

Sequential Gene Function in the Initiation of *Saccharomyces cerevisiae* DNA Synthesis

LYNNA M. HEREFORD†

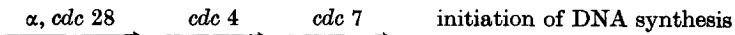
AND

L. H. HARTWELL‡

Department of Genetics
University of Washington
Seattle, Wash. 98195, U.S.A.

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Four steps are known to be required for the initiation of DNA synthesis in *Saccharomyces cerevisiae*. Three of these are mediated by the products of genes *cdc 4*, *7*, and *28* and the fourth is identified by the inhibition exerted on haploid α cells by the mating pheromone, α factor. These four steps have been ordered by a combination of two methods and found to be:



The two sequencing methods are described in detail. Experiments involving the shift of mutant cells from the restrictive to the permissive temperature in the presence of cycloheximide demonstrated that the protein synthesis requirement for yeast DNA replication can be completed before the *cdc 7*-mediated step.

1. Introduction

Thirty-two gene products that are each required for a specific step in the cell division cycle of *Saccharomyces cerevisiae* have been identified by temperature-sensitive mutations (Hartwell *et al.*, 1973). We sought to determine the order of the steps mediated by three of these gene products. To this end we developed two methods, the rationales of which will be described. The first is termed the reciprocal shift method and is conceptually identical to the procedure used by Jarwick & Botstein (1973) and by Hermann & Wood (personal communication) to order gene-controlled steps in bacteriophage assembly. The second method depends on a comparison of the phenotypes of single and double mutants and is termed the double-mutant method. The two methods are used in this report to investigate the events that lead to the initiation of DNA synthesis.

The interval of the cell cycle that precedes the initiation of DNA synthesis in *S. cerevisiae* is of singular significance because it is within this interval that the developmental potentials of the cell are directed. A haploid cell of *S. cerevisiae* at a position in the cell cycle just before the initiation of DNA synthesis is able to undertake one of three possible developmental programs. It may initiate DNA synthesis and begin

† Present address: Department of Biology, University of California at San Diego, La Jolla, Calif. 92037, U.S.A.

‡ To whom reprint requests should be addressed.

the mitotic cycle, it may fuse with a cell of opposite mating type to create a zygote, or it may enter the quiescent state of stationary phase. However, once the cell has initiated DNA synthesis, it appears to be committed to the mitotic cycle, as it does not undertake either of the other two developmental pathways until the cycle is completed (Reid, personal communication; Pringle & Maddox, personal communication). For these reasons we view the initiation of DNA synthesis in *S. cerevisiae* as an act of differentiation and the subsequent steps of the cell cycle as elements of a developmental program. The central importance of this particular event to growth control and differentiation in higher and lower organisms has been emphasized many times (Prescott, 1969; Holtzer, 1970; Helmstetter, 1969; Donachie & Masters, 1969), and we suspect therefore that the genetic dissection of the events involved in the initiation of DNA synthesis in yeast will have broad significance.

2. Methods

(a) Strains and media

The following strains were used in the reciprocal shift experiments: H185.3.4 (*cdc28-1*); H135.1.1 (*cdc 4-3*); and 4008 (*cdc 7-4*). All three strains are of *a* mating type and carry the markers *ade*₁ *ade*₂ *ura*₁ *tyr*₁ *his*₇ *lys*₂ *gal*₁. H185.3.4 carries an additional *leu*₁ marker. The lineage of these strains has been discussed elsewhere (Hartwell, 1973). The restrictive temperature is 36°C for H135.1.1 and 38°C for H185.3.4 and 4008 (Hartwell, 1973). The particular alleles in these strains were chosen for their quick reversibility after a shift from 36°C or 38°C down to 23°C.

The strains carrying double *cdc* mutations were constructed by appropriate crosses. Although these strains are not isogenic, we have previously shown that variation of the genetic background between strains used for out-crosses and the alleles presently used does not result in significant morphological differences (Hartwell *et al.*, 1973).

(b) α Factor

α Factor was purified according to the procedure of Bücking-Throm *et al.* (1973). The activity of the factor was determined by adding various dilutions of α factor to cultures of strain A364A at a standard concentration of 2×10^6 cells/ml. The increase in unbudded cells with time was then scored. Cells can recover from α factor (Throm & Duntze, 1970), and the recovery time is directly proportional to the α factor concentration (L. Wilkenson, personal communication). Therefore a concentration of α factor was chosen that produced greater than 95% unbudded cells and that inhibited budding for at least 8 h.

(c) Analytical techniques for reciprocal shift experiments

Cell morphology, cell number and the incorporation of [$2\text{-}^{14}\text{C}$]uracil into DNA were determined by standard procedures (Hartwell, 1970). The reciprocal shift experiments were carried out in medium containing 6.7 g yeast nitrogen base (Difco), 10 g succinic acid, 6 g NaOH, 2 g glucose, 0.005 g uracil, 0.01 g adenine and 1 g yeast extract in 1 liter of water at a final pH of 5.8. Amino acids at a concentration of 0.04 g/l were added according to the requirements of the strain. YM-1 medium (Hartwell, 1967) was used for the double-mutant experiments.

3. Results

(a) Rationale of inferring order from reciprocal shifts

Assume that two steps, A and B, are each mediated by a gene product and that both steps are necessary to complete a developmental sequence. Step A may be related to step B in one of four ways (Table 1). (1) They may be related in a *dependent* sequence such that B cannot occur until the prior completion of A. (2) They

TABLE 1

Sequencing by reciprocal shifts

Relation	Completion of developmental program		
	1st incubation	restrict A, permit B permit A, restrict B	restrict B, permit A permit B, restrict A
(1) Dependent A B →→→→		—	+
(2) Dependent B A →→→→		+	—
(3) Independent A → B →		+	+
(4) Interdependent A,B →→		—	—

may be related in a *dependent* sequence such that A cannot occur without the prior completion of B. (3) The two steps may be *independent*; either A or B can occur in the absence of the other. (4) They may be *interdependent* such that neither can occur in the absence of the other. Now assume that each step can be reversibly and independently blocked. It is then possible to distinguish between the four possibilities by doing two experiments.

In the first experiment the organism is placed at the restrictive condition for A (A is blocked) and the permissive condition for B at the outset of the developmental program until such time as both steps A and B would have been completed in an unperturbed culture (first incubation). The A block is then reversed by shifting from the restrictive to the permissive condition for A and, at the same time, B is blocked by shifting from the permissive to the restrictive condition for B (second incubation). If the developmental program is completed during the second incubation then step B was completed during the first incubation without the completion of step A and models (1) and (4) are ruled out. If the developmental program is not completed during the second incubation then step B was not completed during the first incubation and models (2) and (3) are eliminated.

