Bud Formation by the Yeast *Saccharomyces cerevisiae* Is Directly Dependent on "Start"

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ABSTRACT Cells of the yeast *Saccharomyces cerevisiae*, which bear a *cdc4* gene mutation, arrest early in the cell cycle but continue to produce buds in a periodic fashion. We show here that this periodic bud formation by cells already arrested at the *CDC4* step is inhibited if the cell cycle regulatory step "start" is also specifically blocked by mutation or by the presence of the yeast mating pheromone α-factor. Thus, the characteristic periodic bud formation by *cdc4* mutant cells requires the continued ability to perform start. This finding raises questions concerning the nature of start; these issues are discussed.

The unicellular yeast *Saccharomyces cerevisiae* reproduces by bud formation. Bud formation is an event of the yeast cell division cycle, and as such is regulated and coordinated with other gene-mediated cell cycle events (19). To be able to form a bud, a cell must first perform the cell cycle regulatory step "start." This dependency relationship is borne out by the observations that cells do not bud when the start event is blocked (7). Blockage of almost any other cell cycle step by a temperature-sensitive cell division cycle mutation causes a cell to arrest in the cell cycle as a singly budded cell. One notable exception concerns cells bearing a *cdc4* cell cycle mutation (5). A cell arrested at the *cdc4* block point and therefore unable to complete the cell cycle not only buds but puts out buds repetitively (5, 7). Like any buds, these buds on *cdc4* mutant cells are dependent for their formation on the *CDC24* gene product (7), which in turn has been shown to be specifically involved in the budding process (7).

Cells arrest as unbudded cells if the start event is blocked by conditions that specifically affect start (6): start mutations or the presence of the yeast mating pheromone α-factor. These methods of blocking start block bud formation indirectly by prohibiting cells from initiating the cell cycle. We report here a more direct role for start in bud formation by cells that have already initiated the cell cycle. We found that for singly budded *cdc4* mutant cells arrested in the cell cycle at the *cdc4* block point, further periodic bud formation was inhibited by conditions that block start.

MATERIALS AND METHODS

Strains: *Saccharomyces cerevisiae* strains GR1 (*MATα his6 ura1*) and GR2 (*MATa his6 ura1*) have been described (14). The *cdc4-6* mutant strain 19021 (*MATa cdc4-6 cdc14-6 ts230 his7 ura1 ade1,2 lys2 tyr1 gal1*) has been described (8) and was obtained from L. H. Hartwell, University of Washington. Strains ST34 (*MATa cdc28-15 lys2 tyr1 cyh2*) and SR661-2 (*MATa cdc36-16 ura1 trp1*) have also been described (20) and were obtained from S. I. Reed, University of California, Santa Barbara. Strain EP-12 [*MATα cdc4-6 cdc14-6 ts230 his(6,7) ura1*] is a recombinant of strains 19021 and GR1; strain A12-34 (*MATa cdc28-15 cdc4-6 lys2 tyr1*) is a recombinant of strains ST34 and EP-12; strain A12-36 [*MATa cdc36-16 cdc4-6 cdc14-6 ts230 his(6,7) ura1 trp1*] is a recombinant of strains SR661-2 and EP-12. Strain GR428.10.2 [*MATα cdc28-15 cdc4-6 his(5,6,7) ura1*] was from a cross between strain A12-34 and a *cdc4-6 his(5,6,7) ura1 ade2* strain and was constructed for use in label incorporation experiments. Strain GJ100 (*MATa cdc4-6 ura1 trp1*) and 111XD [*MATα cdc4-6 his(1,5,7)*] were derived from strain A12-36 by multiple matings.

Strain 19021, as originally isolated, was shown to contain a second *cdc* mutation, *cdc14-6*, hypostatic to the *cdc4-6* mutation (8). The derivative strain EP-12 also bears both of these *cdc* mutations. The *cdc14-6* mutation is hypostatic because by itself it causes cell cycle arrest in the second cycle after shift to the nonpermissive temperature (unpublished observations), whereas the *cdc4-6* mutation alone in other strains causes the same type of first-cycle arrest exhibited by strains 19021 and EP-12 (unpublished observations). Therefore, cells of strains 19021 and EP-12 arrest at the epistatic *cdc4-6* block before they have the opportunity to reach the second-cycle *cdc14-6* block. We found that the strains 19021 and EP-12 contain yet a third *cdc*-like mutation, *ts230*. This new mutation is also hypostatic to the *cdc4-6* mutation because it, too, by itself produces a second-cycle arrest (unpublished observations).

