DETERMINATION OF THE ORDER OF GENE FUNCTION IN THE YEAST NUCLEAR DIVISION PATHWAY USING *cs* AND *ts* MUTANTS

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ABSTRACT

Cold-sensitive (*cs*) and heat-sensitive (*ts*) conditional-lethal mutations that affect specifically the cell division cycle of budding yeast (*Saccharomyces cerevisiae*) were used to determine the order of gene function. Reciprocal temperature-shift experiments using *cs-ts* double mutants revealed a detailed order of function among genes whose execution points and mutant phenotypes are very similar. The data suggest that the nuclear branch of the overall cell-cycle pathway itself contains at least one branch.

THE cell division cycle of the budding yeast *Saccharomyces cerevisiae* involves more than 50 genes whose products appear to be required specifically to carry out steps in the pathways of cell division (HARTWELL, CULOTTI and REID 1970; HARTWELL *et al.* 1973, 1974; MOIR *et al.* 1982). Functions for some of the gene products have been deduced from the phenotypes of conditional-lethal cell-division-cycle (*cdc*) mutations. Temperature-shift experiments using heat-sensitive (*ts*) *cdc* mutants have allowed the definition of the "execution point" (*i.e.*, the latest time at which an essential *cdc* gene product can act to allow passage to the next cell cycle): the execution point appears to be approximately the same for different alleles of the same *cdc* gene (HARTWELL *et al.* 1973). Some information about the organization of *cdc* functions into dependent pathways has been obtained by observing the phenotypes of double-mutant strains and by the use, in conjunction with the *cdc* mutations, of two specific inhibitors of the progress of the cell cycle (α-factor, which causes cells to accumulate at the "start," and hydroxyurea, which causes reversible inhibition of DNA synthesis; HEREFORD and HARTWELL 1974; HARTWELL 1976). This kind of analysis has resulted in a comprehensive view of the cell cycle genes as specifying functions that comprise dependent pathways containing at least one branch (HARTWELL *et al.* 1974; HARTWELL 1974, 1978).

Nevertheless, many different *cdc* genes are functionally indistinguishable from each other by methods used thus far: such genes appear identical in their terminal mutant phenotype, in their execution points, and in other order of function relative to the defects caused by treatment with α-factor or hydroxyurea.

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The accompanying paper (MOIR *et al.* 1982) describes the isolation of cold-sensitive (*cs*) *cdc* mutations, many of which define new *cdc* genes. These have made feasible the application to the yeast cell cycle of the reciprocal temperature-shift method. By means of *cs* and *ts* double mutants, this general method yields information about the order in which two genes function. (JARVIK and BOTSTEIN 1973).

The reciprocal temperature-shift method takes advantage of the possibility in *cs-ts* double mutants of blocking the function of two gene products independently. If one incubates cells at the high nonpermissive temperature (during which the gene bearing the *ts* mutation fails to function), allows the cell cycle to arrest, and then shifts to the low nonpermissive temperature (during which the gene bearing the *cs* mutation fails to function), progress to the next cell cycle will occur if and only if the gene carrying the *cs* mutation can act *before* the gene carrying the *ts* mutation. Reversing the order of incubation at the two temperatures should have the opposite result if the two genes in which the mutations lie are part of a dependent pathway. The possibilities and their interpretation are summarized in Table 1, from which it can be seen that the method not only yields information about the order of gene function when the two genes lie on a dependent pathway, but also allows one to decide whether or not two gene functions are arranged in such a dependent pathway. The same logic was used by HEREFORD and HARTWELL (1974) to order the function of *cdc* genes relative to the block imposed by treatment with α-factor; HARTWELL (1976) used the method to order *cdc* functions relative to the block imposed by treatment with hydroxyurea. In all these cases the important feature is the ability to impose blocks at possibly different points in the cell cycle independently of one another.

This paper describes the application to the yeast cell cycle of the reciprocal temperature-shift method using *ts* and *cs cdc* mutations. The results indicate that the nuclear division branch of the cell-cycle pathway is itself branched.

TABLE 1

Results expected in reciprocal shift experiments using cs-ts cdc *double mutants*

Dependency relationship	Result of shift 17° → 37°	37° → 17°
Dependent $\xrightarrow{ts}\xrightarrow{cs}$	+	—
Dependent $\xrightarrow{cs}\xrightarrow{ts}$	—	+
Independent $\xrightarrow{ts}$ / $\xrightarrow{cs}$	+	+
Interdependent $\xrightarrow{(ts,\ cs)}$	—	—

"+" indicates passage to a second cell cycle (*i.e.*, two arrested cells are found); "—" indicates arrest in the first cell cycle (*i.e.*, one arrested cell is found).