To distinguish between models (1) and (4) or (2) and (3), the reciprocal protocol is carried out. The organism is started at the restrictive condition for B and the permissive condition for A and held there long enough for both steps A and B to have been completed in an unperturbed system (first incubation). The organism is then shifted to the permissive condition for B and at the same time to the restrictive condition for A (second incubation). If the developmental program is completed during the second incubation, then step A was completed during the first incubation without the completion of step B, and the order is consistent with models (1) or (3). If the developmental program is not completed during the second incubation, then step A was not completed during the first incubation and the order is that presented in models (2) or (4). Together the two reciprocal experiments distinguish between all four possible models listed in Table 1.

(b) *Restrictive conditions*

The first restrictive condition that we have used is the restrictive temperature for three mutants that are temperature sensitive for the initiation of DNA synthesis. The three mutants (defective respectively in genes *cdc* 28, *cdc* 4 and *cdc* 7) are members of a collection of cell division cycle mutants (Hartwell *et al.*, 1970, 1973). Evidence that cells carrying any one of these mutations arrest before the initiation of DNA synthesis has been presented elsewhere (Hartwell, 1973).

The second restrictive condition is the presence of α factor. α Factor is a substance produced by cells of α mating type which Bücking-Throm *et al.* (1973) have shown arrests cells of α mating type before the initiation of DNA synthesis. This substance is thought to have a role in the synchronization of the haploid cell cycles before cell fusion during zygote formation.

Since each of the four restrictive conditions results in the arrest of cells at a specific point in the cell cycle, the population of cells assumes a homogeneous morphology. This morphology has been called the *terminal phenotype*. This fact permits one to assess the fraction of the population arrested at a particular block, the utility of which will become apparent in subsequent sections.

(c) *Sequence of the cdc 4 and α factor-sensitive steps*

Cells carrying the *cdc* 4 mutation were aligned at the α factor block by growing them for three hours at the permissive temperature in the presence of α factor. At this time ongoing DNA synthesis had been completed, but subsequent rounds of DNA synthesis were not initiated as evidenced by a comparison of DNA synthesis in the α factor-treated culture with a control culture (Fig. 1(a)). Cell counts revealed that by three hours the population had undergone one cell division and microscopic examination showed that greater than 90% of the cells had accumulated as single unbudded cells, the terminal phenotype of α arrested cells (Bücking-Throm *et al.*, 1973). The factor was then removed and cells were either shifted to the restrictive temperature or maintained at the permissive temperature.

Cells maintained at the permissive temperature initially, underwent one synchronous round of DNA synthesis after which synchrony decayed but DNA synthesis continued (Fig. 1(a)). Budding and cell division paralleled the pattern of DNA synthesis: phase contrast microscopy showed that a normal budding cycle accompanied DNA synthesis and cell counts demonstrated that the first round of DNA synthesis was followed by a synchronous cell division, which was completed by six hours. Subsequent budding and cell division showed a decay in the synchrony of both these processes. As determined by the criteria of DNA synthesis, cellular morphology and cell division, cells carrying the *cdc* 4 mutation can resume normal cell cycles after removal of α factor from which we conclude that the α factor block is reversible, in agreement with the previous findings of Bücking-Throm *et al.* (1973).

In contrast to the behavior of cells maintained at 23°C, cells shifted to 36°C failed to undergo significant DNA synthesis (Fig. 1(a)). Furthermore, the cells did not divide, and gradually acquired the terminal phenotype characteristic of the *cdc* 4 mutation, a population of cells with elongated buds (Hartwell *et al.*, 1973). The terminal morphology as well as the failure to observe a round of DNA synthesis or cell division after removal of α factor and a shift to the restrictive temperature establishes that the *cdc* 4 gene product did not complete its function while cells were arrested at the α factor block. This result is a specific consequence of the *cdc* 4 mutation since a shift

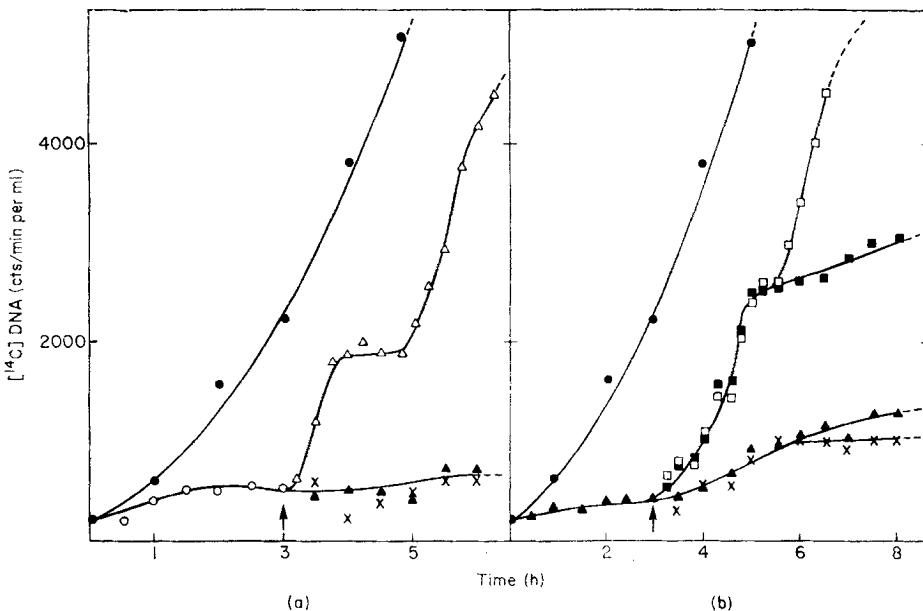


FIG. 1. (a). DNA synthesis patterns following a shift from the α factor block to the *cdc 4* block. A logarithmic phase culture (2×10^6 cells/ml) of H135.1.1 (*cdc 4-3*) growing at 23°C was labeled with $1 \mu\text{Ci}$ [$2\text{-}^{14}\text{C}$]uracil/ml (final spec. act. 35 mCi/mmol). After 30 min (0 time on the graph) the culture was split. One portion received α factor (—○—○—) and the other did not (—●—●—). After growth at 23°C for 3 h (time indicated by arrow), the culture with α factor was divided into 3 portions. The α factor was removed from all 3 portions by filtration of the cultures through a Millipore filter (size HA; Millipore Corp., Bedford, Mass.) followed by extensive washing with non-radioactive medium adjusted to 23°C or 36°C depending on the temperature of the ensuing incubation. One culture was incubated at 23°C (—△—△—), a second received $100 \mu\text{g}$ cycloheximide/ml and was incubated at 23°C (—×—×—), and the third was incubated at 36°C (—▲—▲—). All 3 cultures contained [$2\text{-}^{14}\text{C}$]uracil (final spec. act. 35 mCi/mmol) and samples were removed at various times and analyzed for the incorporation of radioactivity into DNA. The incorporation curves for each of the three cultures were corrected for minor differences in cell number, which existed between them and the original α factor-containing culture.