Media and Chemicals: The complex medium YM-1, the defined medium YNB, and the solid YEPD medium have been described (12). The yeast mating pheromone α-factor was prepared as described (1), or kindly supplied by T. R. Manney (Kansas State University). The chemicals *o*-phenanthroline and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO), and [^{14}C]uracil was obtained from New England Nuclear (Boston, MA).

Cellular Parameters: Cells were counted using a Coulter Counter (Coulter Electronics, Hialeah, FL), and cell morphology was assessed as described (4). At least 200 cells were scored for each assessment. Because the buds formed by strains bearing the *cdc4-6* mutation have unusual morphologies at the nonpermissive temperature, a structure was scored as a bud if a distinct constriction was present at its base, where it joined the parent cell body. This criterion was used to distinguish budded cells from the characteristic pear-

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shaped cells (6) found in cultures arrested at start. The nonpermissive temperature for cells bearing the *cdc28-15* mutation was 38°C (20); at this temperature the cellular deformations typically caused by start mutations such as *cdc28-15* were minimized. Label incorporation procedures were as described (14).

RESULTS

Multiple Budding Is Inhibited by Mating Factor

Like all other periodic cell cycle events, bud formation depends on start, because cells specifically blocked at the start event by yeast mating pheromones or by start mutations do not bud (7). If in like fashion the additional bud formation events in cells already blocked at the *CDC4* step are dependent upon start, then blocking start by start mutations (6, 20) or by the presence of mating pheromone (1) should prevent the further budding events characteristic of the *cdc4* mutant phenotype.

Cells bearing the *cdc4-6* allele were studied, since such cells at the nonpermissive temperature were found to produce bud-like structures from several sites on the cell surface; this property made multiple budding events easy to discern. Exponentially growing cultures of the *cdc4-6* mutant strains GJ100 and 19021 (8) were first placed at the nonpermissive temperature. After a 2-h incubation, most cells were blocked at the *CDC4* step (as indicated by cell phenotypes). Each culture was then split and to one portion was added the mating pheromone α-factor to a concentration that arrested and held control populations of cells at start for at least 8–10 h (data not shown). After a further 4-h incubation, 51% of strain GJ100 cells in the absence of α-factor exhibited the characteristic *cdc4* phenotype of multiple buds (Fig. 1*A*). After the same incubation period, but in the presence of α-factor, virtually all the cells were budded, although now only 13% of cells exhibited multiple buds (Fig. 1*B*). Similar values (88 and 38%, respectively) were found for cells of strain 19021 incubated for 6.5 h after α-factor addition. Undoubtedly, some of the budded cells that did go on to form multiple buds in the presence of α-factor were already committed to second bud formation at the time of α-factor addition. Mating factor thus decreased multiple budding of these *a* mating type *cdc4* mutant cells.

Only yeast cells of the *a* mating type (*MATa*), like strains GJ100 and 19021 used here, show specific responses to α-factor. Accordingly, no decreases in multiple budding were found when cells of strains 111XD (Fig. 1, *C* and *D*) or EP-12, *cdc4-6* mutant strains of the opposite (α) mating type, were exposed to α-factor. Mating factor, which blocks start, thus specifically decreases the ability of cells to bud repetitively at the *cdc4* block.

The inhibition of budding caused by α-factor was reversible. The presence of limiting concentrations of α-factor, which only arrested control cells at start for a short time, in similar fashion only transiently blocked the ability of *cdc4* mutant cells to produce multiple buds (data not shown). Thus, cells treated with α-factor retain the potential to produce multiple buds.

Other *cdc* mutations present in strains 19021 and EP-12 (see Materials and Methods) do not alter the characteristic budding behavior of *cdc4-6* mutant strains or the abrogation of budding when the start event is specifically blocked.

