COLD-SENSITIVE CELL-DIVISION-CYCLE MUTANTS OF YEAST: ISOLATION, PROPERTIES, AND PSEUDOREVERSION STUDIES

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ABSTRACT

We isolated 18 independent recessive cold-sensitive cell-division-cycle (*cdc*) mutants of *Saccharomyces cerevisiae*, in nine complementation groups. Terminal phenotypes exhibited include medial nuclear division, cytokinesis, and a previously undescribed terminal phenotype consisting of cells with a single small bud and an undivided nucleus. Four of the cold-sensitive mutants proved to be alleles of *CDC11*, while the remaining mutants defined at least six new cell-division-cycle genes: *CDC44*, *CDC45*, *CDC48*, *CDC49*, *CDC50* and *CDC51*.——Spontaneous revertants from cold-sensitivity of four of the medial nuclear division *cs cdc* mutants were screened for simultaneous acquisition of a temperature-sensitive phenotype. The temperature-sensitive revertants of four different *cs cdc* mutants carried single new mutations, called *Sup/Ts* to denote their dual phenotype: suppression of the cold-sensitivity and concomitant conditional lethality at 37°. Many of the *Sup/Ts* mutations exhibited a cell-division-cycle terminal phenotype at the high temperature, and they defined two new *cdc* genes (*CDC46* and *CDC47*). Two cold-sensitive medial nuclear division *cdc* mutants representing two different *cdc* genes were suppressed by different *Sup/Ts* alleles of another gene which also bears a medial nuclear division function (*CDC46*). In addition, the cold-sensitive medial nuclear division *cdc* mutant *cs*H80 was suppressed by a *Sup/Ts* mutation yielding an unbudded terminal phenotype with an undivided nucleus at the high temperature. This mutation was an allele of *CDC32*. These results suggest a pattern of interaction among *cdc* gene products and indicate that *cdc* gene proteins might act in the cell cycle as complex specific functional assemblies.

GENETIC analysis of cell division in the budding yeast *Saccharomyces cerevisiae* has resulted in the identification of more than 40 essential genes whose products appear to be needed primarily or exclusively to carry out steps in the pathway(s) of cell division (HARTWELL, CULOTTI and REID 1970; HARTWELL *et al.* 1973, 1974; HARTWELL 1974). Genes devoted primarily to cell division were recognized by the phenotypes of conditional-lethal (temperature-sensitive) mutations; at nonpermissive temperature, *cdc* (for *c*ell-*d*ivision-*c*ycle) mutants arrested their growth with a particular morphology, indicating an interruption in normal cell division in every cell at the same point in the cell cycle (HARTWELL, CULOTTI and REID 1970). Temperature-shift experiments with strains carrying one or more *cdc* mutations allowed the definition of dependent pathways of gene function; the notion of "execution point," *i.e.*, the last moment

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in the cell cycle at which an essential *cdc* gene product can act to allow passage to the next cell cycle, was also defined by temperature-shift experiments (HARTWELL, CULOTTI and REID 1970). Further resolution of the order in which some *cdc* genes function in the cell cycle was made possible by ordering mutant execution points relative to the action of α-factor (HEREFORD and HARTWELL 1974) and the DNA synthesis inhibitor hydroxyurea (HARTWELL 1976), each of which, like the *cdc* mutations, causes cell division to arrest at a particular point in the cell cycle.

At the resolution afforded by these methods, many *cdc* genes appear to act at the same point in the cell cycle. Many gene products may be involved in large complexes and may act together; or they may be involved in one or more pathways of dependent steps, all of which can occur within a short time.

To improve our understanding of the cell division process in yeast, we isolated *cdc* mutations with the conditional-lethal phenotype of cold sensitivity. When cultures of strains bearing these mutations are shifted to their nonpermissive temperature (17°), the cells cease division and display essentially the same array of cell-cycle-specific morphologies associated with the well-known temperature-sensitive *cdc* mutations.

Previous work with *cdc* mutants of yeast suggested that the cell cycle can be thought of as one or more morphogenetic pathways involving the function of many proteins serially or in concert. One way such systems could be analyzed genetically (particularly in the absence of biochemical identification of the proteins involved) was developed in studies of bacteriophage assembly (JARVIK and BOTSTEIN 1975): mutations (*cs* or *ts*) affecting one gene product could often be suppressed by mutations that affected a second interacting gene product. The suppressor mutation itself could also result in a conditional-lethal (*ts* or *cs*) phenotype. Screening for *cs* suppressors of *ts* mutations (or *vice versa*) could permit removal of the original mutation and characterization of the suppressor mutation with respect to its own conditional-lethal phenotype (JARVIK and BOTSTEIN 1975). We report here the application of this kind of analysis to the yeast cell cycle, using the *cs cdc* mutations we isolated. Since there are so many *cdc* genes in which mutations produce an apparent block in nuclear division, a process clearly involving a morphogenetic event (mitosis), we chose the *cdc* mutations with this phenotype for the pseudoreversion analysis described below.

The availability of cold-sensitive (*cs*) as well as temperature-sensitive (*ts*, meaning heat-sensitive) *cdc* mutations has made possible the application to the yeast cell cycle of another genetic method for the analysis of morphogenetic pathways described previously (JARVIK and BOTSTEIN 1973). The accompanying paper (MOIR and BOTSTEIN 1982) describes the application of this method to yeast, using *cs* and *ts cdc* mutations to order steps in the cell cycle by means of reciprocal temperature shifts.

MATERIALS AND METHODS

Media and methods of tetrad analysis: Rich medium (YEPD); minimal medium (SD); and methods of mating, sporulation and tetrad analysis were essentially as previously described

(BRANDRISS, SOLL and BOTSTEIN 1975). The phenotype of each spore was determined by suspending a portion of each spore clone in sterile water contained in a 32-well aluminum block and spotting onto selective media at appropriate temperatures using a 32-point inoculator. This low initial inoculum allowed the most reproducible judgement of cold- and heat-sensitivity.