(b). DNA synthesis patterns following a shift from the *cdc 4* block to the α factor block. A logarithmic phase culture (2×10^6 cells/ml) of H135.1.1 (*cdc 4-3*) growing at 23°C was labeled with $1 \mu\text{Ci}$ [$2\text{-}^{14}\text{C}$]uracil/ml (final spec. act. 35 mCi/mmol). After growth at 23°C for 30 min (0 time on the graph), one portion of the culture was shifted to 36°C (—▲—▲—) and the remaining culture was allowed to grow at 23°C (—●—●—). After 3 h growth (time indicated by arrow), the 36°C culture was shifted down to 23°C . At the time of the shift the culture was split into 3 portions. One portion was allowed to grow unperturbed (—□—□—), one portion received α factor (—■—■—), and the third received $100 \mu\text{g}$ cycloheximide/ml (—×—×—).

of the parental strain from α factor arrest to 36°C inhibits neither DNA synthesis nor cell division (Hartwell, 1973). Therefore, these results are inconsistent with the possibility that the *cdc 4*-mediated step precedes the α factor-sensitive step in a dependent pathway or that the two steps are independent (models (2) and (3), where A is the α sensitive step and B is *cdc 4*, Table 1).

In order to distinguish between the remaining two models ((1) and (4), Table 1), we carried out the reciprocal experiment. Cells carrying the *cdc 4* mutation were arrested at the *cdc 4* block by growing them for three hours at the restrictive temperature. DNA synthesis was arrested at the restrictive temperature as evidenced by a comparison with the control culture at 23°C (Fig. 1(b)). By this time, the population

had doubled and represented a population of cells with elongated buds, the phenotype characteristic of the *cdc 4* mutation (Hartwell *et al.*, 1973). The cells were then shifted to 23°C. At the time of shiftdown, the culture was split in two and one sample received α factor.

Cells shifted back to the permissive temperature without α factor underwent one partially synchronous round of DNA synthesis and began a second (Fig. 1(b)). Cell number followed a similar pattern: cells underwent a partially synchronous doubling, which was completed by five hours. Subsequent increase in cell number ensued but the increase was asynchronous. The kinetics of cell division and DNA synthesis strongly suggest that after arrest at 36°C cells are able to complete one cell cycle and proceed into a second. From these observations we conclude that the *cdc 4* block is reversible.

Cells that received α factor at the time of shift back to 23°C also underwent a synchronous round of DNA synthesis (Fig. 1(b)). In contrast to the control culture, however, only one round of DNA synthesis ensued. Like the control culture, cell number had doubled by five hours, but further increase in cell number did not occur. Microscopic observation showed that by five hours 90% of the cells had accumulated as single unbudded cells, the morphology typical of α arrested cells.

This result is interpreted to mean that the cells completed the α factor-sensitive step while they were at the restrictive temperature, and were able therefore to undergo one round of DNA synthesis and cell division in the presence of α factor before becoming sensitive to its inhibitory effect. We conclude that the α factor-sensitive step and the *cdc 4*-mediated step are not interdependent (model (4), Table 1) but are ordered in a dependent pathway of sequence: $\xrightarrow{\alpha} \xrightarrow{4}$.

(d) Sequence of the *cdc 7* and α factor-sensitive steps

Cells carrying the *cdc 7* mutation were aligned at the α factor block by incubation for three hours in α factor at 23°C. That the cells were at the α factor block was established by the fact that by three hours DNA synthesis was arrested (Fig. 2(a)), the cell number had approximately doubled and greater than 90% of the cells had accumulated as single unbudded cells. The factor was then removed and cells were either shifted to the restrictive temperature or maintained at the permissive temperature.

As shown in Figure 2(a), the patterns of DNA synthesis after removal of α factor were analogous to those observed with the *cdc 4* mutation. At 23°C, cells underwent one synchronous round of DNA synthesis and began a second round. An examination of cell morphology and cell number established that both a normal budding cycle and cell division accompanied the first round of DNA synthesis and we conclude that cells carrying the *cdc 7* mutation are reversibly arrested by α factor, and can therefore resume normal cell cycles after removal of α factor.

Cells shifted to 38°C after removal of α factor failed to undergo significant DNA synthesis (Fig. 2(a)). The cells did not divide but synchronously accumulated as a nearly homogeneous population (89%) of cells each with one large bud, the *cdc 7* terminal phenotype (Hartwell *et al.*, 1973). We conclude that the *cdc 7*-mediated step was not completed while cells were arrested at the α factor block. Therefore the *cdc 7*-mediated step neither precedes the α factor-sensitive step in a dependent pathway nor are the two steps independent (models (2) and (3), where A is the α sensitive step and B is *cdc 7*, Table 1).

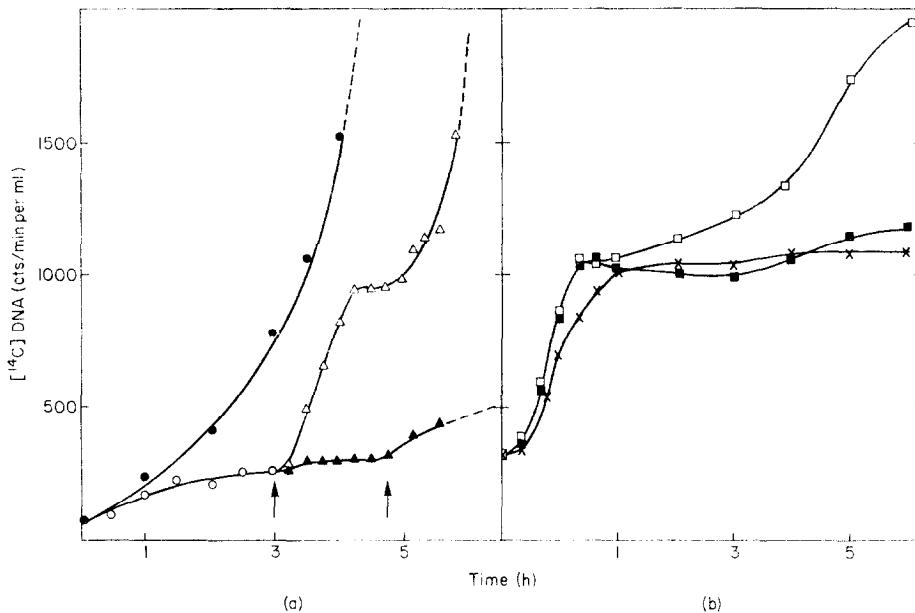


FIG. 2. (a). DNA synthetic patterns following a shift from the α block to the *cdc 7* block. Protocol for strain 4008 (*cdc 7-4*) was identical to that described in the legend to Fig. 1(a) except that the restrictive temperature was 38°C. Accordingly the appropriate media were equilibrated at 38°C. —●—●—, Asynchronous DNA synthesis at 23°C; —○—○—, DNA synthesis at 23°C in the presence of α factor; —△—△—, DNA synthesis at 23°C after removal of α factor; —▲—▲—, DNA synthesis at 38°C after removal of α factor. The first arrow indicates time of removal of α factor.

