A Dependent Pathway of Gene Functions Leading to Chromosome Segregation in *Saccharomyces cerevisiae*

JOHN S. WOOD and LELAND H. HARTWELL
Department of Genetics, University of Washington, Seattle, Washington 98195

ABSTRACT Methyl-benzimidazole-2-ylcarbamate (MBC) inhibits the mitotic cell cycle of *Saccharomyces cerevisiae* at a stage subsequent to DNA synthesis and before the completion of nuclear division (Quinlan, R. A., C. I. Pogson, and K. Gull, 1980, *J. Cell Sci.*, 46: 341-352). The step in the cell cycle that is sensitive to MBC inhibition was ordered in reciprocal shift experiments with respect to the steps catalyzed by *cdc* gene products. Execution of the CDC7 step is required for the initiation of DNA synthesis and for completion of the MBC-sensitive step. Results obtained with mutants (*cdc*2, 6, 8, 9, and 21) defective in DNA replication and with an inhibitor of DNA replication (hydroxyurea) suggest that some DNA replication is required for execution of the MBC-sensitive step but that the completion of replication is not. Of particular interest were mutants (*cdc*5, 13, 14, 15, 16, 17, and 23) that arrest cell division after DNA replication but before nuclear division since previous experiments had not been able to resolve the pathway of events in this part of the cell cycle. Execution of the CDC17 step was found to be a prerequisite for execution of the MBC-sensitive step; the CDC13, 16 and 23 steps are executed independently of the MBC-sensitive step; execution of the MBC-sensitive step is prerequisite for execution of the CDC14 and 23 steps. These results considerably extend the dependent pathway of events that constitute the cell cycle of *S. cerevisiae*.

The reproduction of all eucaryotic cells requires the precise replication and segregation of chromosomes as well as the partitioning of organelles and division of the cell. Each of these processes is likely to be very complicated, requiring the function of hundreds of gene products. Indeed, over 50 genes that each function at a precise stage of the cell division cycle have been detected in the yeast *Saccharomyces cerevisiae* (13) through the isolation of temperature-sensitive lethal mutants.

The successful production of two viable cells from one requires not only that these genes function but that their activity be properly coordinated. Some insight into the coordination of gene function during cell division has been obtained from temporal mapping experiments which define when a temperature-sensitive gene product has completed its normal function (13) and by cataloging the gene products required for completion of each landmark event in the cycle (17). However the most definitive approach to the coordination of events is that which permits the resolution of the dependent pathways in which gene products function. By the method of reciprocal shifts (11, 15, 16) it is possible to discriminate between the four relationships that are possible for two steps in the cell cycle. The methodology requires that it be possible to arrest cells, reversibly and independently, at each of the two steps and entails two series of experiments in which cells are shifted from one arrest point to the other. If this experimental approach demonstrates that one step is prerequisite for completion of the other then a functional coordination within the events of the cell cycle is revealed. This approach has been used to determine the dependent sequence of function for some *cdc* gene products relative to start, the control step in the cell cycle that is inhibited by mating factor (4); for *cdc* gene products relative to DNA replication, the step that is inhibited by hydroxyurea (15); and for some cold-sensitive *cdc* mutants relative to the steps executed by some heat-sensitive *cdc* mutants (D. Moir and D. Botstein, *Genetics*, in press).

The experiments reported here make use of a reversible cell cycle inhibitor, methyl-benzimidazole-2-ylcarbamate (MBC). MBC is known to bind to β tubulin of *Aspergillus nidulans* (6) and *S. cerevisiae* (P. Baum and J. Thorner, personal communication) and to inhibit cell division by virtue of its interaction with β-tublin since resistant mutants in *A. nidulans* (20) and *S. cerevisiae* (D. Botstein, personal communication) occur in the β-tubulin structural gene. We hoped that reciprocal shift experiments with MBC would permit resolution of the dependent steps in the cell cycle subsequent to DNA synthesis where chromosome segregation must occur. We had shown previously

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that *cdc* mutants 5, 13, 14, 15, 16, 17, 20, and 23 completed DNA synthesis but failed to complete nuclear division (10, 11). Byers and Goetsch (5) have demonstrated that *cdc* mutants 13, 16, 17, 20, and 23 arrest with a short spindle while *cdc* mutants 5, 14, and 15 arrest with an elongated spindle; presumably chromosome segregation occurs between these two sets of blocks.

MATERIALS AND METHODS

Strains

Diploid strains were used in the reciprocal shift experiments because they gave a more uniform cell cycle arrest in MBC than did haploid strains. Diploid strains were made by crossing the *cdc* haploid strains isolated by Hartwell, Culotti, and Reid (12) with strain α131-20 (21). The heterozygous diploids were treated with 6,400 rads of x-rays (from a Picker Control Rectifier x-ray Generator, Cleveland, OH) and homozygous *cdc* clones were isolated to make a set of nearly isogenic *cdc* diploid strains. Strains with designations beginning with "D" were a gift from David Schild (University of California); all other strains were constructed by the authors. W828 was made by mating A364A, the parental strain in which the *cdc* mutations were originally isolated, to α131-20. All strains were grown overnight at 23°C to mid-log phase before use. The restrictive temperature for the *cdc* mutations is 36°C.

Media

The minimal liquid medium (M) used has been described elsewhere (J. Wood, in press). The solid medium used for time-lapse photomicroscopy (C-agar) was a defined, complete medium also described elsewhere (26), except that it was solidified with 1% noble agar (Difco Laboratories, Detroit, MI). MBC was added to media after autoclaving to a final concentration of 100 μg/ml from a stock solution that contained 20 mg MBC/ml dimethylsulfoxide. For HU medium, hydroxyurea (HU) was added to media after autoclaving to a final concentration of 0.1 or 0.3 M from a filter sterilized stock solution of 3 M hydroxyurea in water.

Assaying Cell Cycle Parameters

The percentage of unbudded, small budded, or large budded cells was assayed by sonicating the cells for 15 s at 100 W (Braun-Sonic Sonicator model 1510, VWR Scientific, Seattle, WA) to separate clumps and then scoring 100 to 200 cells using a phase contrast microscope. Nuclear morphology was assayed by fluorescent staining of DNA with DAPI (4',6'-diamidino-2-phenylindole-2HCl) as described by Williamson and Fennell (25). DNA synthesis was measured by the incorporation of ^{14}C-uracil into TCA-precipitable counts after NaOH hydrolysis (11). Cell number was monitored by particle count using a Coulter cell counter (Model ZB, Coulter Electronics, Hialeah, FL).