Strains: All new mutants described here were derived by mutagenesis of S288C carrying the spontaneous mutation *his4–619* (strain DBY473, *a gal⁻ mal⁻ his4–619*, provided by G. FINK). Backcrosses were to S288C carrying a spontaneous mutation in *ade2* (strain DB640, **a** *gal⁻ mal⁻ ade2*, provided by G. FINK), wild-type S288C (strain DBY469), or to a strain derived from the isogenic cross DBY473 × DBY640. All mutants derived from EMS mutagenesis were backcrossed at least three times to wild-type S288C or the unmutagenized isogenic derivatives before further analysis.

Mutagenesis: Stationary phase cells of strain DB473 were incubated with agitation at a density of about 10^8 cells/ml in 0.1 M sodium phosphate buffer (pH 7) containing 3% ethyl methanesulfonate (EMS) at 30°. After 60 min, a portion of the suspension was diluted 40-fold into sterile 5% sodium thiosulfate to inactive the EMS. About 10–20% of the cells survived this treatment. These cells were washed twice in sterile water and stored at 4° for no longer than one month. New preparations of mutagenized cells were made frequently since the survival of cold-sensitive *cdc* mutants at 4° could not be predicted.

Isolation of cold-sensitive mutants: Mutagenized cells were grown at permissive temperature (26°) on YEPD solid medium. Plates containing about 100 colonies were replica-plated to YEPD solid medium at 26° and 17°. After two days, the replicas were compared. Colonies that appeared to have grown better at 26° than at 17° (about 5%) were picked, purified by streaking and retested for growth at 17° by spotting a drop of cells suspended in sterile water. Approximately 4% of those retested in this manner were cleanly cold-sensitive (*i.e.*, about 0.2% of the original replica-plated colonies).

Screening of cold-sensitive mutants for cell cycle terminal phenotype: Cold-sensitive mutants were grown overnight at 26° in liquid YEPD and subcultured at a density of about 2×10^6/ml early the next day. After 6–8 hr, when most cultures were in exponential phase, the mutants were again subcultured, this time into YEPD liquid medium at 17° for 18 hr. Cultures were sonicated briefly to disrupt clumps and examined by microscope for a characteristic *cdc* terminal phenotype as described by HARTWELL, CULOTTI and REID (1970). Only mutants that exhibited greater than 80% of the cells with a specific terminal phenotype were retained as cold-sensitive cell-division-cycle mutants.

Isolation of revertants: Spontaneous revertants were isolated as colonies growing on YEPD plates, each seeded with about 10^6 cells of a particular cold-sensitive cell cycle mutant and incubated for two to three weeks at the cold restrictive temperature. To insure independence, each plate was seeded with cells from a different single colony growing permissively on YEPD solid medium, and only one revertant was picked from each plate. Revertants were purified by streaking on YEPD at permissive temperature and then were tested for growth at the high restrictive temperature (35°).

Execution point determination: Execution points of the cold-sensitive *cdc* mutants were determined essentially as described by HARTWELL, CULOTTI and REID (1970) and HARTWELL *et al.* (1973). Exponential phase cultures of each mutant were grown at 26° and sonicated briefly to disrupt clumps. Cells were spotted onto agar slabs cooled to 17°, photographed immediately and then again after 12 hr at 17°. Between 100 and 200 cells were observed for each determination. Calculations were performed exactly as described previously (HARTWELL *et al.* 1973).

Nuclear stain: The nuclear morphology of arrested *cdc* mutants was determined using the fluorescent nuclear dye DAPI and the vital straining method of WILLIAMSON and FENNEL (1975). Cells from permissively grown cultures in exponential phase (YEPD + 1 μg/ml DAPI) were resuspended in fresh medium containing 1 μg/ml DAPI and shifted to 17° for 5 hr. These arrested cultures were sonicated briefly and washed three to four times with sterile distilled water. Temperature-sensitive revertants of the cold-sensitive mutants were examined in the same manner, except that incubation at the restrictive temperature, 37°, was for 3 hr. Longer incubation (such as 5 hr) usually led to poorer nuclear fluorescence, possibly due to degrada-

tion of nuclear material. Fluorescence of cell nuclei was examined using a Zeiss microscope equipped for epifluorescence (incident excitation illumination with a 50W high pressure mercury light source; exciter filter BP485, chromatic beam splitter FT510 and barrier filter LP520) and photography. Exposures for fluorescence photographs were 7–10 sec (Kodak Tri-X Pan film, ASA 400), using a 100× phase oil immersion objective and a low level of transmitted phase illumination to outline the cell body which does not fluoresce.

RESULTS

Isolation of cold-sensitive cell-division-cycle mutants: Screening of approximately 200,000 mutagenized yeast colonies by replica-plating on rich medium yielded 350 cold-sensitive (*cs*) mutants, *i.e.* mutants unable to grow at 17° but able to grow at 26°. Of these, 42 were found to arrest growth with a particular morphology at the nonpermissive temperature, indicating a *cdc* mutant phenotype. Each of these mutants was crossed to a wild-type strain of opposite mating type which, with the exception of spontaneous auxotrophic mutations, is isogenic to the strain used in the mutagenesis. Diploids were sporulated and the segregation of the cold-sensitivity followed in tetrad analysis. Cold-sensitivity segregated 2:2 in 18 of the 42 candidate *cdc* mutants, indicating a single chromosomal mutation; in each of these 18 cases, the heterozygous diploid grew normally at 17°, indicating that the *cs* mutation was recessive to its wild-type allele.

The 18 *cs cdc* mutations were grouped into three phenotypic classes on the basis of their morphological appearance after incubation in growth medium at the nonpermissive temperature for more than one doubling time (Table 1). The overall appearance in phase-contrast as well as the appearance of the stained nucleus using fluorescence microscopy was scored. Class I mutants (Figure 1b) arrest with a small bud and an undivided nucleus. Class II mutants (Figure 1c) arrest at medial nuclear division, with a single large bud and the nucleus in the

TABLE 1

Classification of cold-sensitive cell-division-cycle mutants

Class I Small bud		Class II Large bud		Class III Multiple buds	
Mutants	Gene	Mutants	Gene	Mutants	Gene
*cs*B71	*CDC49*	*cs*A10	*CDC44*	*cs*C17	*CDC11*
*cs*B84	*CDC49*	*cs*A18	*CDC45*	*cs*N84	*CDC11*
*cs*C8	*CDC49*	*cs*E24	*CDC48*	*cs*P44	*CDC11*
*cs*J26	*CDC49*	*cs*H80	*CDC51*	*cs*Q26	*CDC11*
*cs*L63	*CDC49*	*cs*AA15	Y[a]		
*cs*N40	*CDC49*	*cs*CC30	Z[a]		
*cs*SE129	*CDC49*				
*cs*F81	*CDC50*				

Cold-sensitive *cdc* mutants were classified according to their cellular and nuclear morphologies observed microscopically after 18 hr at 17° (*i.e.*, the terminal phenotype). Nuclear staining was with DAPI as described in MATERIALS AND METHODS. Gene assignments were made based upon complementation results shown in Table 2 and upon the results of crosses described in the text.