(b). DNA synthesis patterns following a shift from the *cdc 7* block to the α block. Since strain 4008 cannot be aligned at the *cdc 7* block by growing cells for one generation at the restrictive temperature (see text), a different experimental design was adopted. At 105 min (time indicated by second arrow on (a)) a portion of the culture that had been shifted to 38°C after removal of α factor (—▲—▲—) was shifted back to 23°C. At the time of shift-back (0 time on (b)), the culture was split. One portion was allowed to grow unperturbed (—□—□—); one portion received α factor (—■—■—); and the third portion received 100 μ g cycloheximide/ml (—×—×—).

To distinguish between the remaining two models ((1) and (4), Table 1), we did the reciprocal experiment. Since the *cdc 7* mutation causes inviability after prolonged incubation at the restrictive temperature (Hartwell, unpublished observations), the experiment was done in a manner designed to limit the time of exposure to the restrictive temperature. An asynchronous population of cells was synchronized by pretreatment with α factor for three hours at 23°C. The factor was then removed and cells were incubated at 38°C for 105 minutes. (105 minutes were chosen since by this time 75% of the cells had budded and therefore at least this fraction was at the *cdc 7* block.) The cells were then shifted down to 23°C. At the time of shift down, the culture was split and one sample received α factor.

Cells shifted back to 23°C in the absence of α factor underwent an initial rapid and synchronous round of DNA synthesis followed by a second partially synchronous round (Fig. 2(b)). Cell counts showed that the first round of DNA synthesis was not immediately followed by a round of cell division. Rather at 2.5 hours, cell number gradually began to increase and by four hours 50% of the cells had divided. Observation

of changes in cellular morphology revealed that the failure of cells to divide after the first round of DNA synthesis was merely due to a delay in cell separation since at about the time of the onset of the second round of DNA synthesis the cells with large buds began to bud again, producing cells with three or four buds. Division of these cells produced cells with small buds. Despite the delay in cell division, the kinetics of DNA synthesis and budding are consistent with the conclusion that arrest at the *cdc 7* block for an interval of at least 105 minutes is reversible and that following arrest at the *cdc 7* block, cells can complete one cell cycle and proceed to a second.

Cells that received α factor at the time of shift back to 23°C also underwent a synchronous round of DNA synthesis (Fig. 2(b)). In contrast to the culture without α factor, only one round of DNA synthesis occurred. Cell division was also delayed in a manner similar to that of the culture without α factor. Morphological observation showed, however, that in contrast to the cells without α factor, in this culture the cells with large buds did not bud again. Instead, as cell separation progressed, unbudded cells were produced which by four hours represented the predominant phenotype (58%). From these data we conclude that cells carrying the *cdc 7* mutation completed the α factor-sensitive step while they were at the restrictive temperature, and were able therefore to undergo one cell cycle in the presence of α factor before becoming sensitive to its inhibitory effect. We conclude therefore that the α factor-sensitive step and the *cdc 7*-mediated step are not interdependent (model (4), Table 1) but are ordered in a dependent pathway of sequence: $\alpha \rightarrow 7$.

(e) Sequence of the *cdc 28* and α factor-sensitive steps

Cells carrying the *cdc 28* mutation were aligned at the α block by incubation for three hours in α factor at 23°C. That the cells were at the α factor block was again established by the fact that by three hours DNA synthesis was arrested (Fig. 3(a)), the cell number had approximately doubled and greater than 90% of the cells had accumulated as single unbudded cells. The factor was then removed and the cells were either shifted to the restrictive temperature or maintained at the permissive temperature.

The patterns of DNA synthesis after removal of the factor were again analogous to those observed with mutants defective in *cdc 4* or *cdc 7*. At 23°C, cells underwent one synchronous round of DNA synthesis, after which a second round began. A normal budding cycle and cell division accompanied the first round of DNA synthesis. We conclude, therefore, that cells carrying the *cdc 28* mutation are reversibly arrested by α factor.

Cells shifted to 38°C after removal of α factor failed to undergo significant DNA synthesis (Fig. 3(a)). The cells did not divide but remained as unbudded cells, the terminal phenotype of the *cdc 28* mutation (Hartwell *et al.*, 1973). We conclude that the *cdc 28* gene product did not complete its function while cells were arrested at the α factor block. Therefore the *cdc 28*-mediated step neither precedes the α factor-sensitive step in a dependent pathway nor are the two steps independent (models (2) and (3), where A is the α factor-sensitive step and B is *cdc 28*, Table 1).

To distinguish between the remaining two models ((1) and (4), Table 1), we did the reciprocal experiment. Since the *cdc 28* mutation is leaky (Fig. 3(a)), the experiment was done in a manner analogous to that described above for *cdc 7*. An asynchronous population of cells was synchronized by pretreatment with α factor for three hours at 23°C. The factor was then removed and cells were incubated at 38°C for 90 minutes.

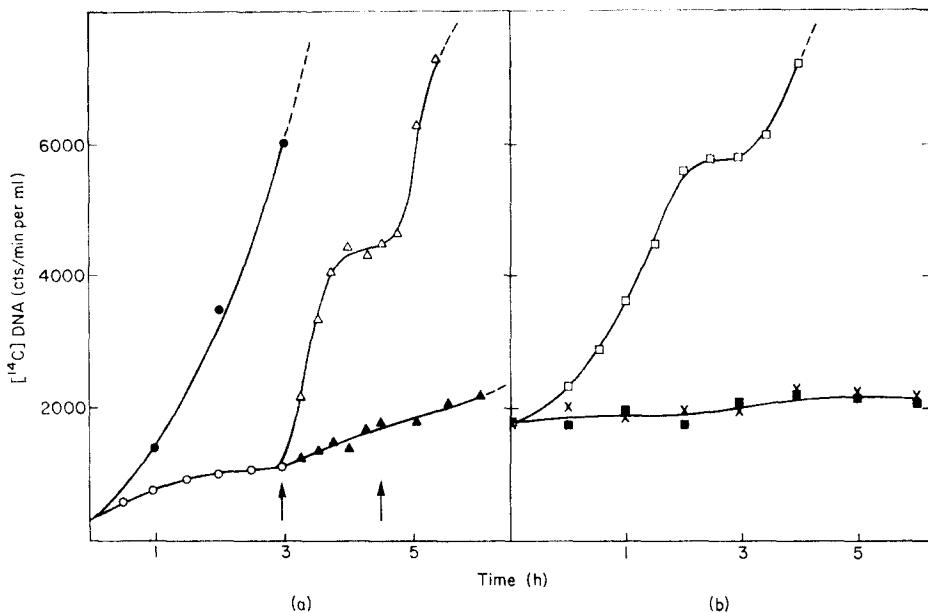


FIG. 3. (a). DNA synthesis patterns following a shift from the α block to the *cdc 28* block. Protocol for strain H185.3.4 (*cdc 28-1*) was identical to that described in the legend to Fig. 1(a) except that the restrictive temperature was 38°C . —●—●—, Asynchronous DNA synthesis at 23°C ; —○—○—, DNA synthesis at 23°C in the presence of α factor; —△—△—, DNA synthesis at 23°C after removal of α factor; —▲—▲—, DNA synthesis at 38°C after removal of α factor. The first arrow indicates time of removal of α factor.