Multiple Budding Is Blocked by Start Mutations

Another way to block the start event is by mutation in any of several *CDC* genes that affect the ability to perform start. Strain A12-34 carries a mutation in the *CDC28* gene (the activity of which defines start; reference 6) and the *cdc4-6* mutation. When exponentially growing cells of this strain were shifted to the nonpermissive temperature, most cells were arrested at start as unbudded cells, while some cells in the population became arrested as budded cells, as expected. These budded cells were those cells which at the time of shift to the nonpermissive temperature had already performed the *CDC28* gene-mediated step but had not yet completed the *CDC4* gene-mediated step. The buds of these cells displayed the morphology typical and characteristic of buds produced by cells bearing only the *cdc4-6* mutation. However, almost all of the budded, arrested cells exhibited only single buds (Table I). Thus, multiple bud formation by cells at the *cdc4* block was inhibited by a *cdc28* start mutation.

In an asynchronously growing population, the time interval between the start event and the *CDC4* gene-mediated step is relatively short; thus, at any one time in such a population, only a few cells have performed start but not the *CDC4* gene-mediated step. To increase the proportion of the population within the cell cycle interval between start and the *CDC4* step at the time of temperature shift, we first synchronized cells of strain A12-34 at start. This was accomplished by treating cells growing at the permissive temperature with α-factor for a period sufficient to produce a population in which 95% of the cells were unbudded. Cells were then removed by centrifugation from medium containing α-factor and resuspended in fresh medium to recover from this arrest at start. At intervals thereafter, portions of the population were shifted to the nonpermissive temperature, incubated for a further 8 h, and then scored for cell morphology. Populations of cells shifted to the nonpermissive temperature showed upon further incubation greater than usual proportions arrested after performance of start but before completion of the *CDC4* gene-mediated step, as evidenced by relatively high proportions of budded cells (Fig. 2*A*). In these budded populations, 90% of the cells exhibited only single buds (Fig. 2*B*). The experimental manipulations themselves did not affect the attainment of a multiple-bud phenotype by cells blocked solely by a *cdc4* mutation. When strains bearing only the *cdc4-6* mutation were treated as described for the double-mutant strain, most cells in the populations displayed more than one bud (Fig. 2*B*).

It was recently noted that one particular start mutation, *cdc28-6* (not studied here), causes cells to be arrested at two points in the cell cycle: at start as unbudded cells, and at another cell cycle point as cells with single buds (17). Although this observation is similar to some of the findings reported here, this type of behavior is probably not involved in our results. For cells bearing only the *cdc28-15* start mutation used in experiments reported here, the protocol with which cells were synchronized at start by treatment with α-factor, released from α-factor-mediated arrest, and then shifted to the nonpermissive temperature at various times after release gave no indication of a second, single-bud arrest phenotype for this mutation (Fig. 2*B*).

The inhibition of budding is not specific to a *cdc28* start mutation. When another double-mutant strain, this time with a mutation in a different start gene, the *CDC36* start gene (*cdc36-16*; reference 20), as well as in the *CDC4* gene (*cdc4-6*), was shifted to the nonpermissive temperature, similar effects were seen. For this strain, too, almost all budded cells had only single buds (Table I). The double-mutant strains so