[a] Mutants in groups Y and Z complement all other *cs cdc* mutants in this table. However, they may or may not be alleles of other previously described *cdc* genes.

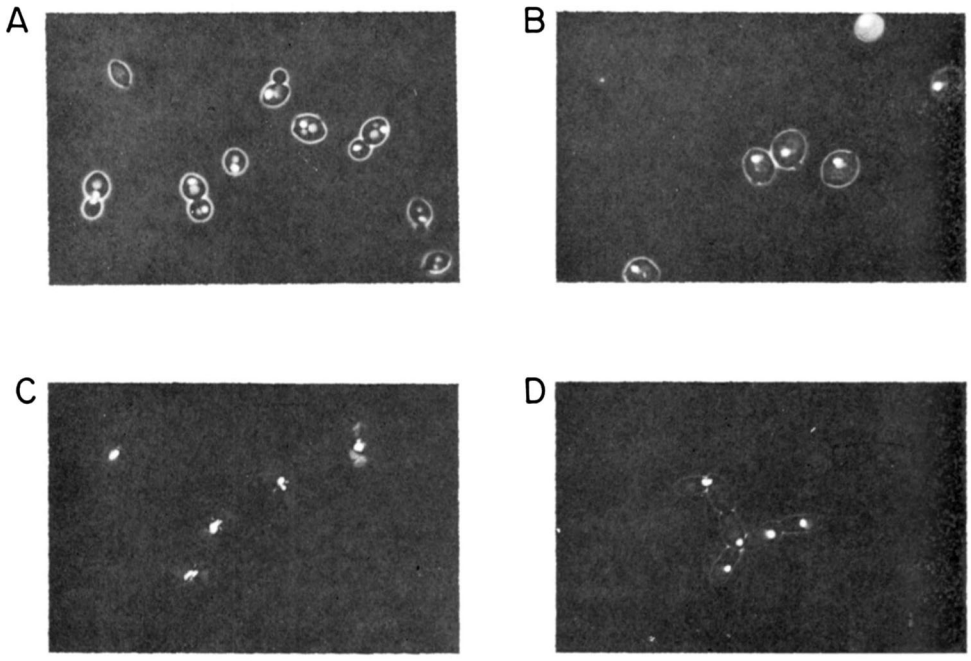

FIGURE 1.—Vital nuclear staining of cells incubated for 5 hr at 17° in the presence of 1 μg/ml DAPI: (A) Diploid cells from the cross between DB640 × DB473, which are isogenic with S288C and are not cold-sensitive; (B) homoalleic cold-sensitive cell-division-cycle diploids carrying the Class I mutation *cs*B84; (C) the Class II mutation *cs*H80; or (D) the Class III mutation *cs*P44.

neck between the mother and daughter. Class III mutants (Figure 1d) exhibit the characteristic cytokinesis terminal phenotype.

Complementation analysis of the cs cdc *mutants*: Complementation tests were carried out between all possible pairs of the 18 *cs cdc* mutants; they fell into nine complementation groups (Table 2). The distribution of mutations into complementation groups is very nonrandom: seven of the eight class I mutants are in a single complementation group and all four class III mutants are in a single complementation group. All of these mutants should be independent of each other; most derive from different mutagenized cultures.

Relationships of cs cdc *mutants to previously defined* cdc *genes*: A representative class III mutant (*cs*P44) was crossed to each of the four previously-described temperature-sensitive cytokinesis mutants (HARTWELL *et al.* 1973) and the segregation of *cs* and *ts* characters followed in tetrad analysis. Tight linkage was found between *cs*P44 and the *ts* mutation *cdc11-1* (PD:NPD:T = 15:0:0), suggesting that the class III *cs* mutations are alleles of the *CDC11* gene.

Linkage tests between *cs cdc* mutations and representative *ts* mutations defining *cdc* genes were carried out for several class II (medial nuclear division) mutants. Representative *ts* mutations from all previously described *cdc* genes showing the medial or late nuclear division phenotype (*i.e.*, *cdc2, 5, 6, 7, 8, 9, 13,*

TABLE 2

Complementation tests between cold-sensitive cell-division-cycle mutants

	mat a:	A10	A18	E24	H80	AA15	CC30	B84	B71	L63	J26	C8	N40	SE129	F81	C17	N84	P44	Q26
	mat α:																		
Class II	A10	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	A18	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E24	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	H80	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AA15	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
	CC30	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
Class I	B84							−	−	−	−	−	−	−	+	+	+	+	+
	B71							−	−	−	−	−	−	−	+	+	+	+	+
	L63							−	−	−	−	−	−	−	+	+	+	+	+
	J26							−	−	−	−	−	−	−	+	+	+	+	+
	C8							−	−	−	−	−	−	−	+	+	+	+	+
	N40							−	−	−	−	−	−	−	+	+	+	+	+
	SE129							−	−	−	−	−	−	−	+	+	+	+	+
	F81							+	+	+	+	+	+	+	−	+	+	+	+
Class III	C17															−	−	−	−
	N84															−	−	−	−
	P44															−	−	−	−
	Q26															−	−	−	−

Each of these cold-sensitive *cdc* mutants was generated by EMS mutagenesis and backcrossed at least three times with an isogenic wild-type strain of opposite mating type carrying a spontaneous *his4* or *ade2* mutation. Spore clones carrying the cold-sensitive mutation and either α *his4* or **a** *ade2* markers were used to perform this complementation test. Growth of the diploids at 17° was scored as shown.