Time-lapse Photomicroscopy

The procedure for time-lapse photomicroscopy of cells was modified from that described previously (12). Cells were sonicated to remove clumps and spotted onto a 10 × 30 × 1-mm thick slab of C-agar on a glass microscope slide. A small piece of fine mesh nylon screen washed in ethanol was placed over the spot of cells; this allowed rapid location of the same field at successive intervals. The agar slab and screen were enclosed within a small plexiglass frame, and a cover slip placed on the frame to prevent the agar from drying out between photographs. The cells were viewed using a microscope with darkfield illumination. For experiments done at 36°C the microscope was placed in an enclosure maintained at this temperature by an Air Curtain Incubator (Sage Instruments, White Plains, NY). Photographs were made with either a Zeiss or Olympus 35 mm camera using Kodak Tri-X film developed normally with Kodak D-76 developer.

Reciprocal Shift Experiments

The procedure used in the reciprocal shift experiments is diagrammed in Table I. In the first set of experiments (MBC → *cdc* shift) exponentially growing cells were incubated at 23°C with (culture B) or without (culture A) MBC. After a 3.5- to 4.5-h incubation, culture B was split into two parts. One part was incubated under the same conditions for a further 8 h (culture B-i) and the other was washed in isotonic saline and resuspended in fresh medium without MBC (cultures B-ii, B-iii, B-iv). Immediately after the cells were resuspended, the culture was divided in half and one half was transferred to 36°C (culture B-iii). The other half was sonicated and spread on two slabs of C-agar for time lapse photography (culture B-ii, incubated at 23°C and culture B-iv, incubated at 36°C). Similar transfers were made at the same time for culture A as indicated in Table I. The experimental culture, culture B-iv, was assayed by photographing fields of cells immediately after transfer from 23°C with MBC to 36°C without MBC, and the same fields were photographed again 5.5 to 8.5 h later. 100 to 300 individual cells were located in the initial set of pictures and the same cells were scored from the second set of pictures for whether they had divided or not. All of the *cdc* mutations used here, as well as hydroxyurea and MBC, arrest cell division after bud emergence, therefore cells were considered to have divided during the second incubation if they formed two cells, each with a bud, between the time of the first and second photographs. All other cultures were controls. Culture A-iv was used as a control for culture B-iv; 100 to 300 cells were assayed for their ability to divide at 23°C. The turbidity (OD_{660}) of the A-i culture was measured at intervals to determine the doubling time of the strain at 23°C and to make certain that the cells used were in exponential growth phase. Culture A-ii was scored for percent budded cells to assure that the strain was sensitive to MBC arrest; culture A-iii confirmed that the strain was temperature-sensitive and arrested with the correct *cdc* terminal phenotype. Culture B-i was scored for percent budded cells to make sure that cells remained arrested in MBC over the time span of both the first and second incubations. Since reversibility of the MBC block was crucial to the experimental design, the percent of cells that was still capable of cell division after incubation in MBC and transfer to permissive

TABLE I

Reciprocal Shift Protocol

First incubation				Second incubation			
Culture	Temp	MBC	Medium	Culture	Temp	MBC	Medium
Experiment 1: MBC → *cdc*							
A	23	−	Liquid	A-i	23	−	Liquid
				A-ii	23	+	Liquid
				A-iii	36	−	Liquid
				A-iv	36	−	Solid
B	23	+	Liquid	B-i	23	+	Liquid
				B-ii	23	−	Solid
				B-iii	36	−	Liquid
				B-iv	36	−	Solid
Experiment 2: *cdc* → MBC							
A	23	−	Liquid	A-i	23	−	Liquid
				A-ii	36	−	Liquid
				A-iii	23	+	Liquid
				A-iv	23	+	Solid
B	36	−	Liquid	B-i	36	−	Liquid
				B-ii	23	−	Solid
				B-iii	23	+	Liquid
				B-iv	23	+	Solid

conditions (23°C without MBC) was assayed in culture B-ii by time-lapse photography. Culture B-iii was used to show that cells which had been exposed to MBC in the first incubation were still temperature sensitive and had the appropriate *cdc* phenotype when shifted to 36°C without MBC.

The second series of experiments (*cdc* → MBC) were exactly parallel to the first set but with the order of the incubations reversed (Table I). Cells were first arrested at the *cdc* block by high temperature, then shifted to low temperature with addition of MBC to determine whether cells blocked at the *cdc* block had completed the MBC sensitive step. Many of the *cdc* mutants lose viability rapidly at 36°C and could not be held at the restrictive temperature long enough for all the cells from an asynchronous population to arrest at the *cdc* block. Therefore, the length of the 36°C incubation was less than a full cell cycle time and thus, some fraction of cells in the population were not arrested at the *cdc* block. Whether these cells divided when shifted to MBC depended only on their position in the cycle relative to the MBC execution point, and not on the relationship between the MBC block and the *cdc* lesion. Thus these cells were a source of "noise" in the experiments. The fraction of such cells in the culture depended on the *cdc* and MBC execution points, the cell doubling time, and the length of time incubated at 36°C. These fractions were calculated as shown in the Appendix and were sufficiently small to avoid ambiguity in interpreting the results in all but one case.

Reciprocal shift experiments with MBC and HU were also done in an identical manner to that used for MBC and the *cdc* mutations except that all cultures were incubated at 23°C, with 0.1 to 0.3 M HU, as indicated, used to arrest cells rather than a 36°C incubation. Cells arrested with HU remained viable; therefore, in both the MBC to HU and HU to MBC shift experiments, the first incubation (3.5 to 4.5 h) was for more than a full cell cycle to insure that all cells were arrested at the block before shifting them to the second incubation.

In one experiment with strain W817H4-2 a spontaneous *Mata/Mata* homozygous isolate of strain W817H4-1, the cells were first synchronized with 10^4 U of partially purified α-factor (4) for 3 h at 23°C (by which time 96% of the cells were unbudded). The α-factor was then removed, the cells were resuspended in HU medium and incubated at 23°C for 4 h. The cells were then transferred to MBC medium as in the preceding shift experiments.