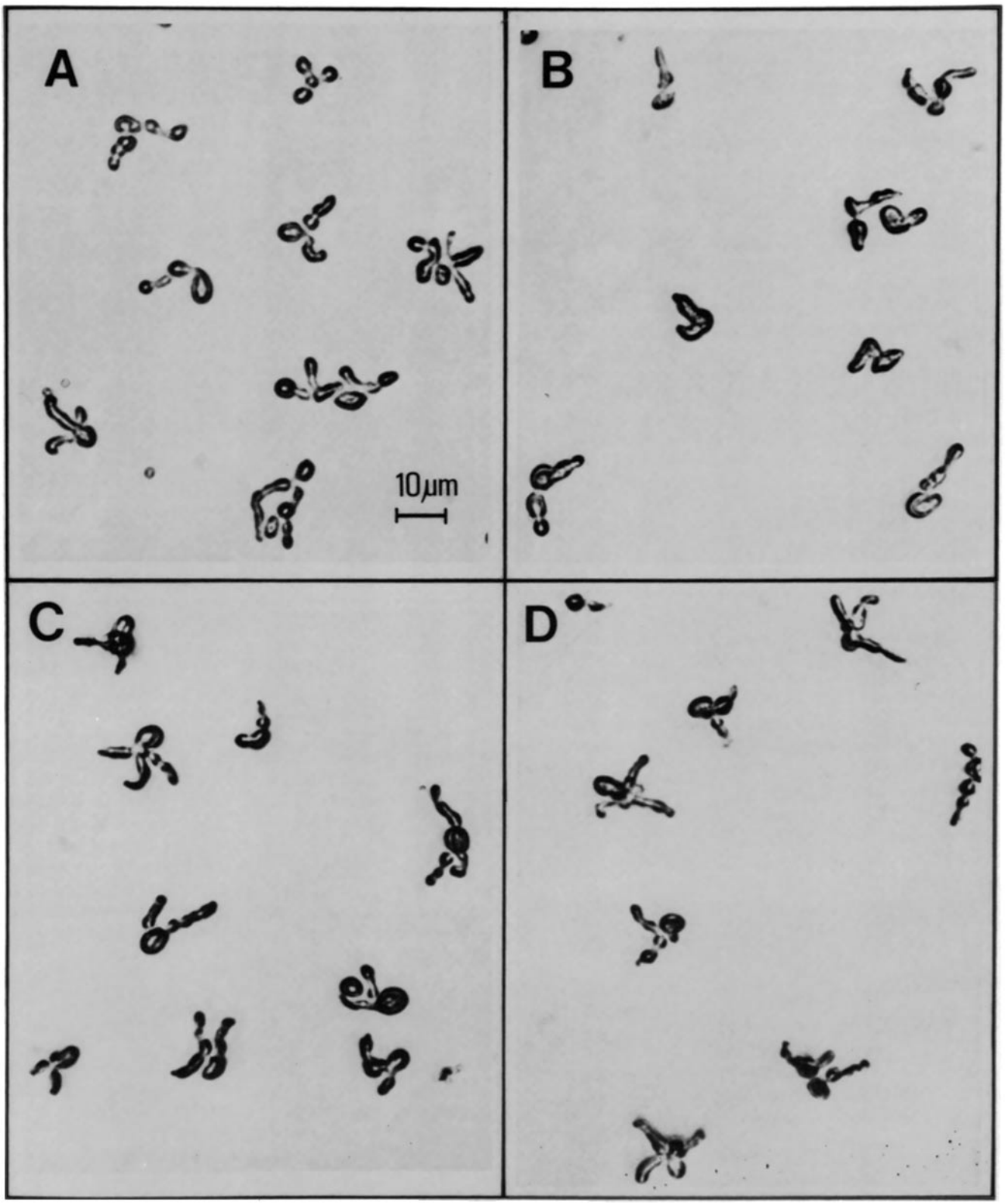

FIGURE 1 Effect of α-factor on the *cdc4-6* mutant cell phenotype. Exponentially growing cells of the *MATa cdc4-6* strain GJ100 or the *MATα cdc4-6* strain 111XD were placed at the nonpermissive temperature. After a 2-h incubation α-factor was added to a portion of each culture. Incubation was continued for an additional 4 h, at which time cells were sampled for cell counting as described (4) and photographed on an agar surface. *A*, *MATa* cells − α-factor; *B*, *MATa* cells + α-factor; *C*, *MATα* cells − α-factor; *D*, *MATα* cells + α-factor.

TABLE I

Arrest Phenotypes of cdc4 and Start Single- and Double-Mutant Strains

Strain	Percent budded	Percent budded population with multiple buds
19021 (*cdc4-6*)	99	82
ST-34 (*cdc28-15*)	6	<2*
SR661-2 (*cdc36-16*)	10	<2*
A12-34 (*cdc28-15 cdc4-6*)	27	7
A12-36 (*cdc36-16 cdc4-6*)	54	8

Exponentially growing cultures were shifted to the nonpermissive temperature and incubated for 6 h before cell phenotypes were scored.
* None observed.