14, *15*, *16*, *17*, *18*, *20*, *21*, *23*, *26*, *27* and *30*) were tested against the class II *cs* mutants *cs*A10, *cs*A18, and *cs*H80 (data not shown). Recombinant spores, (*i.e.* cs^+ ts^+) were found at a frequency of about 0.25, the value expected if the *cs* and *ts* mutations are unlinked. Since the class II *cs* mutations all complement each other, these data suggest that these three *cs* mutations define new *cdc* genes which display the medial nuclear division terminal phenotype. The new genes are *CDC44* (represented by *cs*A10, which becomes *cdc44–1*), *CDC45* (*cs*A18 becomes *cdc45–1*), and*CDC51* (*cs*H80 becomes *cdc51–1*).

We isolated a linked *ts* pseudorevertant (see below) of the class II mutant *cs*E24 that showed the medial nuclear division terminal phenotype at the new nonpermissive (35°) temperature. This new *ts* mutation is recessive and complements *ts* mutations representative of all the previously isolated *cdc* genes showing the medial nuclear division terminal phenotype. For this reason, the gene containing *cs*E24 has been designated *CDC48* (*cs*E24 becomes *cdc48–1*). Two additional new *cdc* mutations were found as unlinked *ts* pseudorevertants of a *cs cdc* mutation (*cdc45–1*) as described below; since these recessive *ts* mutations display a medial nuclear division terminal phenotype at their nonpermissive temperature and complement each other and mutations in all other *cdc* genes displaying a similar mutant phenotype, they have been assigned new *cdc* gene numbers: *CDC46* and *CDC47*.

The class I mutants have no exact phenotypic analogue among the previously described *cdc* mutations. Linkage tests with a *ts* allele of *CDC1* with a somewhat similar phenotype were carried out (data not shown), with the result that no linkage was found. Therefore the class I mutations define (at least provisionally) two additional *cdc* genes: *CDC49* (represented by *cs*B71, which becomes *cdc49–1*) and *CDC50* (*cs*F81 becomes *cdc*50–1).

Only some of the mutations discussed in this report can be assigned to old or to new *cdc* genes with the data obtained thus far. For this reason, and because the formally proper *cdc* nomenclature does not indicate whether mutants are *cs* or *ts*, the trivial names of the mutations already assigned to *cdc* genes (*i.e.* *cs*A10) will still be used wherever the context makes them preferable to the proper names (*i.e. cdc44–1*).

Execution points of the cs cdc *mutants*: The execution points for several mutations in class I and class II were determined by the temperature-shift photomicroscopic method first developed by HARTWELL *et al.* (1973). Class I mutants from either complementation group exhibit execution points early in the cell cycle close to the time of bud emergence (Table 3). Class II mutants exhibit execution points distributed from 0.1 to 0.7 (where 1.0 represents the length of a complete cycle). This range of values is similar to that found by HARTWELL *et al.* (1973) for *ts* mutations with medial nuclear division phenotype.

Reversion studies of cs cdc *mutants*: Spontaneous revertants of four cold-sensitive *cdc* mutants were selected at 17° and screened for concomitant acquisition of a temperature-sensitive growth phenotype (*i.e.*, failure to grow at 35°). These revertants were crossed to isogenic wild-type strains in order to assess linkage of the suppressor with the newly acquired *ts* mutation and the original *cs* mu-

TABLE 3

Execution points of cold-sensitive cell-division-cycle mutants

Class	Mutation	Execution point
I	*cs*L63 (*cdc49–5*)	0.21, 0.28
I	*cs*B84 (*cdc49–2*)	0.22
I	*cs*F81 (*cdc50–1*)	0.30
II	*cs*A10 (*cdc44–1*)	0.71, 0.67
II	*cs*A18 (*cdc45–1*)	0.48
II	*cs*H80 (*cdc51–1*)	0.37
II	*cs*E24 (*cdc48–1*)	0.25
II	*cs*AA15	0.10

Each EMS-generated cold-sensitive *cdc* mutant was backcrossed at least three times to an isogenic wild-type strain. The strains examined here carried, in addition to the cold-sensitive mutation, *α his4* markers. Execution points were determined photomicroscopically as described in MATERIALS AND METHODS.

tation. In all cases examined, the former two phenotypes (suppression of cold-sensitivity and the new temperature-sensitive lethality) were always linked, suggesting that they are the result of a single mutation. For this reason, these mutations will be referred to as Sup/Ts mutations, to emphasize the point that a single lesion apparently causes the two phenotypes of temperature-sensitivity and suppression of cold-sensitivity.

Reversion of cs*E24* (cdc48): *generation of linked (apparently intragenic) suppressors*: Among cold-insensitive revertants of the cold-sensitive *cdc* mutant *cs*E24, four independently-derived temperature-sensitive strains were found (Table 4). All four contain efficient suppressors of the cold-sensitivity of the original *cs*E24 phenotype as judged by growth on plates and in liquid culture at 17°. The Sup/Ts mutations appear tightly linked to the cold-sensitive mutation (*csE24*) they suppress: for example, no cold-sensitive spores were found among 33 tetrads of a cross of one revertant strain (rE24–15) with wild type, even though the temperature-sensitivity segregated 2:2 in all cases. An alternative to the linked Sup/Ts mutation hypothesis is that the revertant might contain two separate mutations, an intragenic simple reversion of the original *cs*E24 mutation and an unlinked *ts cdc* mutation. To rule out this possibility, eight tetrads from a cross of the revertant rE24–15 with *cs*E24 were analyzed with the result that only parental ditype (2*ts*:2*cs*) tetrads were obtained, showing that the *ts* lesion is tightly linked to the original *cs* mutation.

The temperature-sensitivity which accompanied the suppression of cold-sensitivity in all four cases affects the same function, because all four Sup/Ts mutations fail to complement each other with respect to their temperature-sensitive phenotype (failure to grow at 35°), although the temperature-sensitivity is recessive to wild type in each case.