RESULTS

Stage Specific Arrest by MBC

4–6 h after addition of 100 μg MBC/ml to an exponentially growing culture, division was arrested with nearly all cells containing a single large bud. Examination after staining the cells with DAPI to reveal nuclei demonstrated that each budded cell contained a single nucleus located either in the bud or the mother cell (the two were not discriminated) but not located in the neck separating the mother and bud (data not shown). The cells remained division arrested for 10–14 h after the addition of MBC, by which time they had become greatly enlarged. Cells treated with MBC continued to incorporate radiolabel from ^{14}C-uracil into DNA at the same rate as a control culture for ~4 h; DNA synthesis was inhibited only after division was arrested in all cells (Fig. 1). In contrast, hydroxyurea, an inhibitor of DNA elongation (22), stopped incorporation after <2 h. These results indicated that MBC did not directly inhibit DNA synthesis, in agreement with earlier reports (8, 9), and must, therefore, act directly on nuclear division. The execution point (13) for completion of the MBC-sensitive step was calculated from the percent of total cells able to divide after a shift from 23°C to 23°C plus MBC (culture *A-iv*, Experiment *2*, Table I) for each of the *cdc* strains tested. The values ranged from 0.26 to 0.36, with an average value of 0.31, shortly after bud emergence. In one strain, W817 H4-1, both the MBC and HU execution points were measured; the execution point for MBC was 0.35 while that for hydroxyurea was 0.32; thus the two execution points are nearly the same.

Reciprocal Shift Experiments

EXPERIMENTAL RATIONALE: The purpose of these experiments was to determine the dependent relationships between the steps executed by *cdc* gene products and the step

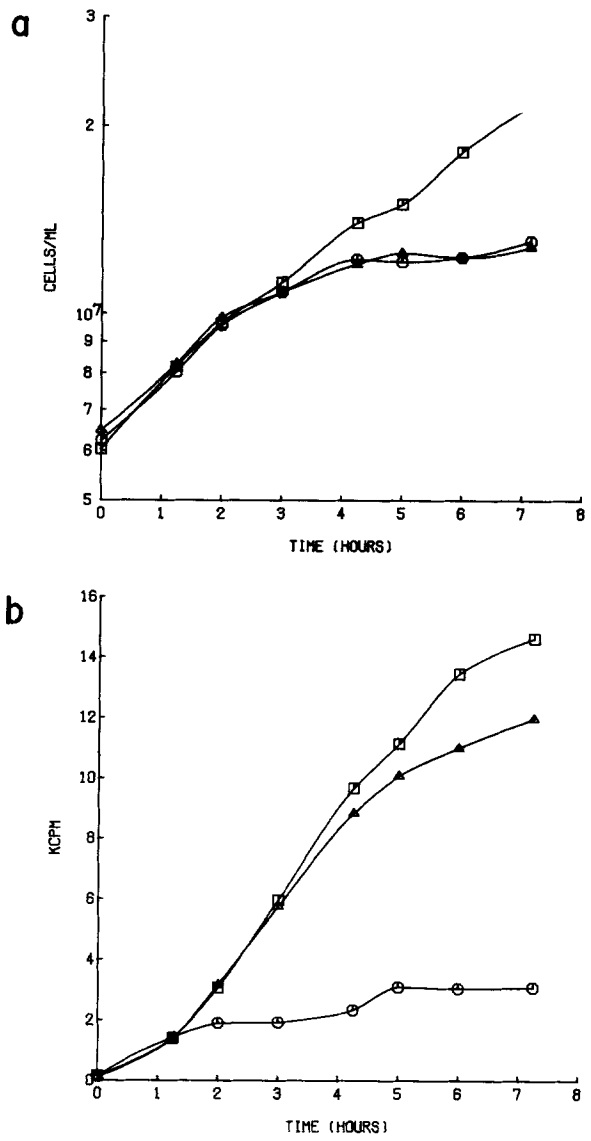

FIGURE 1 MBC and hydroxyurea inhibition of cell division and DNA synthesis. 1 μCi ^{14}C-uracil/ml was added to one half of an exponentially growing culture of a prototrophic diploid strain (C276) in minimal medium. 1 h later (T = 0) both cultures were divided into three parts; one part was kept as a control (□); 20 μg MBC/ml was added to the second part (△); hydroxyurea to 0.3 M final concentration was added to the third part (○). At approximately hourly intervals aliquots were taken from all cultures and assayed for cell number (*a*) or for incorporation of radioactive label into DNA (*b*).

that is sensitive to inhibition by MBC. For purposes of nomenclature, when we use a temperature-sensitive strain with a temperature-sensitive lesion in the *cdc*13 gene to inhibit a particular step in the cycle at the restrictive temperature, the mutant is designated *cdc*13; however, our conclusions relate to the order of function of the gene product at the permissive temperature and we designate the step in question as CDC13. Four possible dependent relationships (Table II) were distinguished using reciprocal shift experiments as follows: In the first series of experiments (MBC to 36°C shift), cells were arrested with MBC at the permissive temperature for the *cdc* lesion. Whether the cells had completed the CDC step before, or during, the arrest at the MBC block was determined by removing the MBC and shifting the cells to 36°C, the restrictive

temperature for the *cdc* mutation. Cells able to complete the CDC step while blocked with MBC would divide once when shifted from the MBC block to the *cdc* block. This result indicated that the CDC step either occurred before the MBC-sensitive step (possibility *i*, Table II), or was independent of the MBC step (possibility *iii*). If cells shifted from MBC to 36°C did not divide, the CDC step was after (possibility *ii*), or interdependent with (possibility *iv*), the MBC-sensitive step. These two pairs of possible relationships were distinguished by the second series of experiments, which imposed the blocks in the reverse order (36°C to MBC shift). *cdc* strains were arrested at 36°C without MBC, then the temperature was lowered and MBC was added. If cells were able to divide after this shift from the *cdc* block to the MBC block, then the MBC-sensitive step must have occurred before the CDC step (possibility *ii*), or independent of it (possibility *iii*). If the cells failed to divide upon a shift from 36°C to MBC, then the MBC-sensitive step was after (possibility *i*), or interdependent with (possibility *iv*), the CDC step. Thus, the results of these two experiments distinguished the four possible relationships between the MBC-sensitive step and a particular CDC step. Reciprocal shift experiments with MBC and HU were done in the same manner except that cells were arrested by adding HU to the medium rather than by shifting them to 36°C.