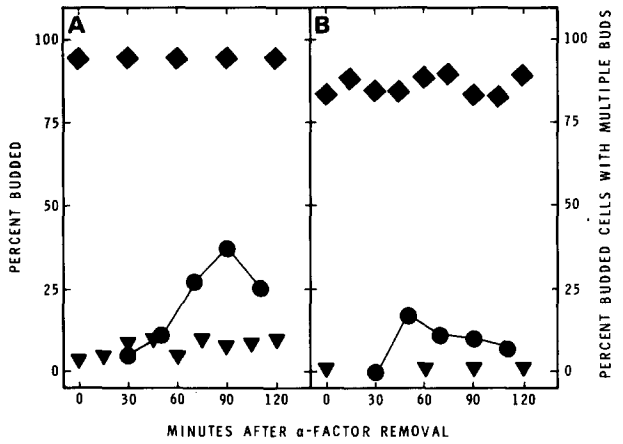

FIGURE 2 Effect of a *cdc28* mutation on the phenotype of cells arrested by a *cdc4* mutation. Cultures of strains A12-34 (*MATa cdc28-15 cdc4-6*), ST34 (*MATa cdc28-15*), and 19021 (*MATa cdc4-6*) growing exponentially in YM-1 medium were treated with α-factor for 2 h to arrest cells at "start." Cells were then removed from α-factor by centrifugation, washed, suspended in fresh medium, and incubated at 22°C. At the times indicated, fractions of each cell population were shifted to 38°C and incubated for a further 9 h before terminal phenotypes were assessed. *A*, proportions of cells exhibiting a budded terminal phenotype. *B*, proportions of those cells with budded terminal phenotypes that exhibited multiple buds. ●, *cdc28-15 cdc4-6* double mutant cells; ▼, *cdc28-15* mutant cells; ♦, *cdc4-6* mutant cells.

far described here are all *MATa*; however, the effects of start mutations on multiple budding at the *cdc4* block are not mating type specific. Double-mutant *cdc36-16 cdc4-6* strains of mating type α, as well as *MATα cdc28-15 cdc4-6* strains, all behaved similarly; in these genetic backgrounds, the start mutations blocked budding of cells arrested at the *cdc4-6* block (data not shown).

We conclude that the inability to perform start, by virtue of a *cdc28* or a *cdc36* mutation, renders cells already blocked by mutation at the *CDC4* step unable to produce more than one bud.

Periodic Bud Formation Is Dependent on Growth

The start event has been shown to be dependent on continued growth (19), so repetitive budding by *cdc4* mutant cells should reflect continuing growth activity at the nonpermissive temperature. Protein accumulation continues at the nonpermissive temperature in *cdc4* mutant cells (12). Fig. 3 shows that RNA accumulation also continued in *cdc4-6* mutant cells at rates comparable with those for wild-type strain GR2 cells (Fig. 3*A*) as those cells rapidly assumed a new, accelerated cell division rate at 36°C (data not shown). Furthermore, the *cdc4-6* mutant cells accumulated labeled uracil throughout the 5-h duration of the experiment (Fig. 3*B*); by that time, most cells were displaying multiple buds (Fig. 4). Since most of the RNA accumulated in cells is ribosomal RNA, it can be inferred that synthesis of ribosomal RNA was not significantly impaired during the production of multiple buds.

Uracil incorporation rates were similarly maintained by double-mutant cells blocked both in the *CDC4* step by the *cdc4-6* mutation and in the start event by the *cdc28-15* mutation (Fig. 3*C*). Thus, it is unlikely that inhibition of multiple budding resulted from some growth defect in this strain, and this supports the conclusion that the effects on budding are responses to the specific inhibition of start itself.

Slowing those growth activities that have been shown to affect start should also affect the periodicity of bud formation by *cdc4* mutant cells. To examine this situation, cells of strain EP-12 were grown for several generations in medium contain-