MATERIALS AND METHODS

[5,6–^{3}H]-uracil was purchased from New England Nuclear and N-hydroxyurea from Boehringer/Mannheim. Purified α-factor (CIEJEK, THORNER and GEIR 1977) was the generous gift of JEREMY THORNER.

Media and methods of mating, sporulation and tetrad analysis were essentially as previously described (BRANDRISS, SOLL and BOTSTEIN 1975; MOIR *et al.* 1982, accompanying paper).

Strains: The cold-sensitive *cdc* mutants *cs*A10 (*cdc44–1*) and *cs*A18 (*cdc45–1*) were derived from S288C as described in the accompanying paper (MOIR *et al.* 1982). The temperature-sensitive allele of *cdc16*, *cdc16–201*, was isolated and characterized exactly as described for the cold-sensitive *cdc* mutants except the restrictive temperature was 35°. The execution point for *cdc16–201* was determined by the method of HARTWELL, CULOTTI and REID (1970) to be 0.5, essentially the same value as previously determined for *cdc16–1* (HARTWELL *et al.* 1973). All mutants, including the temperature-sensitive *cdc* mutants DB375 (*cdc9–1*) and DB380 (*cdc14–1*) obtained from L. H. HARTWELL, were backcrossed to S288C at least four times before *cs-ts* double mutants were constructed by crossing the two haploids, sporulating and dissecting the four-spored asci.

In vivo *radioactive labeling of DNA*: Exponential phase cultures of the strain to be labeled were adjusted to 2×10^{6} cells/ml in labeling medium (HARTWELL 1970; supplemented with 40 μg/ml each of adenine and histidine) without ^{3}H-uracil, and 6 ml cultures were incubated with 30 U/ml α-factor for 3 hr at 26°. At that point, more than 90% of the cells were arrested as unbudded cells with a "schmoo" morphology. The culture was filtered through a Millipore HAWP 0.45 μ filter to remove α-factor, and the cells were washed from the filter with an equal volume of nonradioactive medium. Cells were placed in 6 ml of labeling medium containing 500 μCi ^{3}H-uracil (830 mCi/mmole). The culture was divided into three equal portions: one part was incubated at 26° for 4 hr, one at 17° for 8 hr, and one at 26° for 0.5 hr followed by 17° for 7.5 hr. Aliquots (0.1 ml) were withdrawn periodically and added to 0.01 ml fresh 11 M NaOH. After incubation overnight at room temperature in covered micro test tubes, samples were neutralized with 0.01 ml 10 M HCl. Most of each sample (0.11 ml) was withdrawn and pipetted directly onto 24 mm filter paper circles (Schleicher and Schuell 895-E), which had been previously soaked in 10% trichoroacetic acid containing 0.1 mg/ml uracil and then air dried. After the sample soaked in, but before it dried, the filters were dropped into ice-cold 10% trichloroaretic acid containing 0.1 mg/ml uracil (250 ml for up to 50 filters) and washed batchwise for 15 min on ice. The trichloroacetic acid was poured off and the washing repeated three more times with 5% trichloroacetic acid containing 0.1 mg/ml uracil. Finally, filters were washed in 95% ethanol, aid dried under a heat lamp (10 min), and counted in a scintillation counter. In all experiments, less than 5% of the added label was incorporated into RNA and DNA.

Reciprocal temperature shifts: An exponential phase culture in YEPD medium of a *MAT***a** *cs-ts* double cell-division-cycle mutant was adjusted to 2×10^{6} cells/ml, and a 3 ml portion was incubated with 30 U/ml α-factor for 3 hr at 26°. At the end of this incubation, more than 80% of the cells were synchronized at the beginning of the division cycle (at the unbudded stage as judged by microscopic examination). The culture was filtered through a Millipore HAWP 0.45 μ filter to remove α-factor and the cells were washed twice with 5 ml of YEPD medium. After resuspension in 3 ml of fresh YEPD and brief sonication to disrupt clumps, the culture was incubated for at least 230 min at 17° or 100 min at 37° to arrest cells at the first conditional block. A control experiment with strain DB640 (*MAT***a** *ade2*; otherwise isogenic to S288C) demonstrated that under these conditions cells complete one division cycle in 220 min at 17° and in 120 min at 37°. (These values are essentially the same as the doubling times for exponential cultures of DB640 at 17° and 37°; therefore, synchronization with α-factor does not alter the cell division time requirement.)

