor was quite different. When exponentially growing a cells were exposed to alpha factor, they arrested and formed characteristic shmoo structures (unbudded cells with elongated protrusions). Up to 83 to 90% shmoos were formed after 6 h in alpha factor. However, no increase in

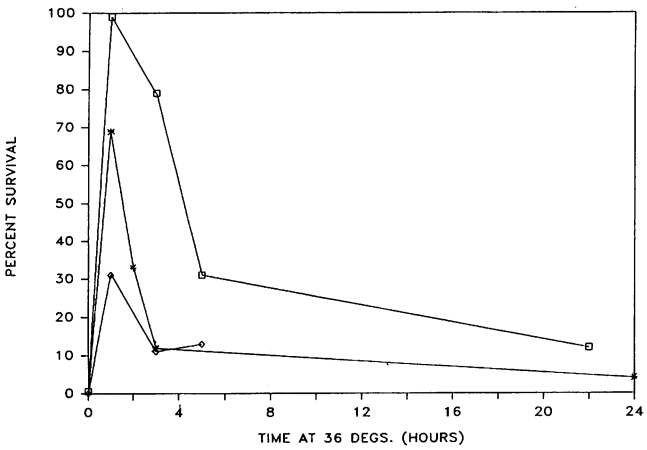

FIG. 4. Thermotolerance of A364a (*) and cell division cycle mutants *cdc7* (◇) and *cdc36* (□) as a function of time at 36°C before heat shock. Cells growing at 23°C in mid-log phase were shifted to 36°C for the times indicated. After incubation at 36°C, samples were heat shocked at 50°C for 10 min, diluted, and plated. Survival was determined after growth for 4 days at 23°C, with 100% being the viable-cell number at the time of the shift to 50°C.

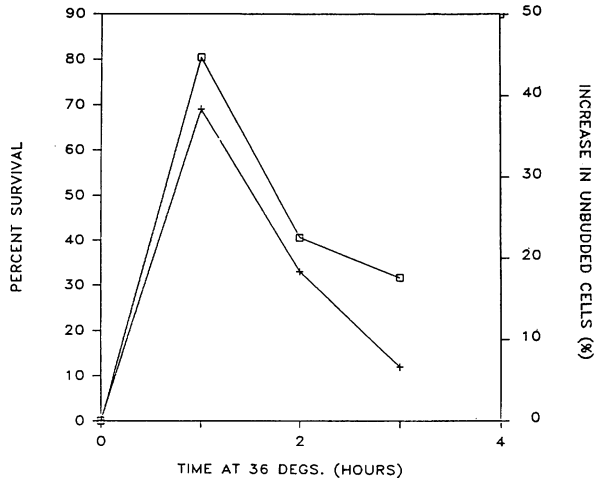

FIG. 6. Morphology and thermotolerance acquisition during a heat shock at 36°C. Cells were shifted from 23 to 36°C, and samples were taken after the times indicated. One sample was subjected to a 10-min heat shock at 50°C, and the viability was then determined (+). A second sample was examined under the microscope, and the increase in percentage of unbudded cells was determined (□).

thermotolerance to a 10-min heat shock at 50°C was observed concomitant with the arrest. Survival was less than 1% before alpha-factor exposure and remained at this level throughout.

Thermotolerance of *cdc* mutants arrested as unbudded cells. We investigated temperature-sensitive cell division cycle (*cdc*) mutants which arrested as unbudded cells. The determination of the thermotolerance of temperature-sensitive cell division mutants was complicated by the necessity of a temperature shift to the restrictive temperature (36°C) to produce the arrest. Parental cells when shifted to 36°C acquired a transient thermotolerance to a subsequent challenge for 10-min at 50°C as shown above. The survival with no preincubation at 36°C was less than 1%, whereas 69% of the cells survived the heat shock after 1 h of pretreatment at 36°C. A364A cells that were grown at 36°C had a higher thermotolerance than those grown at 23°C (4% survival),