RESULTS OF THE MBC → CDC SHIFT: The results of the first experiment for 14 different *cdc*'s are in Table III (experiment *1*). The percent of budded cells which divided after a shift from MBC to 36°C corrected for viability of the MBC treated cells is shown in Fig. 2. Between 88% and 100% of the MBC arrested, viable, budded cells of *cdc*'s 2, 6, 7, 8, 9, 13, 16, 17, 20, and 21 divided when shifted to 36. 85% of the budded cells shifted from MBC to HU divided. Therefore, these *cdc*'s

TABLE II

Dependent Relationships between Different Cell Cycle Blocks and Ability to Divide in Reciprocal Shift Experiments

	Dependent relationships		Cells able to divide during the second incubation	
			Experiment 1 MBC → *cdc*	Experiment 2 *cdc* → MBC
i	*cdc* → MBC →	MBC block dependent and after the *cdc*	Yes	No
ii	MBC → *cdc* →	MBC block dependent and before the *cdc*	No	Yes
iii	MBC ⇒ *cdc*	MBC block independent of the *cdc*	Yes	Yes
iv	MBC → *cdc*	MBC block interdependent with the *cdc*	No	No

TABLE III

Percent of Budded Cells That Produce Two Cells in Second Incubation

		Experiment 1: MBC → *cdc*			Experiment 2: *cdc* → MBC		
Cycle block	Strain	Culture A-iv (23°C → 36°C)	Culture B-iv (MBC → 36°C)	Culture B-ii (MBC → 23°C)	Culture A-iv (23°C → MBC)	Culture B-iv (36°C → MBC)	Culture B-ii (36°C → 23°C)
cdc2-1	D370HB-1	95	94	88	81	47	74
cdc5-1	D473HB-1	25*	38*	85*	84	81	87
cdc6-1	D327HA-1	90	94	89	83	47	78
cdc7-4	W826HA-1	91	95	88	81	31	87
cdc8-1	D198HC-1	62	77	77	77	18	61
cdc9-2	W805HA-1	93‡	98‡	92‡	75	41	79
cdc13-1	D428HA-1	76	88	91	90	92	96
cdc14-1	D7041HA-1	25	3	91	89	90	87
cdc15-1	W806HA-2	32*	26*	85*	90	88	93
cdc16-1	W803HA-1	92	78	89	87	79	96
cdc17-1	W817H4-1	93	90	84	85	20	68
cdc20-1	W801HA-1	90	85	94	75	91	89
cdc21-1	D17026HB-1	54	76	83	85	41	94
cdc23-1	W804HA-1	60	3	82	85	65	61
		23°C → HU	MBC → HU	MBC → 23°C	23°C → MBC	HU → MBC	HU → 23°C
0.1 M HU	W817H4	80	76	88	66	37	98
0.2 M HU	W828				81	31	87
0.3 M HU	W817H4-2§				—	12	86

* Average of three experiments.

‡ Average of two experiments.

§ Presynchronized with α-factor (see text).

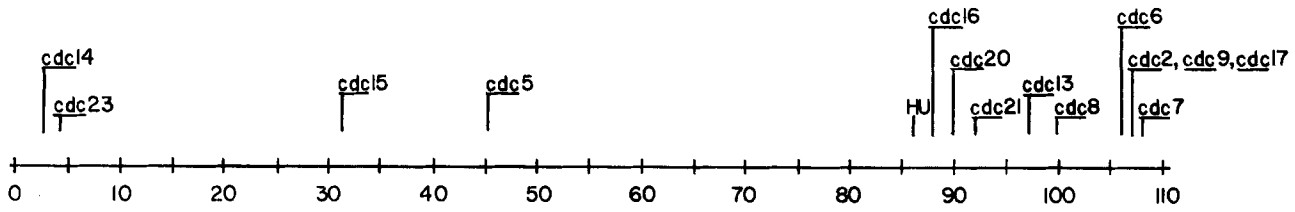

FIGURE 2 Results of MBC → *cdc* shift experiments after correction for viability. The percent of budded cells which divided after the shift from MBC to 36°C was corrected for the viability of the MBC treated cells by dividing the values for culture B-*iv* (Experiment *1*, Table III) by the percent of cells able to divide upon a shift to permissive conditions (culture B-*ii* in Experiment *1*, Table III).

and HU each arrested cells at a step located before, or independent of the MBC-sensitive step. <5% of the budded cells of *cdc*14 and *cdc*23 were able to divide and form new buds upon the shift from MBC to 36°C, indicating that these CDC steps were located interdependent with, or after the MBC block. The fact that a significant percentage of budded cells of *cdc*5 and *cdc*15 divided (30 and 45%, respectively) when shifted from MBC to 36°C was not a consequence of a leaky block at the MBC-sensitive step during the first incubation in these strains (culture *B-i*, Experiment *1*, Table I).

RESULTS OF THE CDC → MBC SHIFT: The results of the 36°C to MBC shift experiments are listed in Table III (experiment *2*) and plotted in Fig. 3 after correction for viability. For comparison, the percent of budded cells expected to divide if the CDC step was located either before or after the MBC-sensitive step are also presented for each *cdc* mutant (calculated as shown in the Appendix). For *cdc*'s 5, 13, 14, 15, 16, and 20, the percent of budded cells able to divide was close to that expected if the MBC-sensitive step was before or independent of these CDC steps. The CDC23 step must also be before or independent of the MBC block since 100% of the budded cells were able to divide when shifted to MBC, even though only 73% were expected to. This excess over the expectation probably indicated that *cdc*23 cells were able to progress through the cell cycle to the *cdc* execution point faster after the switch to 36°C than they could at 23°C (see Appendix). The CDC7 and CDC17 steps must occur before, or interdependent with, the MBC-sensitive step since the percent of budded cells that divided in MBC after incubation at 36°C was close to the value expected if the CDC step occurred before the MBC sensitive step. Four mutants, *cdc*'s 6, 8, 9, and 21, and cells blocked with HU, gave a value for the budded cells that divided after the shift to MBC that was intermediate between the two expected values (Fig. 3). A higher concentration of HU was also tested and a similar result was obtained (Table III). Because the expected limits for cells able to divide for *cdc*2 were relatively narrow (Fig. 3), it was not possible to conclude whether the observed value for *cdc*2 was also intermediate or was within experimental error of the value expected if the CDC step was after or independent of the MBC-sensitive step. *Cdc*'s 2, 6, 8, 9, 21 and HU all arrest DNA synthesis, suggesting that the intermediate values observed in the 36°C to MBC sequencing experiments are indicative of a complex relationship between DNA synthesis and the MBC sensitive step (see Discussion).