After arrest at the first conditional block in liquid rich medium, the cells were subjected to the second condition by spotting onto solid rich medium at three different temperatures; incubations of the plates were carried out for 6 hr at 26°, 6 hr at 37° and 18 hr at 17°. The cell

cycle position of each cell was recorded by photography before and after the second incubation period (on solid medium) as previously described by HARTWELL, CULOTTI and REID (1970).

Experiments ordering a cold-sensitive block with the hydroxyurea block were performed in an analogous manner except the appropriate *MAT***a** *cs cdc* mutant was used, and incubations were for 100 min in liquid rich medium containing 0.1 M hydroxyurea and for 6 hr on solid rich medium containing 0.3 M hydroxyurea as described by HARTWELL (1976).

Calculations of the results were as follows: (1) Correction for lethality: the number of cells arrested in the first division cycle of the experimental shifts (*e.g.*, 17° → 37° and 37° → 17°) for the trivial reason that they are dead was calculated by multiplying the percentage of cells that did not recover from the initial conditional block (*e.g.*, 17° → 26° and 37° → 26° control shifts, respectively) by the total number of cells counted in the experimental shift. This value was subtracted from the number of first cycle-arresting cells and from the total number of cells counted (*i.e.*, the cells are dead and thus are omitted from the final calculations) in the experimental shift. (2) Correction for leakiness: Similarly, the number of cells passing to the second division cycle in the experimental shifts (*i.e.*, 17° → 37° and 37° → 17°) for the trival reason that they leaked past the first cycle block was calculated by multiplying the percentage of cells that leaked past the 37° and 17° blocks (*i.e.*, 37° → 37° and 17° → 17° control shifts, respectively) times the total cells counted in the experimental shift. This value was subtracted from the second cycle-arresting cells and the total cells counted (*i.e.*, they are not tightly arrested cells and are omitted from the final calculations) in the experimental shift. Finally, the corrected percentage of cells arresting in the first and second cycles was calculated from these adjusted numbers. In all cases, more than 100 total cells were counted for each shift. Recovery from the initial block was generally greater than 70% (*i.e.*, the lethality estimate was less than 30%) and leakage past the initial block was usually less than 10%.

RESULTS

In the accompanying paper (MOIR *et al.* 1982) cold-sensitive *cdc* mutations (*cs*A10, now called *cdc44-1* and *cs*A18, now called *cdc45-1*) were found which represent new *cdc* genes. These mutants display a medial nuclear division terminal phenotype. Their execution points were found to be late in the cell cycle (about 0.7 for *cdc44-1* and about 0.5 for *cdc45-1*), clearly after DNA synthesis. To test the implication that *cdc44-1* and *cdc45-1* carry out the initiation and elongation functions comprising DNA synthesis at their restrictive temperature (17°), incorporation of radioactive precursors into DNA was measured in whole cells synchronized at "start" with α-factor (BUCKING-THROM *et al.* 1973; HEREFORD and HARTWELL 1974). Measurements were made periodically after removal of the α-factor and incubation of cells at the permissive temperature (26°) and the restrictive temperature (17°). The results (Figure 1) show clearly that both mutants initiate and apparently complete one round of DNA synthesis at the restrictive temperature. While the time required to complete one round of DNA synthesis at 17° is somewhat longer than the doubling time of cells growing exponentially at 17° (see MATERIALS AND METHODS), this may be because DNA synthesis was measured in a minimal medium (HARTWELL 1970) while the exponential growth and all ordering experiments reported here were done in rich medium (YEPD). Control experiments (not shown) using wild-type strain DBY640, isogenic to the mutants used in this study (MOIR *et al.* 1982), indicate that 70% of the cells synchronized with α-factor have completed a cell division and are at the small bud stage of a second cell cycle by 270 min after