(b). DNA synthesis patterns following a shift from the *cdc 28* block to the α block. Protocol for strain H185.3.4 (*cdc 28-1*) was the same as that described in the legend to Fig. 2(b) except that the time at which portions of the 38°C culture were shifted back to 23°C was 90 min (time indicated by second arrow on (a)). Time of shift back is 0 time on (b) and the curves are as follows: —□—□—, DNA synthesis in cells shifted to 23°C ; —■—■—, DNA synthesis in cells shifted to 23°C plus α factor; —×—×—, DNA synthesis in cells which received $100 \mu\text{g}$ cycloheximide/ml 5 min before the shift to 23°C .

(90 minutes were chosen since controls have established that when wild-type cells are shifted to 38°C after arrest with α factor more than 90% of the cells have budded by this time.) The cells were then shifted down to 23°C . At the time of shift down, the culture was split and one sample received α factor.

Cells shifted back to 23°C in the absence of α factor underwent a partially synchronous round of DNA synthesis, after which a second round began (Fig. 3(b)). Cell counts failed to show any increase in cell number. However, observation of cell morphology showed that the population of unbudded cells went through a normal budding cycle. By two hours, 80% of the cells had accumulated as cells with one large bud. By four hours, 40% of these cells had budded again. Although the reason for the absence of cell division following arrest at the *cdc 28* block is not understood, the budding pattern as well as the pattern of DNA synthesis suggest that cells can recover from the *cdc 28* block, with respect to these two processes.

Cells shifted back to 23°C in the presence of α factor failed to undergo significant DNA synthesis (Fig. 3(b)). Again there was no increase in cell number. Morphological observation revealed that the cells failed to bud and gradually elongated, characteristic of the *cdc 28* terminal phenotype.

From the observation that in *cdc 28* neither DNA synthesis nor budding occurs when cells are shifted from the α factor block to the *cdc 28* block or from the *cdc 28* block to the α factor block we conclude that the *cdc 28*-mediated step and α factor-sensitive step act in an interdependent pathway (model (4), Table 1). Moreover, since we have shown that the α factor-sensitive step precedes both the *cdc 4* and *cdc 7*-mediated steps, these results suggest that the *cdc 28*-mediated step also precedes the steps mediated by the *cdc 4* or *cdc 7* genes.

(f) *Rationale of inferring order from double mutants*

The reciprocal shift method is applicable only for conditional blocks that can be independently imposed. With this method it was possible to infer the order of the *cdc 4*, *7* and *28*-mediated steps relative to the α factor-sensitive step; however, it was not possible to determine the order of the *cdc 7* and *cdc 4*-mediated steps since the restrictive condition for each of these blocks is the same.

The order of the *cdc 7* and *cdc 4*-mediated steps can be inferred by a comparison of the phenotypes of the respective single and double mutant strains. Consider again the four ways in which two steps, A and B, can be related in a developmental program (Table 2). The basic reasoning for using double mutants to infer order is as follows. If two mutant strains complete the same step(s) at the restrictive temperature they will have the same phenotype. If two mutant strains do not complete the same step(s) at the restrictive temperature they may, but need not necessarily, show different phenotypes. The first assumption is only rigorously correct if the mutant gene products are completely non-functional at the restrictive temperature (i.e. they are amorphic alleles).

Consider the situation where two single mutants have different phenotypes, as in the case of mutant strains carrying the *cdc 4* and *cdc 7* mutations. Since the two single mutants have different phenotypes their relation cannot be one of interdependence (model (4)) since in both single mutants neither step A nor step B would occur at the restrictive temperature. Hence, models (1), (2) and (3) in Table 2 remain as possibilities. If A precedes B in a dependent pathway (model (1)), then the double mutant

TABLE 2
Sequencing by double mutants

Relation	Steps completed in mutant			Phenotype of AB
	A	B	AB	
(1) Dependent	A → →	neither	A	neither =A
(2) Dependent	B → →	B	neither	neither =B
(3) Independent	A → B →	B	A	neither unique
(4) Interdependent	A,B →	neither	neither	neither =A =B

should have the same phenotype as the A mutant since in both the double mutant and the single A mutant, neither steps A or B will be completed. By the same reasoning, if B precedes A in a dependent pathway (model (2)), the double mutant should have the same phenotype as the single B mutant.

However, it is not always possible to distinguish one of the dependent relations (models (1) or (2)) from an independent relation (model (3)). In theory, if the two steps are independent, a double mutant might have a phenotype distinct from either of the single mutants since in either single mutant, one of the two steps is occurring while in the double mutant neither step occurs. However, in practice there is no assurance that our resolution is sufficient to reveal the expected phenotypic difference, and for this reason, if the double mutant is identical in phenotype to one of the single mutants the result is consistent with either an independent relation (model (3), Table 2) or one of the two possible dependent relations (models (1) or (2), Table 2).

(g) *Phenotypes of double-mutant strains*

Mutations in each of the three genes, *cdc 28*, *cdc 4* and *cdc 7* produce a distinct terminal phenotype at the restrictive temperature. The cells carrying these lesions have an essentially normal phenotype at the permissive temperature (Plate I(a)). A mutation in gene *cdc 28* results in a failure of bud emergence and in the initiation of DNA synthesis (Hartwell, 1973). If an asynchronous culture of cells carrying the *cdc 28* mutation is shifted to the restrictive temperature most of the cells are unbudded after one generation time (Plate I(b) and Table 3). After longer intervals at 36°C the cells continue to elongate and branch; and by this time, some of the *cdc 28* mutant cells are similar in appearance to *cdc 4* mutant cells (Plate I(c) and Table 3). The phenotypes of the two mutants are best distinguished by observing the fraction of unbudded cells after one cycle time at 36°C, which is high for the *cdc 28* mutation and low for the *cdc 4* mutation (Table 3). The morphological effects of the *cdc 28* mutation at the restrictive temperature are almost indistinguishable from that produced by the action of the α factor on α cells. This result is consistent with the conclusion from the reciprocal shift protocol that the *cdc 28*-mediated step and the α factor-sensitive step are interdependent.