*Cdc*5 cells frequently divided several times after being held at the restrictive temperature and then shifted to MBC. None of the other mutants or the parent strain divided more than once in the same experiment. To quantitate this phenomenon an experiment was done arresting *cdc*5 cells at high temperature for different lengths of time before shifting to MBC, and the number of budded cells which could divide more than once

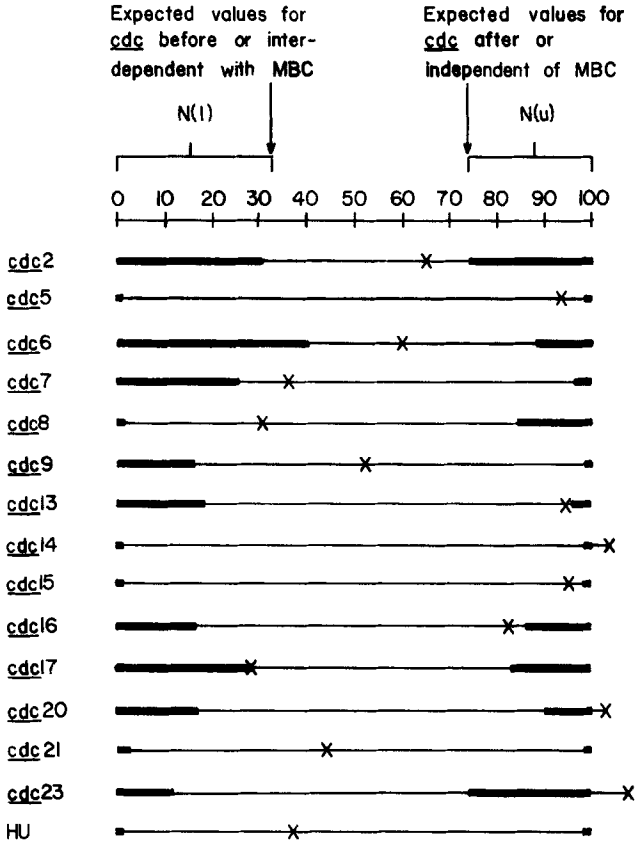

FIGURE 3 Results of *cdc* → MBC shift experiments corrected for viability. The percent of budded cells that divided after the shift from 36°C to MBC was corrected for the viability of the 36°C treated cells by dividing the values for culture B-*iv* (Experiment *2*, Table III) by the percent of cells able to divide upon a shift to permissive conditions (culture B-*ii* in Experiment *2*, Table III). *X*, observed value. Heavy bars, *N(I) or N(u)* calculated as shown in the Appendix.

was scored (Fig. 4). *Cdc*5 cells that had been incubated at 23°C divide at most once when incubated with MBC. However, after 90 min at 36°C (when approximately all of the cells in the population should have reached the *cdc*5 block) 56% of the budded cells divided at least twice in MBC. By 3 h at 36°C, 70% of the cells were able to divide twice or more. Some budded cells went through at least three divisions (>8 cells); clusters larger than 8 cells were too crowded to count accurately. An examination of *cdc*5 cells shifted from 36°C to MBC in liquid medium demonstrated that these apparent multiple divisions were in fact complete cell divisions: after sonication between 73% and 79% of the cells were single cells with a large bud, characteristic of the MBC arrest phenotype. Since *cdc*5

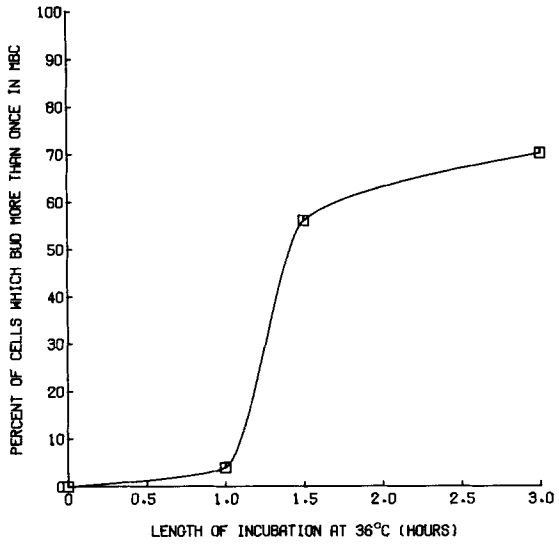

FIGURE 4 *Cdc*5 multibudding in MBC after 36°C incubation. Exponentially growing cells of a *cdc*5 strain (D473HB-1) were transferred to 36°C and incubated as indicated before shifting to an agar slab at 23°C containing 100 μg MBC/ml. Immediately after transfer to the agar slab fields of cells were photographed. After incubation for 8 h the same fields were photographed again and the percent of initially budded cells that budded more than once in the presence of MBC was scored.

TABLE IV

Dependent Relationships Determined by Reciprocal Shift Experiments

Cycle block	Relationship to MBC block	
cdc2	Independent	
cdc7	Before	*cdc* → MBC
cdc13	Independent	
cdc14	After	MBC → *cdc*
cdc16	Independent	
cdc17	Before	*cdc* → MBC
cdc20	Independent	
cdc23	After	MBC → *cdc*

cells did eventually arrest division in MBC, the *cdc*5 mutation did not make the cells permanently insensitive to MBC. These results indicated that *cdc*5 cells at the restrictive temperature accumulate the potential to go through multiple rounds of division in the presence of MBC.