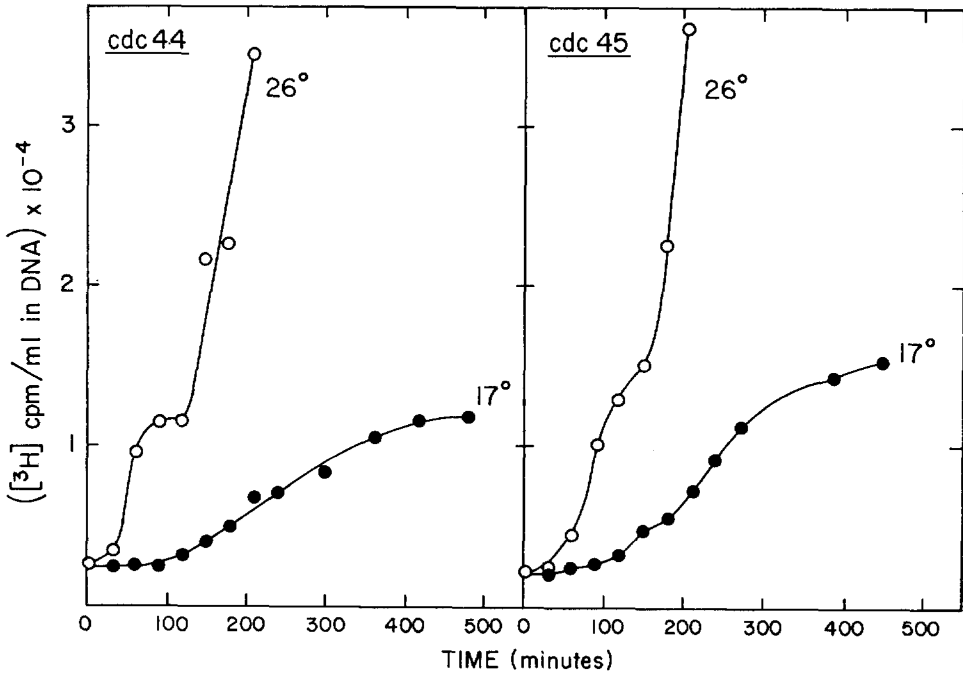

FIGURE 1.—Incorporation of radioactivity into DNA by *cdc44-1* and *cdc45-1* at permissive (26°) and nonpermissive (17°) temperatures. Cells synchronized with α-factor were incubated at the indicated temperature in the presence of [^{3}H]-uracil as described in MATERIALS AND METHODS. The alkali-resistant, acid precipitable radioactivity is plotted as a function of time after removal of α-factor and addition of the radioactive uracil. Less than 5% of the total radioactivity added was incorporated into acid-precipitable form in each experiment.

shift to 17° in rich medium. Thus, recovery from α-factor arrest is rapid under our conditions, and prior arrest with α-factor has little or no effect on cell doubling times after removal of the peptide.

Protocol for reciprocal temperature-shift experiments: The protocol for reciprocal shift experiments used in all the experiments presented in this paper are described in detail in MATERIALS AND METHODS. Briefly, exponentially growing *MATa* haploid cells of the appropriate genotype (*i.e., cs-ts* double mutants, in most cases) were treated with purified α-factor (CIEJEK, THORNER and GEIER 1977) which has the effect of reversibly arresting their growth at the beginning ("start") of the cell cycle (BUCKING-THROM *et al.* 1973). The α-factor was washed away and the cells incubated under one of the nonpermissive conditions (*i.e.*, 17° to block a *cs* function, 37° to block a *ts* function, or hydroxyurea at 26° to block DNA synthesis) in liquid medium. The length of this *first* nonpermissive incubation varied in different experiments; in all cases the incubation was longer than a single cell cycle at that temperature. Cells were then *shifted* to the *second* nonpermissive condition by spotting onto solid medium after brief sonication to separate clumps of cells. The individual cells were followed by photo-

microscopy to determine whether they arrested in the first cell cycle (*i.e.*, only single arrested cells were ever seen) or were able to pass to a subsequent cell cycle (*i.e.*, two arrested cells were seen).

In conjunction with each reciprocal shift experiment, control experiments were carried out simultaneously to measure lethality during the first nonpermissive incubation (seen as arrest in the first cycle when permissive conditions were provided in the second incubation) and to measure leakiness (seen as passage to a second cell cycle when the same nonpermissive condition was maintained in both incubations). With one exception (below), lethality was always less than about 30% and leakiness was always less than about 10%. The observed fraction of cells undergoing the reciprocal shifts which pass to a second cell cycle is the primary datum desired for each experiment; this number was corrected for lethality and leakiness as described in MATERIALS AND METHODS.