These data indicate that each of the four pseudorevertants of *cs*E24 independently suffered a Sup/Ts mutation which lies in a single gene tightly linked to the original *cs* mutation. It seems likely that the original *cs* and the Sup/Ts both

lie in the same gene, and that in these cases the pseudo-reversion is intragenic. This idea is supported by the observation that the pseudorevertant strains exhibit the medial nuclear division terminal phenotype at 35°, which is the same phenotype displayed by the original *cs*E24 mutant strain at 17°. Since, as mentioned above, the Sup/Ts mutation complements *ts* mutant representatives of all previously described *cdc* genes with the same terminal phenotype, *cs*E24 and its Sup/Ts derivatives define a new *cdc* gene, *CDC48*.

Revertants of cs*A18* (cdc45)*: Extragenic suppressors, some of which define new* cdc *genes*: The cold-sensitive *cdc* mutant *cs*A18 (which defines the new gene *CDC45*) yielded seven independently derived temperature-sensitive revertants (Table 4). The Sup/Ts mutations fall into two classes: those which efficiently suppress cold-sensitivity and themselves exhibit a medial nuclear division terminal phenotype at 35°, and those which suppress cold-sensitivity only weakly and do not display any cdc phenotype. The efficient suppressors among the Sup/Ts mutants are recessive with respect to their temperature-sensitive phenotype (see below) and fall into two different complementation groups represented by the Sup/Ts mutations in revertant strains rA18–95 and rA18–100. Since these new *ts* lesions complement *ts* representatives of all other *cdc* genes

TABLE 4

Temperature-sensitive revertants of cold-sensitive cell-division-cycle mutants

cs mutants (*cdc* gene)	Number of independent revertants examined	ts revertants found	*sup/ts cdc* gene	*sup/ts* linkage to *cs* marker	*sup/ts* cell cycle terminal phenotype at 37°	Efficiency of suppression
*cs*A18	100	rA18–50	*CDC46*	None	Large bud	Efficient
(*cdc45*)		rA18–69	*CDC46*	None	Large bud	Efficient
		rA18–74	*CDC46*	None	Large bud	Efficient
		rA18–95	*CDC46*	None	Large bud	Efficient
		rA18–100	*CDC47*	None	Large bud	Efficient
		rA18–66	—	None	None	Weak
		rA18–79	—	None	None	Weak
*cs*E24	143	rE24–6	*CDC48*	Tightly linked	Large bud	Efficient
(*cdc48*)		rE24–15	*CDC48*	Tightly linked	Large bud	Efficient
		rE24–23	*CDC48*	Tightly linked	Large bud	Efficient
		rE24–107	*CDC48*	Tightly linked	Large bud	Efficient
*cs*CC30	192	rCC30–161	*CDC46*	None	Large bud	Efficient
		rCC30–252	*CDC46*	None	Large bud	Efficient
*cs*H80	400	rH80–16	*CDC32*	None	Unbudded	Weak
		rH80–119	—	None	None	Weak
		rH80–391	—	None	None	Weak

Spontaneous temperature-sensitive revertants from cold-sensitivity were isolated from four cold-sensitive class II *cdc* mutants as described in MATERIALS AND METHODS. In all cases, a single *sup/ts* mutation was responsible for both suppression of cold-sensitivity and the temperature-sensitive phenotype. Linkage of this *sup/ts* mutation to the original *cs* mutation was determined by tetrad analysis of crosses to a wild-type isogenic strain. Exponential cultures of the revertants growing at 26° were shifted to 37° for 6 hr and examined microscopically for a characteristic *cdc* terminal phenotype. Efficient suppression of cold-sensitivity could be scored in three to four days at 17° while weak suppression required at least seven days for unambiguous scoring.

displaying the medial nuclear division phenotype, they define (see above) two new *cdc* genes: *CDC46* and *CDC47*, respectively.

The efficiently-suppressing Sup/Ts mutations are unlinked to the original *cs*A18 mutation. A cross between the revertant rA18–95 (which contains the original *cs*A18 mutation as well as a Sup/Ts lesion) and an isogenic wild-type strain was carried out. The results (Table 5) show that the two lesions expected to be in the revertant (*i.e.* the *cs*A18 mutation and the Sup/Ts mutation) segregate independently. From the phenotypes of the spores in nonparental ditype (NPD) and tetratype asci it is possible to infer that the Sup/Ts mutation confers upon cells bearing it the temperature-sensitive phenotype even in the absence of the original *cs*A18 mutation. Linkage of the suppressor character with the temperature-sensitivity of the Sup/Ts mutation was confirmed by crosses of spores from PD asci carrying both the *cs* and Sup/Ts mutations back to the original *cs*A18 strain. In tetrads from this cross all spores receive a *cs* mutation, and temperature-sensitivity is always found in conjunction with phenotypic cold-insensitivity. Table 5 also shows the results of a cross between the revertant rA18–100 and wild type. Again the *cs* and Sup/Ts lesions are shown to be unlinked, although the temperature-sensitivity of the Sup/Ts mutation in the absence of the original *cs* mutation is somewhat diminished, suggesting that interaction of the two mutations is required for full expression of the temperature-sensitive phenotype. In both cases, the Sup/Ts mutation, even in the absence of the original *cs* mutation, displays the medial nuclear division terminal phenotype at the nonpermissive temperature.

TABLE 5

Tetrad analysis of cross between pseudorevertants and wild type

Ascus type	Number found	Phenotypes found	Inferred genotype
Cross 1: rA18–95 (*cs*A18, *sup/ts*) × wild type			
PD	10	2 Ts : 2 +	2 *cs*, *sup/ts* : 2 +
NPD	3	2 Ts : 2 Cs	2 *sup/ts* : 2 *cs*
T	24	2 Ts : 1 Cs : 1 +	1 *cs*, *sup/ts* : 1 *sup/ts* : 1 *cs* : 1 +
Cross 2: rA18–100 (*cs*A18, *sup/ts*) × wild type			
PD	4	2 Ts : 2 +	2 *cs*, *sup/ts* : 2 +
NPD	4	2 Ts : 2 Cs	2 *sup/ts* : 2 *cs*
T	13	2 Ts: 1 Cs : 1 +	1 *cs*, *sup/ts* : 1 *sup/ts* : 1 *cs* : 1 +
Cross 3: rCC30–161 (*cs*CC30, *sup/ts*) × wild type			
PD	3	2 Ts : 2 +	2 *cs*, *sup/ts* : 2 +
NPD	2	2 leaky Ts : 2 Cs	2 *sup/ts* : 2 *cs*
T	11	1 leaky Ts : 1 Ts : 1 Cs : 1 +	1 *sup/ts* : 1 *cs*, *sup/ts* : 1 *cs* : 1 +
Cross 4: rH80–16 (*cs*H80, *sup/ts*) × wild type			
PD	3	2 Ts : 2 +	2 *cs*, *sup/ts* : 2 +
NPD	4	2 Ts : 2 Cs	2 *sup/ts* : 2 *cs*
T	5	2 Ts : 1 Cs : 1 +	1 *cs*, *sup/ts* : 1 *sup/ts* : 1 *cs* : 1 +