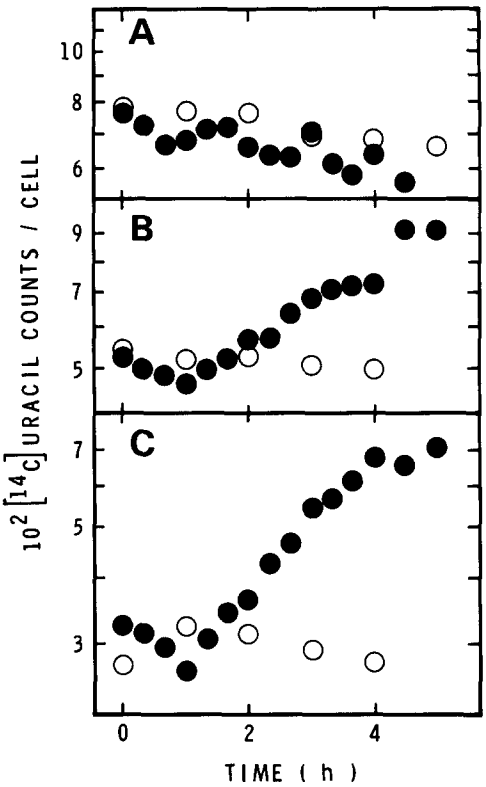

FIGURE 3 RNA accumulation by cells at the nonpermissive temperature. Cells of strains GR2, EP-12 (*cdc4-6*), and GR428.10.2 (*cdc4-6 cdc28-15*), all auxotrophic for uracil, were grown at 23°C for at least five generations in YM-1 medium containing 0.02 μCi [^{14}C]uracil/ml. Exponentially growing cells of each strain were shifted to 36°C at time zero. At intervals samples were removed for cell concentration determinations. To quantitate label incorporation into RNA, other samples were removed and mixed with equal volumes of ice-cold 10% trichloroacetic acid containing 20 μg uracil/ml to precipitate macromolecular material; these precipitates were collected, and radioactivity in the precipitate from each sample was quantitated by liquid scintillation counting. *A*, strain GR2; *B*, strain EP-12 (*cdc4-6*); *C*, strain GR428.10.2 (*cdc4-6 cdc28-15*). ●, radioactivity accumulated by cells at 36°C; ○, radioactivity accumulated by cells at 23°C.

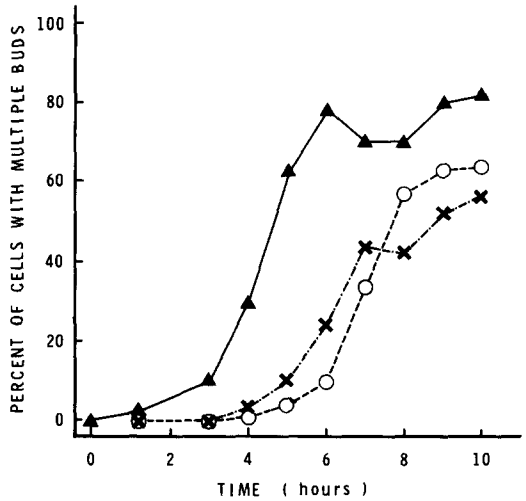

FIGURE 4 Effect of growth inhibition on multiple budding of *cdc4-6* mutant cells. Cells of strain EP-12 were grown for several generations in YNB medium containing either 0.1 μg cycloheximide/ml or 1 μg *o*-phenanthroline/ml. At time zero cultures were shifted to 36°C, and samples were removed periodically for determination of cell morphology. ▲, cells in medium without inhibitors; O, cells in medium containing *o*-phenanthroline; ×, cells in medium containing cycloheximide.

ing limiting concentrations of cycloheximide or *o*-phenanthroline. The degree of cell cycle inhibition produced by these treatments was assessed by monitoring effects on start. At higher concentrations, these compounds indirectly block start by affecting either protein synthesis (9) or precursor ribosomal RNA metabolism (13). At the low concentrations used here, however, the start event was not completely blocked. Generation times at the permissive temperature were increased threefold, and the proportions of unbudded cells in the treated populations were 65–70%, compared with 40–45% for untreated cells (data not shown). The increased proportions of unbudded cells suggested that these conditions not only affected growth, but also partially impaired the start event. When under these growth conditions *cdc4* mutant cells were grown at the permissive temperature and then shifted to the nonpermissive temperature, the kinetics of budding were affected; production of multiply budded cells was delayed (Fig. 4).

DISCUSSION

Requirements for Budding

Cells arrested at the *cdc4* block point continue new bud formation at the nonpermissive temperature (7). Here we report that being at the *cdc4* block point is not in itself sufficient for multiple bud formation. To form the second bud, a cell at the *cdc4* block point must also be in a unique cellular state with respect to start. This conclusion derives from the budding behavior of cells blocked both in the *CDC4* step and in the performance of start (see Table I and Fig. 2). Therefore, there must be two inputs to bud emergence, one from the cellular condition attained prior to the *CDC4* step and one from start.