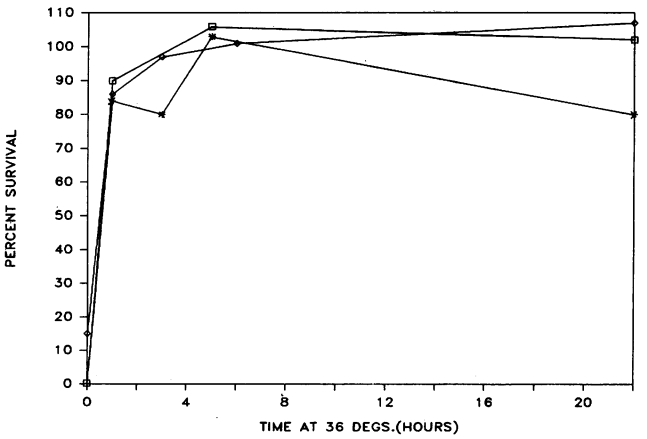

FIG. 5. Thermotolerance of cell division cycle mutants *cdc25* (□), *cdc33* (*), a, and *cdc35* (◇) as a function of time at 36°C before heat shock. Cells growing at 23°C in mid-log phase were shifted to 36°C for the times indicated. After incubation at 36°C, samples were heat shocked at 50°C for 10 min, diluted, and plated. Survival was determined as described in the legend to Fig. 4.

confirming the findings of Walton and Pringle (16). However, the steady-state level of thermotolerance of cells grown at 36°C was much lower than the peak of the transient level caused by a shift from 23 to 36°C (Fig. 4).

All of the mutants tested showed this immediate increase in thermotolerance after a shift from 23 to 36°C. However, the thermotolerance displayed as these *cdc* mutants were maintained at 36°C and allowed to arrest fell into two quite distinct patterns (Fig. 4 and 5).

Mutants *cdc7* and *cdc36* showed the same pattern of thermotolerance acquisition and decay as the parental strain A364A (Fig. 4). After a shift from 23 to 36°C, the cells rapidly acquired thermotolerance, reaching a maximum at about 1 h. The thermotolerance then decayed and by 24 h was only slightly higher than the uninduced level.

Mutants *cdc25*, *cdc33*, and *cdc35* showed a dramatically different pattern (Fig. 5). The acquisition of thermotolerance was similar in magnitude and induction time to that in the parental strain. However, the thermotolerance did not decay as cells became arrested. They maintained protection against the 10-min heat shock at 50°C for at least 24 h at 36°C.

Morphology and thermotolerance acquisition during a 36°C heat shock. Yeast cells have been shown previously to acquire thermotolerance during a brief preincubation at 36°C (10, 13; Fig. 4). We examined the time course of the acquisition of thermotolerance and determined the effect of the preincubation at 36°C on cell cycle position. During the first hour at 36°C, there was a large increase in survival after a 10-min challenge at 50°C (from 0.1 to 69% survival; Fig. 6). The thermotolerance then declined over the next 3 h. There was also an 80% increase in the percentage of cells found in the unbudded stage of the cell cycle during the first hour after the shift from 23 to 36°C (Fig. 6). The increased level of unbudded cells declined from this peak at 1 h, reaching 32% of the 23°C level by 3 h after the shift to 36°C. The heat shock, which induced a transient thermotolerance, also induced a transient increase in unbudded cells.

We showed that there were two populations of cells in exponentially growing SKQ2n cultures (Fig. 1). We also showed that the thermotolerance of cells could be changed

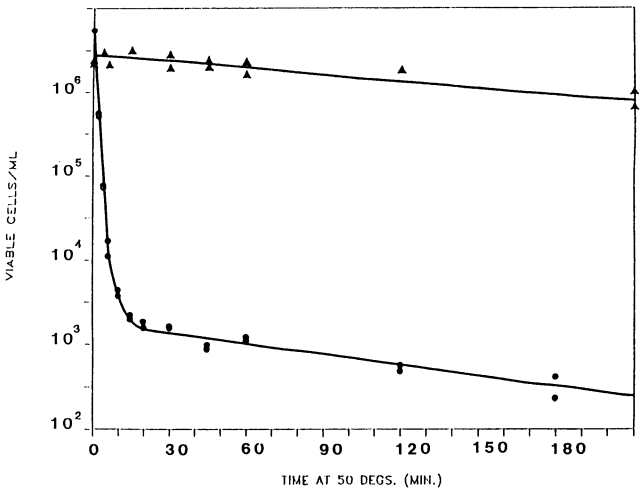

FIG. 7. Viability of logarithmically growing and arrested *cdc25* cells as a function of time at 50°C. *cdc25* cells growing logarithmically at 23°C were shifted to 50°C, and samples were taken at the times indicated (●). *cdc25* cells arrested at 36°C were shifted to 50°C, and samples were taken at the times indicated (▲). Viability was determined by colony counts as described in Materials and Methods.

by arresting them as unbudded cells. It is of interest to compare the kinetics of killing of arrested thermotolerant cells with that of exponentially growing cells. The comparison is shown for *cdc25* in Fig. 7. One culture was grown at 23°C and then shifted to 50°C for the times indicated. The other culture was grown to mid-log phase at 23°C and then shifted to the restrictive temperature of 36°C for 20 h before the heat challenge at 50°C. The cells shifted from 23 to 50°C showed a biphasic curve similar to that seen with the diploid strain SKQ2n, with approximately 0.04% of the cells in the thermotolerant state. After arrest for 20 h at 36°C, the culture showed only one population with a thermotolerance similar to that of the more heat-resistant subpopulation of the logarithmically growing culture.