Mutations in the *cdc 4* gene result in continued bud emergence, at periodic intervals of about one cycle time, without cell division at the restrictive temperature (Hartwell, 1971). The buds continue to elongate and cells are produced that contain as many as five long buds on a single parent cell (Plate I(d) and Table 3).

Cells carrying the *cdc 7* mutation arrest development with a single large bud (Plate I(e) and Table 3). The parent cell and bud continue to enlarge at the restrictive temperature.

Since each of the phenotypes produced by the lesions in these three genes result in a distinct cellular type it is unlikely that any two of these gene-mediated steps are related in an interdependent relation. In order to gain some insight into their dependent relations we have constructed double-mutant strains and examined their phenotypes.

Asynchronous populations of double-mutant strains were shifted to the restrictive temperature and the phenotypes of the cells were compared with those of the single mutants at successive two-hour intervals (Table 3). If the two gene-mediated steps are related in a dependent pathway then most of the double mutant cells should assume the phenotype characteristic of the first step, since few cells in the asynchronous

TABLE 3
Phenotypes of single and double-mutant strains

Mutant gene	Time at 36°C (h)	Cellular phenotype				
		A	B	C	D	E
28	0	31	48	21	0	0
	2	79	8	10	1	2
	4	21	1	2	30	46
4	0	45	39	16	0	0
	2	36	25	3	35	1
	4	10	3	4	79	4
	6	11	1	1	85	2
7	0	33	50	17	0	0
	2	30	33	37	0	0
	4	11	6	83	0	0
	6	4	1	95	0	0
28 and 4	0	27	48	24	1	0
	2	75	13	12	0	5
	4	77	3	3	0	17
28 and 7	0	30	50	19	0	1
	2	69	16	6	0	9
	4	60	1	1	5	33
4 and 7	0	33	49	18	0	0
	2	27	27	7	35	4
	4	8	5	3	81	3
	6	8	1	2	85	4

Asynchronous populations of mutant cells growing at the permissive temperature (23°C) in YM-1 medium were shifted to the restrictive temperature (36°C). The strains were the same as those in legend to Fig. 4. One-ml samples were withdrawn at various times and diluted into 9 ml of 0.15 M-NaCl containing 3.7% formaldehyde. The samples were sonicated and scored for the various morphological types by phase-contrast microscopy. Two hundred cells were scored for each sample. A, unbudded cells; B, cells with normal buds less than half the length of the parent cell; C, cells with normal buds larger than half the length of the parent cell; D, cells with one or more elongated buds (characteristic of the *cdc* 4 lesion); E, cells with unusual morphologies different from A through D (characteristic of the *cdc* 28 lesion).

population will be between the two successive steps in the G1 portion of the cycle at the time of the shift.

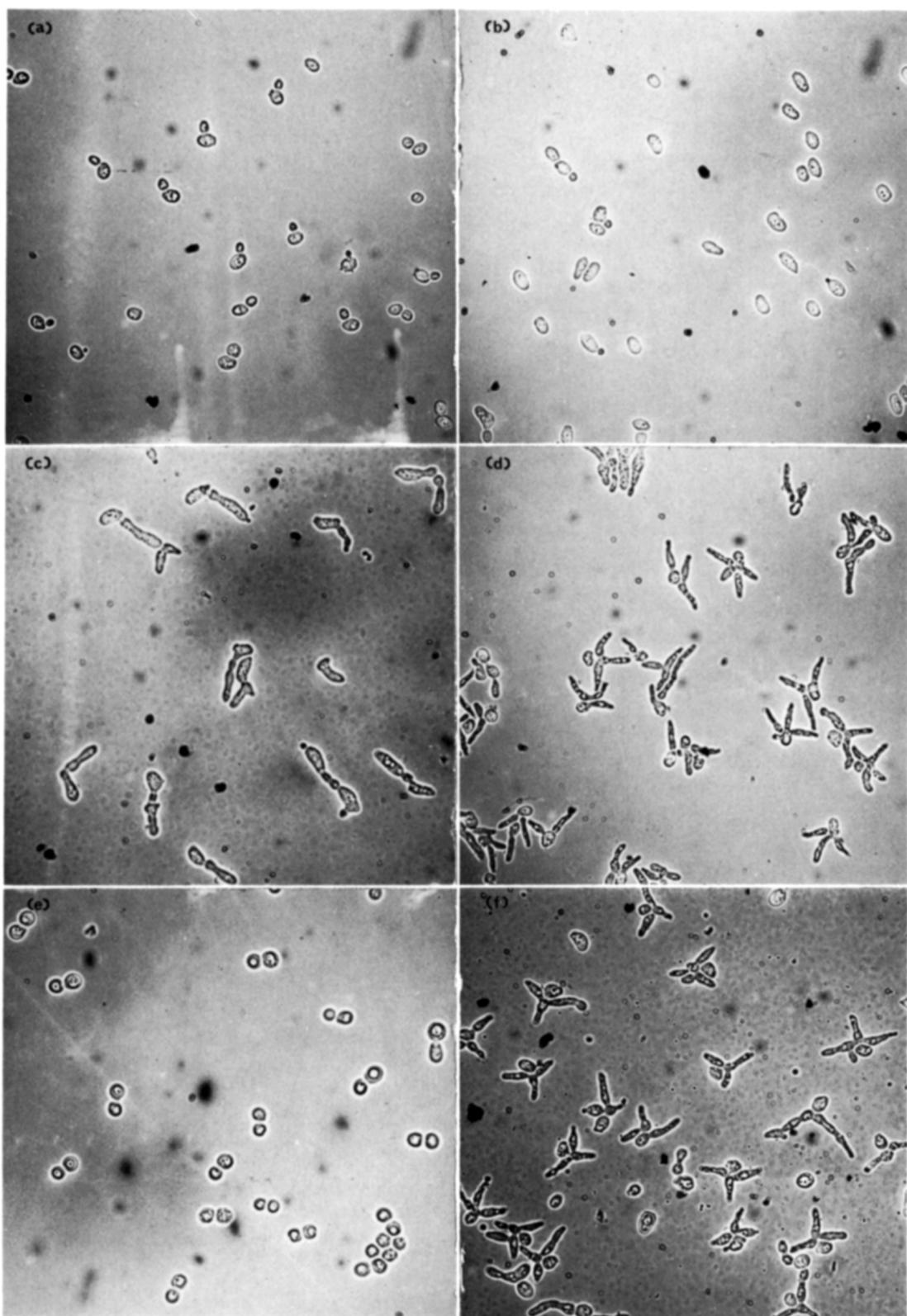
The *cdc* 28-*cdc* 4 and *cde* 28-*cde* 7 double-mutant strains showed phenotypes like that of the *cde* 28 mutant (Table 3). This result is consistent with the conclusions derived from the reciprocal shift protocol that the *cde* 28 step precedes the *cde* 4 and *cde* 7-mediated steps in a dependent pathway. The *cde* 4-*cde* 7 double mutant strain has the phenotype of the *cde* 4 single mutant (Plate I(f) and Table 3). This result is inconsistent with the hypothesis that the *cde* 7-mediated step precedes the *cde* 4-mediated step. We conclude that the *cde* 4 and *cde* 7-mediated steps are either related in a dependent pathway in the sequence *cde* 4 then *cde* 7 or that they are independent of one another. For reasons that will be explained below we favor the former hypothesis.