The conditions used to block start do not appear to affect budding by acting on the bud formation process itself. This conclusion can be inferred from the behavior of cells which, at the time start-blocking conditions are imposed, have already performed start but have not yet budded. Unbudded cells such as these go on to bud normally in the presence of start-blocking conditions (3, 9, 21). Moreover, if bud formation were differentially sensitive to start-blocking conditions, then some cells would be expected to arrest with the configuration of *cdc24* mutant cells specifically defective in bud formation, able to complete the nuclear cell cycle and produce daughter nuclei but without forming buds. However, the absence of unbudded but binucleate cells upon treatment with *o*-phenanthroline or α-factor, or upon exposure of start mutant strains to nonpermissive temperatures (1, 8, 13, 20), suggests that bud formation itself is not sensitive to start-blocking conditions. Because these conditions do not themselves affect bud formation, it follows that bud formation by *cdc4* mutant cells is affected through the inhibition of start.

Budding by *cdc4* mutant cells was affected by conditions that slow growth and thus indirectly affect start (Fig. 4), but it is also clear that inhibition of budding caused by specifically blocking start is not because of indirect growth effects. Quite the contrary, cells enlarge and accumulate both RNA and protein when treated with α-factor (22; and our unpublished results), or when blocked by any of a number of start mutations, such as those in the CDC28 and CDC36 start genes (20; Fig. 3*C*; and our unpublished results).

Implications for Start

Each time a cell initiates the cell cycle, a growth requirement for start, seen as a critical cell size associated with cell cycle initiation, must be met (19). It has been argued that a growth-dependent or size-dependent cell cycle event such as start is best modeled on theoretical grounds as resulting from the synthesis of an unstable effector molecule, which upon achieving a critical concentration triggers completion of that cell cycle step (23). Recent speculations on the physical changes represented by the start event have centered on the yeast spindle pole body. This structure, which is the microtubule-organizing center associated with the nuclear envelope, is duplicated early in the cell cycle in a start-dependent fashion. It has been suggested (2, 19) that start could occur when spindle pole body components accumulate to an amount sufficient to duplicate the mass of the spindle pole body.

Start is that cell cycle step sensitive to mating pheromones and to *cdc* start mutations such as *cdc28* (6, 19). Using this functional definition of start, it has been found that cells blocked at the *CDC4* step have already performed start for that cell cycle, because these cells, if subsequently allowed to complete the *CDC4* step, can go on to synthesize DNA in the presence of start-blocking conditions (11). These *cdc4* mutant cells blocked in the cell cycle can repeatedly perform bud formation, a cell cycle activity dependent on start (7). However, we show here that on functional grounds the start event necessary for formation of the first bud is not in itself sufficient for formation of the next bud by these mutant cells. These results have caused us to reconsider the properties of the start event.

Three interpretations of the results presented here will be considered in turn. Each interpretation is consistent with theoretical and physical models for the start event, but each entails different properties for start.

The first interpretation is that in *cdc4* mutant cells the start event can recur to permit each new bud formation event. Since cells unable to complete the *CDC4* step are not pro-

gressing through the cell cycle, the repetitive budding behavior of these cells suggests that start still occurs periodically in a growth-dependent fashion once each cell cycle time. In this case, novel dependency relationships involving start must be formulated; the start event would not be dependent on the prior completion of any *CDC4*-dependent step of the previous cell cycle.

If start is independent of other cell cycle events, then the process resulting in start, shown here to be continuously active in *cdc4* mutant cells, is not regulated by the cell cycle. It is already well known that continuous processes of growth, which themselves do not appear to be directly affected by the cell cycle (12; manuscript in preparation), are involved in start regulation. Thus, the activity regulating the start event could simply be an aspect of growth; no further regulatory relationships for start need be postulated.

In a second interpretation, the start event could still retain a dependency on the previous cell cycle, and yet be able to be completed repeatedly in *cdc4* mutant cells blocked in the cell cycle, if start dependency relationships are conceptualized in a more complex way. For example, the particular process that results in the start event could be periodic in the cell cycle, activated by some previous cell cycle event and remaining active until after completion of the *CDC4* step. In this way the start event could be dependent on some other cell cycle step and yet could be repeatedly completed by *cdc4* mutant cells. A cell cycle precedent for physiological changes behaving in this way is provided by histone H2B mRNA production in these yeast cells. The production of histone mRNA is periodic in the cell cycle; it is blocked by start-blocking conditions, maintained in mutant cells blocked in the *CDC7* cell cycle step, and ceases after the *CDC7* step has been completed (10). If the process resulting in the start event is periodic in the cell cycle, however, then additional cell cycle relationships must be postulated to regulate this periodicity.