Reciprocal shift experiments involving hydroxyurea: Since we showed above that both the *cs* mutations *cdc44–1* and *cdc45–1* are able to synthesize DNA at the nonpermissive temperature, and because the execution points found for these mutants are so late in the cell cycle (MOIR *et al.* 1982, accompanying paper), it was expected that if the *cs* mutations were in a dependent relationship with DNA replication, the replication would be prerequisite to the *cs* function. Thus, reciprocal shift experiments with hydroxyurea (a reversible inhibitor of DNA synthesis [SLATER 1973] used previously by HARTWELL [1976] in such experiments) would serve as a kind of control for our protocol involving prior synchronization with α-factor. The results of such experiments are given in Table 2. In the case of *cdc44*, 83% of the cells shifted from 17° to hydroxyurea (HU)

TABLE 2

Reciprocal shift experiments involving hydroxyurea

Experiment	Duration first block (min)	Total cells examined	Shift*	% arrest in first cycle uncorrected	corrected†	% passage to second cycle†
cdc44 (*cs*)	230	263	17° → HU	26	17	83
hydroxurea (HU)		196	17° → 26°	15		
		296	17° → 17°	91		
	100	266	HU → 17°	97	100	0
		220	HU → 26°	11		
		292	HU → HU	83		
cdc 45 (*cs*)	230	282	17° → HU	43	40	60
hydroxyurea (*HU*)		247	17° → 26°	6		
		346	17° → 17°	79		
	100	310	HU → 17°	100	100	0
		214	HU → 26°	5		
		261	HU → HU	99		

* All incubations with hydroxyurea were at 26°. Where a temperature is indicated, incubation was in the absence of HU.

† Corrections for lethality during the first incubation and leakage were carried out as described in MATERIALS AND METHODS.

passed to a second cell cycle whereas none did so when the shift was from HU to 17°. This result clearly indicates that the HU-sensitive step is prerequisite to the cold-sensitive step; DNA synthesis is required before the *CDC44* function in a dependent pathway. In the case of *CDC45*, exactly the same conclusion can be drawn since shift from cold to HU allows 60% of the cells to pass to a second cell cycle as opposed to none when the shift is from HU to cold; DNA synthesis is required before the *CDC45* function in a dependent pathway.

The percentage of cells which passed to the second cell cycle in the permissive shifts (cold to HU) in these experiments was less than 100%. It is possible that the correction for cell death is an underestimate. Alternatively, there may be some interaction between the drug and some consequence to the cell resulting from prior loss of the *cs cdc* function (see also DISCUSSION). Following HARTWELL (1976), we take passage to a second cycle as the definitive parameter (as opposed to first cycle arrest, which might reflect simple lethality beyond that indicated by our control experiments). In view of the fact that prolonged incubations at the first block (to insure that all functions controlled by the second block have sufficient time to act) frequently result in unacceptably high lethality, we found (as did HARTWELL 1976) that it was necessary to compromise. Given these constraints, it seems reasonable to accept numbers in the range of half the cells passing to a second cell cycle as indicating that the shift is a permissive one, again following the interpretation of HARTWELL (1976).

Reciprocal shift experiments involving cdc14: Table 3 shows the results of reciprocal shift experiments using *ts-cs* double mutants. The *cs* mutations are again *cdc44–1* and *cdc45–1*, which are defective in medial nuclear division

TABLE 3

Reciprocal shift experiments involving cdc14

Experiment	Duration first block (min)	Total cells examined	Shift	% arrest in first cycle uncorrected	corrected*	% passage to second cycle*
cdc44 (*cs*)	240	228	17° → 37°	99	100	0
cdc14 (*ts*)	240	163	17° → 26°	7		
	240	335	17° → 17°	92		
	120	151	37° → 17°	10	5	95
	120	151	37° → 26°	5		
	120	159	37° → 37°	96		
cdc45 (*cs*)	240	238	17° → 37°	97	98	2
cdc14 (*ts*)	240	265	17° → 26°	43		
	240	281	17° → 17°	95		
	270	242	17° → 37°	96	95	3
	270	203	17° → 26°	59		
	270	250	17° → 17°	95		
	110	218	37° → 17°	12	5	95
	110	182	37° → 26°	8		
	110	220	37° → 37°	98		

* Corrections for lethality during the first incubation and leakage were carried out as described in MATERIALS AND METHODS.