(h) Requirements for protein synthesis

Protein synthesis is required for the initiation of DNA synthesis in *S. cerevisiae* but not for the completion of replication once the initiation step(s) have occurred (Hereford & Hartwell, 1973; Williamson, 1973; Slater, personal communication). We

PLATE I. Morphologies of single and double-mutant strains. Cells from the experiment described in the legend to Table 3 were placed on a thin film of agar, overlaid with a coverslip, and photographed by bright-field illumination.

- (a) Strain 4C1A7C1a29 carrying mutations in genes *cdc* 4 and *cdc* 7 at the permissive temperature.
- (b) Strain 28C1a2 carrying a mutation in gene *cdc* 28 after 2 h at 36°C.
- (c) Strain 28C1a2 carrying a mutation in gene *cdc* 28 after 6 h at 36°C.
- (d) Strain 4C1a1 carrying a mutation in gene *cdc* 4 after 6 h at 36°C.
- (e) Strain 7C4a2 carrying a mutation in gene *cdc* 7 after 6 h at 36°C.
- (f) Strain 4C1A7C1a29 carrying mutations in genes *cdc* 4 and *cdc* 7 after 6 h at 36°C.



have attempted to locate the site(s) of this requirement within the sequence of gene-mediated steps by determining whether an inhibition of protein synthesis prevents the ensuing round of DNA synthesis after cells have been aligned at the α factor-sensitive and temperature-sensitive blocks. The data from these experiments are included in Figures 1(a), 1(b), 2(b) and 3(b). Protein synthesis is required for DNA synthesis after arrest at the α factor, the *cdc 28*, and the *cdc 4* blocks, but not after arrest at the *cdc 7* block. Cesium chloride gradients have demonstrated that more than 90% of the DNA made in a *cdc 7* mutant is of nuclear density following a shift from the restrictive temperature to the permissive temperature in the presence of cycloheximide (data not shown).

These results are consistent with the placement of the requirement for protein synthesis after the *cdc 4*-mediated step and before the *cdc 7*-mediated step, but do not rigorously rule out an assignment before the *cdc 4* step for the following reason. One might argue that the inability of the *cdc 4* mutant to undergo DNA synthesis after a shift from restrictive to permissive temperature in the absence of further protein synthesis is an artifact of the irreversible thermal denaturation of the *cdc 4* gene product, which must then be resynthesized at the permissive temperature before DNA synthesis can occur. However, since four independently isolated alleles of the *cdc 4* gene show the same requirement for protein synthesis after a shift from the restrictive to the permissive temperature while three independently isolated alleles of *cdc 7* do not (Hereford, unpublished observations), we feel justified in tentatively placing the requirement for protein synthesis after the *cdc 4*-mediated step, and before the *cdc 7*-mediated step.

4. Discussion

(a) *Interpretation of the sequencing results*

The two methods that we have used for determining the order of gene-mediated steps should have broad applicability in developmental genetics. However, several considerations must be borne in mind in applying these methods.

The reciprocal shift method requires that one be able to block independently and reversibly the two steps under consideration. Independence can be achieved by using a heat and a cold-sensitive mutant (Jarwick & Botstein, 1973), an inhibitor and a temperature-sensitive mutation (present work), or two inhibitors. It is of course important to know that a single step is being blocked at a time. With mutants and with inhibitors it is possible to ascertain that a single gene product is being affected; however, one must generally assume that this gene product has but one function. Control experiments can establish that the restrictive conditions are reversible and in the present experiments this condition was met.

In addition to the two initial criteria, which must be established before the reciprocal method can be meaningfully used, two additional considerations must also be taken into account. First, the cells must be left at the restrictive condition in the first incubation long enough for both steps to have been completed in an unperturbed culture. Here we have used three criteria to show that this condition obtains: DNA synthesis, cell number and the terminal phenotype. In addition, the observation that reversal of each of the blocks results in partial synchrony for the ensuing replication cycle provides corroborative evidence that the majority of cells in the population are arrested at a unique point in the cell cycle. Since each of the three steps under

consideration normally occurs during a very short interval of the cell cycle these observations demonstrate that sufficient time had been allowed for both steps to have been completed in an unperturbed system.

Second, after the shift protocol has been carried out, one must ascertain whether or not the developmental program has been completed. In the present studies we have used the ability of the cells to complete one round of DNA synthesis as an assay. This analysis suffers from the criticism that the patterns of DNA synthesis which we observe may be fortuitous and may not in reality represent the completion of a normal round of replication. However, the fact that cells can continue through subsequent cell-cycle events (i.e. cell division, or budding, or both), after arrest at each of the restrictive conditions makes it likely that the DNA synthetic period was successfully completed.

As pointed out by Jarwick & Botstein (1973), even if all of the above conditions are met, erroneous answers may be obtained as a result of the artificial situation imposed by the reciprocal shift protocol itself. The protocol requires separating in time, two steps, A and B, which may follow one another very rapidly *in situ*. If step B is dependent upon some change brought about by step A, but this change is short-lived, then the sequencing protocol will provide an incorrect answer of interdependence (Table 1), whereas the true situation will be one of dependence. In our experiments, this possibility must be considered for the *cdc 28*-mediated and α factor-sensitive steps.

The double-mutant method suffers from a limitation not found in the reciprocal shift method, namely that the two blocks defined by the two mutations must produce phenotypes that can be readily and unambiguously identified. In the present study we have been able to exploit the fact that the three mutations in question each produce distinct terminal phenotypes. Furthermore, an assumption inherent in the double-mutant rationale is that the mutant-gene product is completely non-functional at the restrictive condition (i.e. an amorphic allele). While this assumption is difficult to verify and should be strongly suspect with temperature-sensitive mutations it is likely to be true in cases where several independently isolated alleles display the same phenotype. This is the case for two of the three genes used in the present study, *cdc 4* and *cdc 7*. Finally, the double mutant method does not have quite the resolving power of the reciprocal shift method. For reasons that were pointed out earlier, although it is possible to distinguish the two possible dependent relations it is not always possible to distinguish an independent relation from one of the dependent relations.

Using both sequencing approaches we have been able to order a series of three steps that are required for the initiation of DNA synthesis in *S. cerevisiae*. It must be emphasized that the derived sequence is consistent with a variety of biochemical mechanisms. A dependence of one event upon another may be due to a dependence for the synthesis of the gene product in question or for the ability of the gene product to do its function. We will be able to decide between these two alternatives only when we know whether the particular temperature-sensitive or inhibitor-sensitive steps act at the level of synthesis or function of the relevant gene products.