DISCUSSION

The results presented above lead to two conclusions regarding the cell cycle in *S. cerevisiae*. First, the experiments presented here support the conclusion that a G0 state exists in yeast cells and that increased thermotolerance is a characteristic of the G0 state. By G0 we mean a distinct physiological state outside of the normal cell cycle. Cells paused at some specific point as unbudded cells within the normal cell cycle are not by this definition considered in the G0 state. The experiments presented here also suggest that in an exponential culture some cells are in the G0 state. The population of cells that we observed in this thermotolerant state in an exponentially growing culture was extremely small (approximately 0.1%). If this is an obligatory part of each cell cycle, then this percentage is a reflection of the time spent in the state by each cell as it cycles. The time (0.1% of the cell cycle time) is too short for synthesis or expression of specific cellular functions defining a distinct physiological state. It is for this reason that we feel that this population represents not a subdivision of G1 on the normal pathway but a branch point off the cell cycle path into which some small percentage of cells in an exponentially growing culture enter in a stochastic way.

The experiments with sulfur-starved cells suggest that cells which arrest at a point in G1 from which entry into G0 is possible make the transition to G0. Sulfur-starved cells continued to acquire increased thermotolerance for several hours after their unbudded arrest was complete (Fig. 3). This hypothesis is supported by experiments by Iida and Yahara (5), who showed an increased lag time for recovery of cell division the longer cells are starved of sulfur.

The second conclusion that we draw from the work presented here is that cells can transit into G0 only at a certain point(s) in the cell cycle. Not all arrested unbudded cells were able to enter this state. Neither alpha-factor cells nor arrested *cdc36* cells acquired thermotolerance.

Previous evidence has been presented that suggests distinct states or phases in G1. The evidence was based on morphological (2, 13) and biochemical (5) criteria. The biochemical evidence for multiple states within the unbudded population comes from studies on the protein synthesis patterns of cells arrested by various means (6). Two distinct groups were identified. The first group included cells arrested via sulfur starvation for 6 h and cells containing the mutations *cdc25*, *cdc33*, and *cdc35* after 6 h at the restrictive temperature. Cells in this group showed elevated synthesis of a small set of proteins that included some proteins identified as heat shock proteins (6). The second group included *cdc28*, which like the parent A364A only transiently induced the same set of proteins when shifted to 36°C.

Our groupings based on differential thermotolerance in G1 are consistent with previous divisions of G1 based on morphological and protein synthesis criteria, and we believe this reflects the existence of separate states within G1.

The role of any of the heat shock proteins in either the establishment or maintenance of this thermotolerant state is an intriguing problem. The transcript for the 70-kilodalton heat shock gene has been shown to be cell cycle dependent in sychronized HeLa cells, showing a transiently increased level at the end of the S phase (7). The relationship of the heat shock proteins and thermotolerance has been investigated by several workers. It has been established that a correlation exists between the induction of the heat shock proteins and the acquisition of thermotolerance in exponentially growing cells (8, 10, 13). This correlation also exists for stationary-phase cells, in which high levels of synthesis of a subset of the heat shock proteins are maintained when overall protein synthesis declines dramatically (1). A possible interpretation of the relationship between the synthesis of the heat shock proteins and thermotolerance is that the heat shock proteins are not themselves directly required for thermotolerance but are involved in growth or cell cycle regulation. Heat shock proteins could be negative regulators of cell division and could signal cells to arrest briefly at a specific position in the cell cycle from which the cells can enter the more stress-resistant G0 state. The view is consistent with the observation that *Tetrahymena* spp. can be synchronized by a series of timed heat shocks (17). It is also consistent with our observation that during a protective preincubation of A364A, during which time cells become more thermotolerant, there was a transient increase in the percentage of unbudded cells.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 16252 from the National Institute of Allergy and Infectious Diseases and by a grant from the North Atlantic Treaty Organization.

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