(MOIR *et al.* 1982); the *ts* mutation is *cdc14–1*, which is defective in a late step in nuclear division (HARTWELL *et al.* 1973). The results are clear; essentially none of the cells (less than 3%) pass to a second cell cycle when the shift is from 17° (nonpermissive for *cs*) to 37° (nonpermissive for *ts*) while essentially all the cells (more than 95%) pass to a second cell cycle when the shift is in the other direction. Clearly the cold-sensitive step (*cdc44* or *cdc45*) is prerequisite to the heat-sensitive step (*cdc14*). Both *CDC44* and *CDC45* functions are required before the *CDC14* function on a dependent pathway. These experiments also serve in part as controls, since the phenotypes of the mutations suggest strongly that the order of function is the one we obtain with the reciprocal shift method.

Reciprocal shift experiments involving cdc9 and cdc16: Table 4 shows the results of reciprocal shift experiments using *ts-cs* double mutants where the *ts* mutation is *cdc9–1*, which is defective, like the *cs cdc* mutations, in medial nuclear division. The results, in the case of *cdc44–1*, are incomplete: although the shift from 17° to 37° yields a clear result (99% passage to a second cycle), the reciprocal shift cannot be carried out. With this particular double-mutant, incubation at 37° results in death of all (96%) the cells, even in the case of shift back to 26°, which is permissive for both the *ts* and *cs* mutations. Thus only a partial conclusion can be drawn: the *CDC44* function is not prerequisite to execution of *CDC9*; either the two functions are independent of each other or *CDC9* function is before the *CDC44* function on a dependent pathway.

TABLE 4

Reciprocal shift experiments involving cdc9

Experiment	Duration first block (min)	Total cells examined	Shift	% arrest in first cycle uncorrected	% arrest in first cycle corrected*	% passage to second cycle*
cdc44 (*cs*)	230	172	17° → 37°	18	1	99
cdc9 (*ts*)	230	192	17° → 26°	41		
	230	234	17° → 17°	93		
	100	—	37° → 17°	—		
	100	205	37° → 26°	96		all dead
	100	—	37° → 37°	—		
cdc45 (*cs*)	240	229	17° → 37°	57	51	49
cdc9 (*ts*)	240	268	17° → 26°	30		
	240	258	17° → 17°	91		
	300	182	17° → 37°	48	41	59
	300	165	17° → 26°	25		
	300	229	17° → 17°	92		
	100	177	37° → 17°	84	90	10
	100	157	37° → 26°	26		
	100	99	37° → 37°	83		

* Corrections for lethality during the first incubation and leakage were carried out as described in MATERIALS AND METHODS.

The results with double mutants between *cdc45–1(cs)* and *cdc9–1(ts)* are more straightforward. Cell death is not a large factor (less than 30%) and about half (50% to 60%) of the cells pass to a second cell cycle when the shift is from 17° to 37°, while only 10% pass to a second cell cycle in the reciprocal shift. When the first incubation time is increased in the 17° to 37° shift (Table 4), the percent of passage to the second cycle increases as well, suggesting that the event controlled by the second incubation (*i.e., cdc9* function) will occur at 17° given more time. This was unexpected, since the time of the first incubation (4 hr) is equivalent to a single cell cycle at that temperature for wild-type cells either growing exponentially or previously synchronized with α-factor. Nevertheless, the effect gives increased reason to believe that passage of half the cells to the second cycle should be interpreted as a positive (as opposed to intermediate) result.

The results of these reciprocal shifts suggest that the heat-sensitive step (*cdc9*) is prerequisite to the cold-sensitive step (*cdc45*); *CDC9* function appears to be required before *CDC45* function on a dependent pathway.