Cold-sensitive *cdc* mutants bearing *sup/ts* mutations were crossed to a wild-type isogenic strain of opposite mating type. The diploids were sporulated and subjected to tetrad analysis. Phenotypes were determined by testing growth of the spore clones at 17°, 26° and 37° on solid medium.

The second class of revertants of *cs*A18, those which show weak suppression and no *cdc* terminal phenotype, were difficult to analyze genetically. Spore viability was poor (about 40%) in crosses with wild-type strains. Nevertheless, cold-sensitive segregants were found frequently (about 15% of the spores) indicating that the Sup/Ts mutation is not linked to the original *cs*A18 mutation. These revertants complement all of the efficiently-suppressing Sup/Ts mutations and each other for growth at 35°.

Revertants of cs*CC30*: Another class II cold-sensitive mutant (*cs*CC30, not yet assigned to a *cdc* locus) yielded two independently-derived temperature-sensitive pseudorevertants (Table 4). Both contain efficient suppressors of cold-sensitivity and they do not complement each other for growth at 35°. When one of these revertant strains, rCC30–161, was crossed to an isogenic wild-type strain and subjected to tetrad analysis (Table 5), cold-sensitive spores were found, indicating lack of linkage of the Sup/Ts and the original *cs*CC30 mutations. However, segregation of the temperature-sensitive phenotype is not cleanly 2:2, as was the case for the unlinked suppressors of *cs*A18 described above. The temperature-sensitive phenotype of the Sup/Ts mutation appears to be substantially dependent in these cases upon the simultaneous presence of the original *cs*CC30 mutation. Support for this idea is provided by the results of a cross between rCC30–161 and the original strain carrying only *cs*CC30; in this cross all progeny receive the *cs*CC30 mutation, and the temperature-sensitivity now segregates cleanly 2:2.

The revertants of *cs*CC30 define a *cdc* gene, since they display the characteristic nuclear division terminal phenotype (Figure 2a). However, as shown below, it turns out that the Sup/Ts mutations in these revertants are alleles of *CDC46*, which is a new *cdc* gene already defined by some of the revertants of *cs*A18.

Revertants of cs*H80: An extragenic suppressor in a* cdc *gene having a different terminal phenotype*: Examination of 400 independently-isolated revertants of the class II mutant *cs*H80 (*cdc51*) revealed three that simultaneously acquired a temperature-sensitive growth phenotype. All three of these suppress cold-sensitivity weakly, requiring six days to grow into substantial colonies at 17° instead of the three to four days required by wild-type or well-suppressed cold-sensitive pseudorevertants. Tetrad analysis of crosses of these revertant strains to isogenic wild-type strains revealed the presence of Sup/Ts mutations in each case which are not linked to the original *cs* mutations. The result of one of these crosses rH80–16 × wild type) is shown in Table 5; the analogous crosses with the other revertants gave PD:NPD:T ratios of 1:1:6 (rH80–119 × wild type) and 1:3:4 (rH80–391 × wild type).

The most interesting result obtained with the revertants of *cs*H80 (*cdc51*), a typical class II *cdc* mutant showing the nuclear division terminal phenotype, is that the Sup/Ts mutation in one of its pseudorevertants, rH80–16, displays a different *cdc* terminal phenotype at its nonpermissive temperature (35°). Instead of the nuclear division phenotype, over 90% of the cells arrest at the unbudded stage with a single nucleus (Figure 2b), indicating a defect early in the cell cycle. This Sup/Ts mutation was tested for complementation with pre-

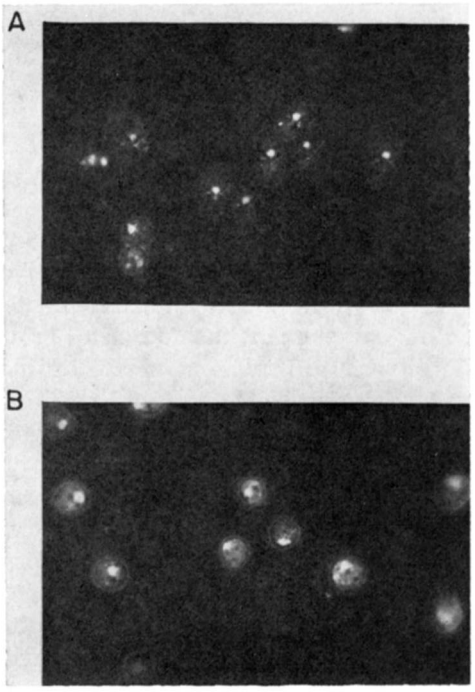

FIGURE 2.—Vital nuclear staining of two temperature-sensitive revertants of cold-sensitive cell-division-cycle mutants arrested for 3 hr at 37° in the presence of 1 μg/ml DAPI: (A) Cells of the diploid rCC30–161–D1, homoallelic for *both* the cold-sensitive mutation *cs*CC30 and the temperature-sensitive suppressor rCC30–161. (B) Cells of the diploid rH80–16–D1, homoallelic for the temperature-sensitive suppressor of *cs*H80, rH80–16.

viously described *cdc* mutations showing the unbudded terminal phenotype and found to be an allele of *CDC32*.

The other two weak suppressors of *cs*H80 do not show any obvious *cdc* phenotype.