The dependent relationships between the MBC-sensitive and CDC steps of the cell cycle are summarized in Table IV. Some of the *cdc*'s gave atypical results and could not be unequivocally located from these experiments.

DISCUSSION

Many of the *cdc* mutants produced results in the reciprocal shift experiments with MBC that were consistent with one of the four dependent relationships (Fig. 5). Function of the *CDC*7 and *CDC*17 gene products is prerequisite for the execution of the MBC sensitive step; the *CDC*13, *CDC*16, and *CDC*20 gene products function independently of the MBC-sensitive step, and execution of the MBC-sensitive step is prerequisite for execution of the CDC14 and CDC23 steps. None of the CDC functions were found to be interdependent with the MBC-sensitive step.

Moir and Botstein have isolated cold sensitive *cdc* mutants and performed reciprocal shift experiments to map them with respect to some of the heat-sensitive *cdc* mutants used here (Genetics, in press). The function performed by the *CDC*45 locus was found to be prerequisite to the CDC14 step and independent of the CDC16 step. Thus the function performed by the *CDC*45 gene product may occupy the same position in the dependent pathway of the cell cycle as the MBC-sensitive step; it is possible that the CDC45 and MBC-sensitive steps are interdependent.

A number of the *cdc* mutants and the chemical inhibitor, hydroxyurea, produced results that were not compatible with one of the four simple dependent relationships. In the second set of experiments, shifting cells from 36°C to MBC, *cdc*'s 6, 8, 9, 21, and possibly *cdc*2, and cells shifted from HU to MBC, gave values for the percent of cells that divided during the second incubation that were intermediate between that expected for steps before (or interdependent with) MBC and that expected for steps after (or independent) of MBC. It is noteworthy that all of these *cdc*'s and HU prevent completion of DNA synthesis (9, 10, 14); this correlation suggested that cells blocked in S phase were heterogeneous for completion of the MBC-sensitive step, with some cells before the MBC-sensitive step and some after, or independent of it. An obvious trivial possibility for such heterogeneity would be a leaky arrest at the first block. Cells might slowly complete DNA synthesis while incubated at 36°C or with HU and, therefore, also execute the MBC-sensitive step. However, control experiments eliminated this possibility; for all of these *cdc*'s all cells shifted from 23°C to 36°C arrested after one or less divisions, and remained arrested for 8 h (culture *A-iv*, Experiment *1*, in Table I). Even at higher concentrations of HU, similar results were obtained. The simplest interpretation of these results is that some DNA replication is necessary for execution of the MBC-sensitive step but completion of DNA replication is not. In an asynchronous population of cells a substantial fraction of the cells (25% to 50%) are in S phase (1, 22, 23); hence cells shifted from an asynchronous population to conditions that are restrictive for DNA synthesis will be arrested at different positions within S phase. To test this idea a culture of cells was synchronized with α-factor and then arrested at the beginning of S phase with HU. A much lower proportion of cells divided upon a shift from this block to the MBC block (strain W817H4-2, experiment *2*, in Table III), than in the experiments with unsynchronized cells (Table III). This result is consistent with the idea that execution of the MBC-sensitive step requires the replication of some unique DNA sequences early in S phase. One attractive model is that the MBC-sensitive step is the attachment of microtubules to the newly replicated centromeres. Although the appearance of distinct sister centromeres occurs late in the mammalian cell cycle (2), it is not yet known for any cell whether centromeric DNA replicates early or late in S phase.

Although we favor the above mentioned hypothesis for the heterogeneity of cells in S phase with respect to execution of the MBC-sensitive step, yet a third possible interpretation cannot be ruled out at this time. In cells that are progressing

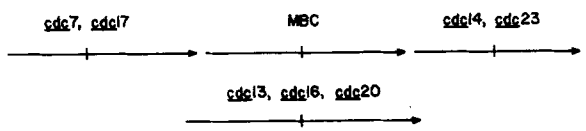

FIGURE 5 MBC and *cdc* dependent relationships.

through the cell cycle normally it might be that the completion of DNA replication activates execution of the MBC-sensitive step. However, in our protocol, dependence is tested by arresting cells in the DNA synthetic period for an abnormally long period of time. Perhaps the normal activation of the MBC-sensitive step upon the completion of replication is not an absolute requirement and the MBC-sensitive step can be executed slowly (or abnormally) in the absence of DNA replication. It should be noted that this possibility is applicable to any case in which the reciprocal shift protocol fails to reveal a dependence of one step upon another since this experimental protocol always entails long delays of cell cycle events.

The work presented here further defines the dependent pathway of events executed by individual gene products during the cell cycle. Ultimately, we would like to be able to describe this pathway in terms of the molecular events occurring at each step. The pathway itself will aid in this goal since knowledge of the molecular event occuring at any one step would suggest the nature of the molecular events that might immediately precede and succeed the event in question. In this context, it is noteworthy that some information is available about the mode of action of MBC.

MBC binds to the β-tubulin of *S. cerevisiae* (P. Baum and J. Thorner, personal communication) and *A. nidulans*, and in the latter case, binds in a competitive manner with colchicine (6). It is clear that MBC inhibits cell division through its action on β-tubulin since MBC-resistant mutants occur in the structural gene for β-tubulin in both *Aspergillus* and *S. cerevisiae* (20 and D. Botstein, personal communication). These facts are consistent with the notion that MBC inhibits some step of chromosome segregation through its action on the microtubules of the mitotic spindle although the precise mode of spindle inhibition is still unclear. The in vivo data from *S. cerevisiae* suggest that MBC acts on an early stage of spindle formation (19). Thus the execution point for MBC (the time in the cycle at which cells can no longer be prevented from dividing) is early in the cycle. If MBC uptake were slow the execution point might appear to be earlier than it actually is; however, other evidence is consistent with the notion of an early execution point. Thus, the initiation of DNA replication probably precedes bud emergence and continues for some time after it (see reference 7 for review) and execution of the MBC-sensitive step appears from our results to require some DNA replication but not the completion of DNA synthesis. Furthermore, in the nucleus of MBC-arrested cells the spindle poles are doubled but the spindle is small or absent (B. Byers, personal communication).