Table 5 shows the results of reciprocal shift experiments using double mutants involving the heat-sensitive mutation *cdc16–201*. The results with the *cdc44–1*(cs) *cdc16–201*(ts) strain was that 30% to 50% of the cells passed to a second cycle when the shift was from 17° to 37° while only 1% passed to a

TABLE 5

Reciprocal shift experiments involving cdc16

Experiment	Duration first block (min)	Total cells examined	Shift*	% arrest in first cycle uncorrected	% arrest in first cycle corrected*	% passage to second cycle*
cdc44 (cs)	230	235	17° → 37°	74	68	32
cdc16 (ts)	230	210	17° → 26°	31		
	230	222	17° → 17°	98		
	270	268	17° → 37°	64	51	49
	270	289	17° → 26°	30		
	270	250	17° → 17°	99		
	100	209	37° → 17°	98	99	1
	100	200	37° → 26°	23		
	100	258	37° → 37°	96		
cdc45 (cs)	240	340	17° → 37°	66	61	39
cdc16 (ts)	240	354	17° → 26°	21		
	240	384	17° → 17°	93		
	270	377	17° → 37°	53	36	76
	270	359	17° → 26°	30		
	270	260	17° → 17°	98		
	120	244	37° → 17°	14	8	92
	120	203	37° → 26°	7		
	120	331	37° → 37°	95		

* Corrections for lethality during the first incubation and leakage were carried out as described in MATERIALS AND METHODS.

second cycle in the reciprocal shift. Although the former percentage is small, again increasing the length of the first incubation resulted in an increased fraction of passage to the second cycle. Taken together with the complete arrest in the first cycle in the reciprocal shift, it seems likely that the heat-sensitive step (*cdc16*) is prerequisite to the cold-sensitive step (*cdc44*); *CDC16* function appears to be required before *CDC44* function on a dependent pathway.

The results with the *cdc45-1 cdc16-201* strain were quite different from any of the above cases. A large fraction of cells proceeded to a second cell cycle regardless of the direction of the shift. In one direction (17° to 37°) there was again an increase (39% to 76%) in passage to the second cycle when the first incubation was lengthened. This result can most readily be interpreted as indicating that the heat-sensitive step (*cdc16*) and the cold-sensitive step (*cdc45*) are not prerequisite to each other; *CDC16* function and *CDC45* function are not on the same dependent pathway, but on parallel independent branches of the nuclear division pathway.

involving cold-sensitive mutations in genes *CDC44* and *CDC45*, respectively. As shown in Table 6 *CDC44* appears to be part of a single unbranched dependent pathway: the HU-sensitive step in DNA synthesis must be executed before *CDC16* (HARTWELL 1976), which in turn must be executed before *CDC44* which itself must be executed before *CDC14*. The position in this pathway of the *CDC9* function is not determinable from our results although these data do indicate that *CDC9* function does not require prior execution of the *CDC44* function.

Table 7 indicates that *CDC45* is, in contrast, part of a branched pathway: the HU-sensitive step in DNA synthesis must be executed before *CDC9* (HARTWELL 1976), which in turn must be executed before *CDC45*. On the other hand, the *CDC16* function and the *CDC45* function may be executed independently, and therefore the pathway must branch. The *CDC45* function is prerequisite to execution of *CDC14*.

DISCUSSION

Tables 6 and 7 summarize the results obtained in reciprocal shift experiments

TABLE 6

Summary of pathway relationships involving cdc44

Experiment	Order of blockage	% passage to second cell cycle	Conclusion
1	*cdc44*-HU	83	Dependent: HU then *cdc44*
	HU-*cdc44*	0	
2	*cdc44-cdc16*	32, 49	Dependent: *cdc16* then *cdc44*
	cdc16-cdc44	1	
3	*cdc44-cdc9*	100	Uncertain: *cdc9* not dependent on *cdc44*
	cdc9-cdc44	all dead	
4	*cdc44-cdc14*	0	Dependent: *cdc44* then *cdc14*
	cdc14-cdc44	95	

TABLE 7

Summary of pathway relationships involving cdc45

Experiment	Order of blockage	% passage to second cell cycle	Conclusion
1	*cdc45*–HU HU–*cdc45*	60 0	Dependent: HU then *cdc45*
2	*cdc45*–*cdc9* *cdc9*–*cdc45*	45, 59 10	Dependent: *cdc9* then *cdc45*
3	*cdc45*–*cdc16* *cdc16*–*cdc45*	40, 76 92	Independent
4	*cdc45*–*cdc14* *cdc14*–*cdc45*	2, 5 95	Dependent: *cdc45* then *cdc14*

The two sets of results can be merged to produce a single pathway, shown in Figure 2. The positions of the *CDC9* and *CDC44* execution points are ambiguous with respect to the branch required to make *CDC45* and *CDC16* independent; otherwise the positions of the functions on this pathway are determined by the experiments reported here and by HARTWELL (1976) and HEREFORD and HARTWELL (1974), who carried out reciprocal shift experiments using HU and α-factor, respectively. Not only is the position of the bifurcation of the branches determined by the shift experiments, but also the position of the rejoining, since it must be before the *CDC14* function which is dependent upon the execution of both *CDC44* and *CDC45*.