Complementation analysis of Sup/Ts mutations: Six Sup/Ts mutations derived as suppressors of four different *cs cdc* mutations were found which themselves display a *cdc* phenotype at their nonpermissive temperature. All were recessive with respect to temperature-sensitivity (Table 6; also below). These six Sup/Ts mutations were tested for complementation pairwise at 37° (Table 6). As expected from the results described above, all the homo-allelic diploids failed to grow at 37°, and the Sup/Ts mutations in rA18–50, rA18–69, and rA18–95 are all members of a single complementation group. However, the analysis also shows the more interesting result that the Sup/Ts mutation in rCC30–161 is also a member of the same complementation group. The original mutations *cs*A18 and *cs*CC30 are independent, they themselves complement for growth at 17° (Table 1), and they are not linked (10 of 25 spores derived from a cross of *cs*A18 and *cs*CC30 were cs^+ recombinants). Thus, it appears that mutations in different *cdc* genes can be suppressed by alleles of a single gene (*CDC46*, defined by the several Sup/Ts mutations).

TABLE 6

Complementation tests of sup/ts *mutations*

mat a:	rA18–95	rA18–50	rA18–69	rA18–100	rE24–15	rCC30–161	wild type
mat α:							
rA18–95	—	—	—	+	+	—	+
rA18–50	—	—	—	+	+	—	+
rA18–69	—	—	—	+	+	—	+
rA18–100	+	+	+	—	+	+	+
rE24–15	+	+	+	+	—	+	+
rCC30–161	—	—	—	+	+	—	+
wild type	+	+	+	+	+	+	+

The *sup/ts* mutations were obtained free of the original cold-sensitive *cdc* mutations by selecting an α *his4 ts* or a *ade2 ts* spore clone from a NPD tetrad as shown in Table 5. An exception is the *sup/ts* mutation in rE24–15 which is tightly linked to the *cs cdc* mutation; separation of the two mutations has not been accomplished. Growth at 37° was scored as shown.

Crosses were performed to determine whether the same Sup/Ts mutation could suppress the *cs* mutations in the two different genes: the Sup/Ts mutation from rA18–95 was crossed with *cs*CC30 and the Sup/Ts mutation from rCC30–161 was crossed with *cs*A18. The results indicated that the Sup/Ts mutations did not cross-suppress; the suppression is apparently allele-specific. In fact, the analysis suggested that the cross-combinations of the *cs* and Sup/Ts tended to be inviable or to grow very poorly at any temperature.

Dominance of the suppression phenotype and recessiveness of the temperature-sensitivity phenotype of Sup/Ts mutations: The Sup/Ts mutations exhibit two phenotypes: temperature-sensitivity of growth and allele-specific suppression of a cold-sensitive cell cycle defect. For cases in which the suppressor is efficient and can easily be segregated free of the original *cs* mutation (Table 5), it is possible to determine separately the dominance of the two phenotypes. An example of this analysis for the Sup/Ts mutation from the pseudorevertant rA18–95 is shown in Table 7. Diploids carrying the Sup/Ts and original *cs* mutations homozygous and heterozygous in all combinations were constructed and tested for growth at 17° and at 35°. The results show that the temperature-sensitivity phenotype is always recessive (lines B, C, and F in Table 7), as is the cold-sensitivity of the original *cs*A18 mutation (line A) but that the suppression phenotype is dominant (line F). Similar results were obtained for the Sup/Ts mutations in rA18–100 and rCC30–161 (not shown) although the somewhat leakier temperature-sensitive phenotype of these mutations in the absence of the original *cs* mutation necessitated the use of 37° as the nonpermissive temperature. It thus appears to be a common property of Sup/Ts mutations that their suppression phenotype is dominant while the temperature-sensitivity is recessive.

DISCUSSION

Cold-sensitive lethal mutations with cdc *phenotypes*: Eighteen independent cold-sensitive cell-division-cycle mutations representing nine different genes were found. Although cold-sensitive mutations appear to be more difficult to

TABLE 7

Dominance tests on the temperature-sensitive suppressor in rA18–95

Strain	Genotype *cs* locus	Genotype *ts* locus	Phenotype Growth at 17°	Phenotype Growth at 35°
Haploids:				
DBY1101 or DBY1102	*cs*	+	−	+
DBY1103 or DBY1104	+	*ts*	+	−
DBY1105 or DBY1106	*cs*	*ts*	+	−
DBY640	+	+	+	+
Diploids:				
A: DBY1101 × DBY640	+/*cs*	+/+	+	+
B: DBY1103 × DBY640	+/+	+/*ts*	+	+
C: DBY1103 × DBY1102	+/*cs*	+/*ts*	+	+
D: DBY1103 × DBY1104	+/+	*ts*/*ts*	+	−
E: DBY1101 × DBY1102	*cs*/*cs*	+/+	+	+
F: DBY1105 × DBY1102	*cs*/*cs*	+/*ts*	+	+
G: DBY1105 × DBY1106	*cs*/*cs*	*ts*/*ts*	+	+
H: DBY1103 × DBY1106	+/*cs*	*ts*/*ts*	+	−

All possible combinations of the cold-sensitive *cdc* mutation *cs*A18 and its temperature-sensitive suppressor rA18–95 (*sup/ts*, or simply *ts* for the purpose of this table) were generated by the appropriate crosses of haploid strains bearing either or both mutations. The haploid strains were obtained by tetrad dissection; their complete genotypes are as follows: DBY1101, *α his4 cs*A18; DBY1102, **a** *ade2 cs*A18; DBY1103, *α his4 sup/ts*; DBY1104, **a** *ade2 sup/ts*; DBY1105, *α his4 cs*A18 *sup/ts*; DBY1106, **a** *ade2 cs*A18, *sup/ts*; DBY640, **a** *ade2*.

obtain than temperature-sensitive ones (from the same mutagenized cultures we routinely found about 0.2% *cs* and about 0.8% *ts*), the frequency of *cdc* mutations among the total conditional-lethals was about the same (about 10%). One of the nine *cdc* genes represented by the *cs cdc* mutations appears to correspond to one already previously identified (*CDC11*). Five others are, by tests of linkage, new *cdc* genes not represented among the previously identified *cdc* genes with a similar mutant phenotype. The remaining three genes represented by *cs cdc* mutations may also be new ones; the linkage analysis for them is not yet complete.