The pathway presented in Fig. 5 is consistent with recent observations by Francis Fabre (personal communication) relating to the position in the cell cycle of chromatid separation. Haploid cells of *S. cerevisiae* are very sensitive to killing by ionizing radiation during G1 and become resistant during S (3), presumably because sister chromatids can cooperate in the repair of x-ray lesions; the cells become sensitive again in G2 when sister chromatids separate. Some of the mutants that we have ordered by reciprocal shifts with MBC have been examined for x-ray sensitivity after cell cycle arrest at 36°C. *Cdc*'s13, 16, and 23 as well as cells arrested with MBC are all x-ray resistant indicating that sister chromatids are still in proximity. *Cdc*14 cells are x-ray sensitive suggesting that sister chromatids are separated. This observation also agrees with the notion that MBC-arrested cells are at an early stage of chromosome separation before segregation of sister chromatids. Finally, MBC-arrested cells lose chromosomes at a very high frequency (>1% per chromosome per viable cell (26) but show little or no change in mutation or recombination frequencies. This result is also consistent with an effect of MBC upon the mitotic spindle.

Results of the shift experiments with *cdc*5 and *cdc*15 did not fit the expectations for CDC steps having one of the four dependent relationships with the MBC block. The shift from MBC arrest to 36°C produced ambiguous results that were not due to leakiness of the MBC block. The heterogeneity of cells within the cycle at the time of MBC treatment could be responsible but this possibility was not tested.

When *cdc*5 cells were first incubated at 36°C and then shifted to MBC medium they underwent multiple rounds of division before arresting. We do not know why this occurred but several explanations are possible. *Cdc*5 blocked cells might induce an enzyme that degrades MBC, become impermeable to MBC, or accumulate proteins (e.g. β-tubulin) that bind MBC.

APPENDIX

Ideally the reciprocal shift protocol is carried out by incubating the population of cells until all of the cells have become arrested at the position of the block imposed during the first incubation before the culture is shifted to the second restrictive condition. However, since the *cdc* mutants lose viability at the restrictive temperature, prolonged incubation at 36°C is not desirable. It is necessary to consider the expected response of an asynchronous population *cdc* mutant cells after incubation at the restrictive temperature for less than a full cycle to decide whether the fraction of cells that have become arrested at the *cdc* block during the first incubation has or has not executed the MBC-sensitive step. The important parameters for this calculation are: Ex(cdc), the excution point of the *CDC* step; Ex(MBC), the execution point of the MBC sensitive step; Ex(bud), the execution point of budding; t, the length incubation at 36°C; T, the cell cycle time. The execution point of the CDC step was calculated from the fraction, F, of total cells which divided upon a shift from 23°C to 36°C (culture *A-iv*, Experiment *1*, in Table I) with the following equation which takes into account the asymmetric age distribution of cells in an asynchronous population (18):

$$Ex = 1 - \log (F + 1)/.301$$

In a similar manner, the execution point of the MBC-sensitive step could be calculated from the fraction of total cells which divided upon a shift from 23°C to MBC (culture *A-iv*, Experiment *2*, in Table I). The execution point of budding was calculated from the fraction of total cells which were budded at the start of the second incubation (culture *A-iv*, Experiment *1*, in Table I). The length of time the cells were held at 36°C was directly measured in each experiment and varied between 80 and 115 min. Since these strains were *ts* and do not go through more than one division at 36°C, the cell cycle time at 36°C could not be measured. The 23°C doubling time of each strain, (between 145 and 230 min) was used as an approximation of the cell cycle time immediately after the shift from 23°C to 36°C; this is probably an underestimate because cells increase their mass more rapidly at 36°C in steady-state conditions but may be a reasonable approximation because cells display a transient lag in mass increase upon a shift from 23° to 36°C. The close agreement between the predicted and the observed values (Fig. 3) suggested that this approximation was relatively accurate in most cases.

In an asynchronous, exponentially growing population of cells the fraction of total cells which are past a particular point, x, in the cyle is (18):

$$N = 2^{(1-x)} - 1,$$

where x equals the fractional position in the cycle between 0 and 1. Distortion of this distribution by unequal cycle times for mother and daughter cells in the population (14) is minimal in rapidly growing diploid strains and has been ignored for the sake of simplicity.

The first possibility we shall consider is that for which execution of the CDC step is a necessary prerequisite for execution of the MBC-sensitive step. If all of the *cdc* mutant cells were arrested at the position of the *cdc* block in the cell cycle after incubation at the restrictive temperature, then none of the cells would divide upon a shift to medium containing MBC at the permissive temperature. However, since the incubation time at the restrictive temperature is less than one complete cycle time some cells will divide and it is this fraction, N(1) (expressed as percent of total cells), that we wish to calculate. In all cases being considered here, Ex(MBC) − Ex(cdc) < t/T. The distribution of cells in an unperturbed population is shown in Fig. 6*a* for comparison to the cases considered below.