(b) Corroborative evidence for the derived sequence

Two lines of evidence confirm the derived sequence of steps. The first is the phenotypes of the mutants with respect to budding. Mutant *cdc 28* and α factor-arrested cells remain unbudded at the restrictive temperature, *cdc 4*-arrested cells bud multiply and *cdc 7*-arrested cells bud once. Since multiple budding is not characteristic of the

normal cell cycle, it is not obvious what the different budding patterns of mutants defective in *cdc 4* or *cdc 7* mean with respect to their relative order in the dependent sequence. However, it is logical that the *cdc 28* block, which arrests cells before bud emergence, should precede in the dependent sequence *cdc 4* and *cdc 7*, which do not.

A second line of evidence derives from the morphology of the spindle plaque in these mutants. The spindle plaque is a structure located within the nuclear membrane from which the microtubules originate that are presumed to have a role in chromosome segregation (Robinow & Marak, 1966). The following sequence of changes in the plaque during the mitotic cell cycle has been established by Byers & Goetsch (1973). Early in the cell cycle a cell contains a single plaque, which then duplicates at about the time of budding to produce two plaques lying side by side in the membrane (a double plaque). The two plaques then separate to opposite sides of the nucleus with a bundle of microtubules passing from one to the other (the complete spindle). At the time of nuclear division, the spindle elongates and the nuclear membrane is pinched in half thereby restoring the single plaque condition.

The four conditions that we have used arrest cells at different stages of this sequence of plaque development: both the *cdc 28* mutant and α factor-arrested cells contain a single plaque, the *cdc 4* mutant contains a double plaque, and the *cdc 7* mutant contains a complete spindle (Byers & Goetsch, 1973). The fact that the order of mutants from earliest to latest stages of plaque development is the same as the order of steps derived from experiments reported here is strong support for both studies.

(c) Role of the α factor and the *cdc 28*, *4* and *7* genes in cell physiology

Several changes in cell phenotype accompany the sequential gene-mediated steps that culminate in the initiation of DNA synthesis (Fig. 4). Before completion of both the α factor-sensitive and *cdc 28*-mediated step, the cell is unbudded, possesses a single nuclear plaque (Byers & Goetsch, 1973), and has the ability to mate with cells of opposite mating type (B. Reid, personal communication). This stage is the earliest period of the cell cycle that we have been able to recognize by mutational blocks and represents a time before commitment of the cell with respect to its developmental potential.

Following the completion of the α factor-sensitive and *cdc 28*-mediated step the cell is committed to the mitotic cycle, since it loses its ability to mate (Reid, personal communication) simultaneously acquires the ability to bud and to duplicate the nuclear plaque (Byers & Goetsch, 1973). As determined by the *cdc 4* terminal phenotype, the ability to bud is unrestricted for cells at this stage of the cell cycle since they undergo several budding events with a periodicity of approximately one cell cycle despite the fact that DNA synthesis and nuclear division do not occur (Hartwell, 1971).

The completion of the *cdc 4*-mediated step imposes a restriction upon the budding

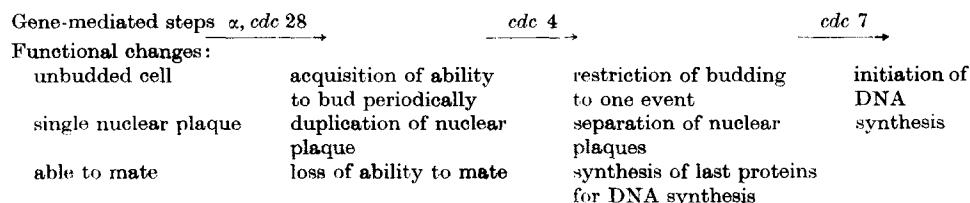


FIG. 4. Gene-controlled events in the G1 period of the yeast cell cycle.

processes such that mutants blocked subsequent to this point (DNA initiation, *cdc 7*; DNA synthesis, *cdc 8*, *cdc 21*; nuclear division, *cdc 13*; and others) bud only once (Culotti & Hartwell, 1971; Hartwell *et al.*, 1973; Hartwell, 1973). Completion of this step also permits the duplicated plaques to separate (Byers & Goetsch, 1973) and allows the cell to synthesize all the proteins needed for DNA replication, since mutants blocked beyond this step (*cdc 7*, 8 and 21) are able to complete DNA synthesis in the absence of further protein synthesis (this report; and Hereford, unpublished observations). Completion of the *cdc 7* step leads to the initiation of DNA synthesis. Mutants blocked in DNA replication (*cdc 8* and 21) do not differ from *cdc 7* in their budding behavior, the morphology of their nuclear plaques (Byers & Goetsch, 1973), or their ability to mate (B. Reid, personal communication).

(d) *Implications of the temporal order for the initiation of DNA synthesis in other systems*

Cells blocked at the *cdc 7* mutation have synthesized all the proteins that are required for the initiation and completion of DNA synthesis. In this context it is interesting to note that the *cdc 7* lesions appear similar to the *Escherichia coli dnaC* DNA initiation defects. Like *cdc 7*, *dnaC* mutants do not require protein synthesis for the initiation of DNA synthesis and, moreover, this property seems to hold for two *dnaC* alleles that have been isolated (Schubach *et al.*, 1973). The other *E. coli* initiation mutant (*dnaA*) parallels the behavior of mutants defective in *cdc 4*, since protein synthesis is required for the initiation of DNA synthesis after arrest at the restrictive temperature and furthermore, this requirement does not seem to be allele specific (Hirota *et al.*, 1970; Abe & Tomizawa, 1971; Keumpel, 1969). Since we have been able to show that the *cdc 4* gene-mediated step precedes the *cdc 7*-mediated step, it would not be surprising if suitable techniques could be developed, to discover a similar dependent relation between the *E. coli dnaA* and *dnaC* initiation mutants.

Recently Hori & Lark (1973) have shown that the inability of Chinese hamster cells to complete the S phase of the cell cycle in the absence of protein synthesis is due to the inhibition of the initiation of replicons, and these authors suggest that replicon initiation in higher eukaryotes may be controlled by the synthesis of a variety of initiators. Since replication of yeast chromosomes also occurs *via* multiple replicons (Newlon *et al.*, 1974), one must conclude that in contrast to higher eukaryotes, the synthesis of proteins responsible for replicon initiation in yeast can occur before completion of the *cdc 7*-mediated step and hence before initiation of DNA synthesis. Furthermore, since all yeast replicons probably do not initiate simultaneously (Newlon, personal communication), programs of replicon initiation cannot be due to programmed synthesis of initiator proteins in this organism.

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