The branch in the nuclear division pathway represents the second branching in the overall array of cell division cycle pathways (HARTWELL 1978). This new branch is contained entirely within the nuclear division branch defined before. At this point in our understanding, the meaning of branching in a pathway must

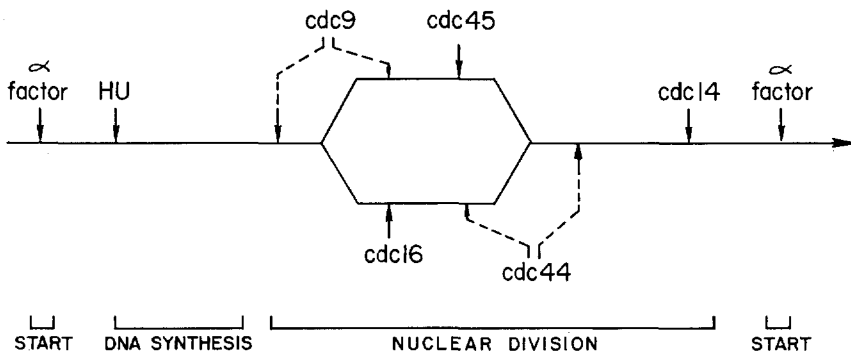

FIGURE 2.—Schematic diagram of the nuclear division pathway deduced from reciprocal shift experiments. The order of gene functions on the dependent pathways are shown by the arrows; dotted arrows indicate alternative possible positions for a gene function. The distances between arrows are arbitrary; the pathway is not drawn to any scale. The branching of the pathway indicates independent function of the gene functions associated with the two branches; execution of functions on one branch is not prerequisite to functions on the other.

remain essentially a speculation. A prominent and attractive possibility is the idea that the two arms of the branch each represent the assembly of different structures which later must cooperate in some essential function. Either assembly can be made in the absence of the other, but both are required for completion of the pathway. This interpretation fits in well with the results of the accompanying paper (MOIR *et al.* 1982), which present genetic evidence for assemblies of *cdc* gene products.

Our results suggest that the reciprocal shift procedure can indeed be used to distinguish order of function and to obtain dependency relationships among mutations with similar phenotypes and affecting functions with execution points quite near each other. On the other hand, all of the ambiguities intrinsic to the use of individual mutations remain: it is not possible to distinguish defects in synthesis of proteins from their activities in the cell cycle without different kinds of experiments.

The most difficult problem in interpreting the temperature shift data concerns the number of experiments in which passage to the second cycle was not complete, but showed intermediate values near 50%. A similar phenomenon was observed by HARTWELL (1976). Usually these values increased when additional time at the first incubation was provided; however, they occasionally failed to reach the high levels (more than 80%) observed in other experiments. Prolonged incubations could not always be used since some mutants rapidly die after one cycle at nonpermissive temperature. We have no convincing explanation for the fact that such long incubations (more than 240 min) are required when a complete cell cycle at 17° takes only 240 min. The problem clearly is not leakage from the point of the first block past the second, since control experiments show that first-cycle arrest is tight for at least 18 hr.

It should also be emphasized that the phenomenon of increased passage to the second cycle with increased length of the first incubation is not observed when the majority of cells show first cycle arrest. Therefore the phenomenon is not simply a general property of the double-mutants; it seems characteristic only for the cases of intermediate (30% to 60%) passage to a second cycle.

The particular case of greatest interest (*CDC16*, which is apparently independent of *CDC45*) is further buttressed by results using a different allele of *CDC16* (*cdc16-1*, from the collection of L. HARTWELL). The appropriate double mutant (*cdc45-1 cdc16-1*) showed increased passage to the second cycle during the shift in question (80% or more; data not shown). Unfortunately, in the reciprocal shift these double mutants were not very reversible (*i.e.*, lethality was high) and were somewhat leaky, making the experiments difficult to interpret.

In summary, we have applied the reciprocal shift method, using *cs-ts* double mutants, to determine order of function and dependent pathway structure in the yeast cell cycle. Application of the method to *cdc* mutations with similar phenotypes and execution points revealed a detailed order of function and a branched pathway structure.

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