Regardless of whether start is independent or dependent on the previous cell cycle, if the start event is periodically completed in *cdc4* mutant cells, then on formal grounds there must be a special physiological state attained directly upon completion of start. There has been up until now no need to postulate such a start-completed state, and completion of start has been considered to lead directly to the *CDC4* step. Being at the *CDC4* step is necessary for bud formation, but results here show that it is not sufficient. If each bud formation event is dependent on a new start event when a cell is already at the *cdc4* block point, then completion of start must lead not to that cell cycle condition directly, but first to this postulated start-completed state.

For repetitive bud formation to be responsive to the completion of start, those properties of the start event that are involved in bud formation would have to be periodically dissipated. Conditions produced by the start event could be irreversibly inactivated with respect to bud formation by the bud formation process itself. This kind of regulation is seen in histone mRNA production. The production of histone mRNA associated with DNA replication in yeast cells ceases as a consequence of DNA replication itself, as cells enter the S phase (10). Alternatively, conditions produced by the start event that are required for bud formation could be intrinsically unstable and unable to persist long enough to allow formation of more than one bud. In either case, reacquisition of this start-completed state would depend on renewed completion of start.

A third interpretation of the results presented here also retains a dependency for start on a previous cell cycle event, but with repetitive bud formation by *cdc4* mutant cells permitted by a single completion of start. Bud formation would be modulated by the activities of the process that normally elicits the start event, and not by completion of the start event itself. An example of an activity producing a product and also regulating another system is provided by the bacterial glutamine synthetase enzyme. In addition to synthesizing glutamine, this enzyme acts as an activator of transcription of the *hut* operons, which are comprised of genes for histidine degradation. Moreover, the ability of glutamine synthetase to activate this transcription is a function of the allosterically regulated activity of that enzyme (15). With respect to yeast, the start event need occur only once to allow cells to reach the "budding window" (7), and in this cell cycle condition, repetitive bud formation could occur as long as processes sensitive to start-blocking conditions were active. One consequence of this hypothesis is that the periodic manner of budding shown by *cdc4* mutant cells has nothing to do with start, but is an intrinsic property of the bud formation process itself. Another consequence is that start-blocking conditions, such as α-factor or *o*-phenanthroline treatment, or the action of a *cdc* start mutation, affect not the product of the activities that result in the start event, but those activities themselves. Moreover, because the periodicity of bud formation can be protracted by exposure to *o*-phenanthroline, the bud formation process must be responsive to the rate of those activities that result in the start event.

These alternative hypotheses of how start is related to the rest of the cell cycle may in part be testable. In particular, the second hypothesis, that the activities which result in the completion of start are themselves periodic in the cell cycle, predicts that for cells blocked at some point in the cell cycle these activities should not be detectable. In turn, this test requires a more general indicator of start than is provided by bud formation, which seems to be permitted only for cells at the *CDC4* step. Just such an indicator may be suggested by recent studies on the expression of the *HO* gene in yeast. Transcripts of this gene are detected in cells blocked at the *CDC4* step, but not in cells blocked at start (16). Transcription of the *HO* gene thus depends only on start or on those activities that result in start, so that *HO* mRNA may be an indicator of those activities.

At present, none of these interpretations can be experimentally ruled out, and none is uniquely supported by other results. The latter two interpretations do not require major reformulations of start dependency relationships and are compatible with the idea that start itself could be dependent on another cell cycle step. The first interpretation, in which the start event in *cdc4* mutant cells recurs periodically in a solely growth-dependent manner, contains few assumptions, but stipulates the novel conclusion that the start event is independent of other cell cycle events. No evidence has yet been put forward that bears directly on the question of whether start itself really is dependent on events of the previous cell cycle. To address dependency issues such as this, order-of-function experiments (18) are usually called upon, but they include the assumption that the cell cycle step in question leads to a cell cycle stage that is stable (18). This requirement is not met for the possibility of an intrinsically unstable start-completed state attained by completion of start independently of other cell cycle activity. For this case, order-of-function

experiments would suggest an apparent but erroneous dependency of start on the previous cell cycle. Unorthodox procedures are thus needed to resolve start dependency relationships.

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