Among the *cs cdc* mutants we frequently found a rare terminal phenotype (small bud and undivided nucleus) as well as the expected more common medial

nuclear division, cytokinesis, and unbudded (which were difficult to analyze genetically and were not included in this study) terminal phenotypes. The only previously described gene with a similar phenotype is *CDC1*, but allelism tests by linkage ruled out the possibility that either of the genes (*CDC49* and *CDC50*) defined by our *cs* mutations is *CDC1*.

Despite the fact that the *cs cdc* mutants exhibited a variety of phenotypes analogous to those reported for the *ts cdc* mutants, the distribution of mutations among genes appears to be quite different. The genes defined by random conditional-lethals of the two types are nearly nonoverlapping. Apparently some genes mutate more readily to a cold-sensitive than a temperature-sensitive form and *vice versa*. Such differences are not entirely unexpected, since proteins that depend largely on hydrophobic interactions for stability might be particularly susceptible to mutation to cold-sensitivity. Some genes (*i.e.*, *CDC11* and *CDC48*) have yielded both *ts* and *cs* alleles. The particular case of *CDC48*, a gene in which we found a *cs* mutation (*cs*E24) which reverts to give intragenic *ts* pseudorevertants, deserves specific mention.

These results correspond well to those found for *cs* and *ts* mutations in bacteriophages, where the distribution into a limited number of genes of conditional-lethals of the two types was also found to be quite different (Scotti 1968; Cox and Strack 1971; Jarvik and Botstein 1975). Even more striking is the case of ribosome assembly in *Escherichia coli*, where extensive searches for *ts* mutants were fruitless while *cs* mutants were readily obtained (Guthrie, Nashimoto and Nomura 1969; Tai *et al.* 1969).

The important conclusion for attempts to study exhaustively processes like the yeast cell cycle is that saturation with mutations of an extensive morphogenetic system is likely to require isolation of mutations with more than one conditional-lethal phenotype. All previous indications had suggested that the number of genes yielding *ts cdc* mutations had nearly been saturated, and that the number of *cdc* genes yet to be found with this phenotype is small. Our results encourage further screening for *cdc* mutations with phenotypes other than temperature-sensitive lethality. However, this very notion that most of the *cdc* genes have already been found which mutate to give a temperature-sensitive lethal *cdc* phenotype is made less likely by our finding that several of the Sup/Ts mutations define new *cdc* genes.

Use of pseudo-reversion methods to study cell cycle mutants: By reversion of existing *cdc* mutations, we obtained many new conditional-lethal mutations which also have the *cdc* phenotype. Although the pseudoreversion method is laborious (many revertants of each *cs* mutant had to be screened to find a Sup/Ts), it is quite efficient in finding new *cdc* mutations in the sense that a majority of the Sup/Ts mutations had a *cdc* phenotype at the new nonpermissive temperature. These results are in accord with the results of Jarvik and Botstein (1975), who originally argued that the number of genes that can mutate to a form which can suppress a particular lesion must be small, and that therefore the pseudoreversion approach is intrinsically efficient for finding mutations affecting a single process even in complex organisms.

Our results also tend to validate the more fundamental supposition of JARVIK and BOTSTEIN (1975) that mutations that simultaneously result in suppression and a new conditional-lethal phenotype will most often lie in genes specifying products involved in the same morphogenetic process. This supposition is supported in particular by our finding of suppressors, in a single gene, of mutations in two different *cdc* genes showing the same mutant phenotype. It seems possible that the number of gene products involved (and that thereby can mutate to a suppressing form) at each step in the cell cycle is relatively small. Thus the pseudoreversion approach may indeed make it possible to saturate with mutations the relatively few genes involved in a single step without making it necessary to isolate large numbers of new *cdc* mutants at random.

One can imagine a "chain of revertants" approach to the cell cycle similar to that proposed by JARVIK and BOTSTEIN (1975). Our results suggest that it is feasible to start with a very small number of mutants affecting a particular step in the cell cycle and find mutations in many of the genes directly involved in this process through a series of pseudoreversion steps. The pseudoreversion approach has the additional advantage that it does not require the suppressor mutations to have an obvious cell-cycle phenotype: one can imagine a protein required for many processes in the cell but which is nevertheless also involved specifically in a protein complex which carries out a step in the cell cycle. A gene specifying such a protein might not often mutate to give a morphologically obvious cell-cycle phenotype, yet it should nevertheless yield Sup/Ts (or Sup/Cs) mutations if one begins with a mutation in an interacting *cdc* gene.

A particular example of such a result is our recovery of a *ts* allele of *CDC32* (which arrests as an unbudded cell) as a pseudoreversion of a *cs* allele of *CDC51* (which arrests at nuclear division). The conclusion is that the products of *CDC32* and *CDC51* intimately interact, despite the differences in the terminal phenotypes of conditional-lethal mutations in those two genes.

Finally, a generalization appears to be emerging from our experience with Sup/Ts (and Sup/Cs) mutations. We have shown above that all the Sup/Ts mutations we found are dominant with respect to their suppressor phenotypes but recessive with respect to their new conditional-lethal phenotype, which is generally retained even in the absence of the suppressed mutation. These results suggest that such suppressors could be selected even in the presence of multiple copies of the gene that is to mutate to a suppressor form. Thus, molecularly cloned genes might be screened for ability to yield suppressors of particular other mutations; positive results might indicate interactions among the genes involved. Promising results have been obtained in suppressing conditional mutations affecting DNA replication in *Salmonella typhimurium* using cloned genes (R. MAURER and D. BOTSTEIN, unpublished results).

In conclusion, we have found that searches for *cdc* mutations with a cold-sensitive phenotype has revealed new *cdc* genes. Suppressors of *cdc* mutations which have acquired a new conditional-lethal phenotype tend to be in other *cdc* genes with similar mutant phenotypes. The results are consistent with the idea that limited numbers of interacting gene products are involved at a given

step in the cell cycle. It may well be that many steps in the cell cycle involve specific complexes of *cdc* gene products in functional assemblies.

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