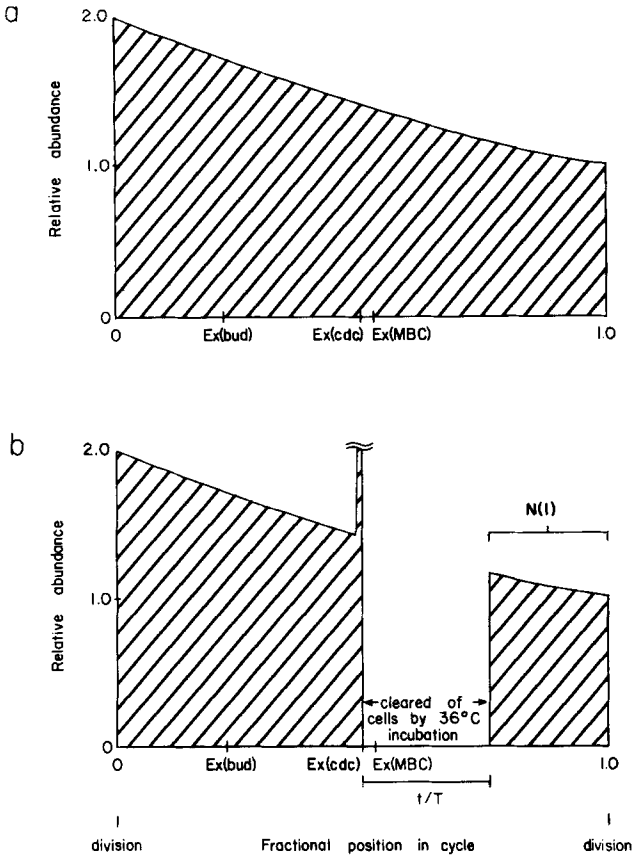

FIGURE 6 Distribution of cells in the cell cycle after incubation at 36°C for $t < T$ where *cdc* block is before the MBC block. (*a*) The steady state age distribution of cells in an asynchronous population (before 36°C incubation) calculated from (18): $Y = e^{\ln 2(1-X)}$, where Y is the relative abundance in the population and X is the fractional position in the cycle. A short incubation at the restrictive temperature for a *cdc* mutation altered this distribution, "clearing" an interval of the cycle of cells as shown. (*b*) Case *i*: for Ex(cdc) + t/T < 1). N(l), the fraction of budded cells past the *cdc* execution point and before cell division.

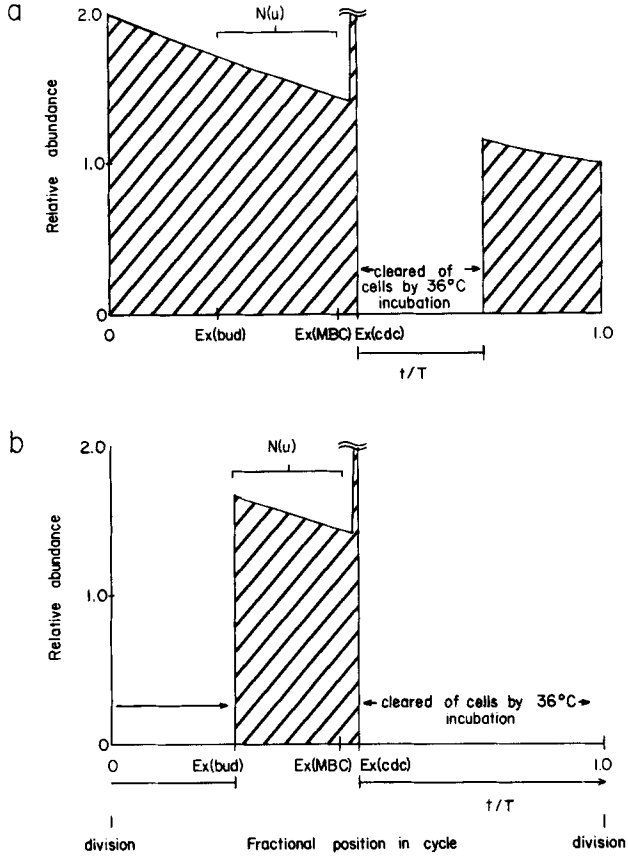

FIGURE 7 Distribution of cells in the cell cycle after incubation at 36°C for $t < T$ where *cdc* block is after the MBC block. See legend to Fig. 6. (*a*) Case *i*: Ex(cdc) + t/T < 1 + Ex(bud). *N(u)*, the fraction of budded cells before the MBC execution point. (*b*) Case *ii*: Ex(cdc) + t/T > 1 + Ex(bud). *N(u)*, the fraction of budded cells before the MBC execution point.

Case *i*: for Ex(cdc) + t/T < 1 (see Fig. 6*b*).

N(1) = percent of cells after the MBC block and before cell division.

$N(1) = [2^{1-(Ex(cdc)+t/T)} - 1]*100.$

$N(1) = [2^{(1-Ex(cdc)-t/T)} - 1]*100.$

Case *ii*: for Ex(cdc) + t/T > 1

N(1) = 0.

If the execution point of the *cdc* block is late in the cycle or the length of the incubation at 36°C is long, such that Ex(*cdc*) + t/T > 1, then all cells past the *cdc* block will have divided before the shift to MBC and N(1) = 0.

The other possibility to consider is that for a CDC step that is not prerequisite for execution of the MBC-sensitive step. If all of the cells were arrested at the *cdc* block they would have executed the MBC-sensitive step and, therefore, would all divide upon a shift from the restrictive temperature to medium containing MBC at the permissive temperature. However since the incubation period at the restrictive temperature is less than one cycle time some of the cells in the population will not have executed the MBC-sensitive step during the incubation period at the restrictive temperature and will not divide upon shifting cells from 36°C to 23°C with MBC. It is this fraction of cells, N(u) (expressed as percent of total cells), that we wish to calculate. In all cases that we consider Ex(MBC) < Ex(cdc).

Case *i*: for (Ex(cdc) + t/T) < 1 + Ex(bud) (see Fig. 7*a*).

N(u) = percent of cells past Ex(bud) and before Ex(MBC).

$N(u) = ([2^{1-Ex(bud)} - 1] - [2^{1-Ex(MBC)} - 1])*100.$

$N(u) = (2^{1-Ex(bud)} - 2^{1-Ex(MBC)})*100.$

Case *ii*: for (Ex(cdc) + t/T) > 1 + Ex(bud) (see Fig. 7*b*).

N(u) = percent of cells past "clearing point" of 36°C incubation and before Ex(MBC).

$N(u) = ([2^{1-(Ex(cdc)+t/T-1)} - 1\} - [2^{1-Ex(MBC)} - 1])*100.$

$N(u) = [2^{2-Ex(cdc)-t/T} - 2^{1-Ex(MBC)}]*100.$

Case *iii*: for (Ex(cdc) + t/T) > 1 + Ex(MBC).

N(u) = percent of cells past "clearing point" of 36°C incubation and before Ex(MBC).

N(u) = 0.

Thus, 0 + N(1) is the expected value in percent for cells able to divide for the *cdc* block before, or interdependent with, the MBC block. Likewise, 100 − N(u) is the expected percent of cells able to divide for a *cdc* block after, or independent of, the MBC block. These are shown schematically in Fig. 3. If the cells do cycle faster at 36°C, contrary to the assumption used in the above equations, this will have the effect of increasing the portion of the cycle cleared of cells by the 36°C incubation (t/T) and decreasing both N(1) and N(u). Therefore, N(1) and N(u) are the maximum or minimum limits, respectively, expected for cells which divide upon shifting to MBC.

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