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Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast; dissociation of the G_1 and G_2 roles of the *cdc2*$^+$ protein kinase

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A cold-sensitive (cs) allele of *cdc2*, a gene that acts in both the G_1 and G_2 phases of the fission yeast cell cycle, has been isolated by classical mutagenesis. Further mutagenesis of a *cdc2*^{cs} strain yielded an extragenic suppressor that rescued the cs cell cycle defect but simultaneously conferred a temperature-sensitive (ts) cdc phenotype. This suppressor mutation was shown to be an allele of *cdc13*, a previously identified gene. A variety of allele-specific interactions between *cdc2* and *cdc13* were discovered. These included suppression of *cdc13*^{ts} alleles by introduction of the *cdc2*$^+$ gene on a multi-copy plasmid vector. *cdc13*$^+$ is required in G_2 for mitotic initiation and was shown to play no role in the G_1 phase of the cell cycle. *cdc2*$^+$, however, is essential in G_1 for DNA replication and in G_2 for mitosis. The newly isolated cs allele of *cdc2* that is rescued by a ts allele of *cdc13* is defective only in its G_2 function. *cdc13*$^+$ cooperates with *cdc2*$^+$ in the initiation of mitosis but not in the regulation of DNA replication. We propose that the *cdc13*$^+$ gene product might be a G_2-specific substrate of the *cdc2*$^+$ protein kinase.

Key words: cell cycle/*cdc13*/*cdc2*/fission yeast

Introduction

The rate of cell division in *Schizosaccharomyces pombe* is regulated by two control points in the cell cycle. One acts in G_1 prior to DNA replication and the other in G_2 controls the initiation of mitosis (reviewed in Fantes, 1984). Several lines of evidence have shown that progression through each of these two rate-limiting steps is dependent on the cell attaining a critical size (Fantes, 1977; Nasmyth *et al.*, 1979). Either the control acting in G_1 or that in G_2 can serve as the primary determinant of the rate of cell division (Nurse, 1975; Fantes and Nurse, 1977; Nurse and Thuriaux, 1977; Nasmyth, 1979); furthermore, cells may enter stationary phase from either G_1 or G_2 (Costello *et al.*, 1986).

The *cdc2*$^+$ gene product is a key component of cell cycle regulation in fission yeast. Analysis of temperature-sensitive (ts) *cdc2* mutants has shown that the activity of this gene is required at both the G_1 and G_2 control points (Nurse *et al.*, 1976; Nurse and Bissett, 1981). *cdc2*^{ts} mutant cells that arrest in G_1 at a nonpermissive temperature can undergo sexual conjugation under appropriate nutritional conditions (Nurse and Bissett, 1981). *cdc2*$^+$ is therefore sometimes described as a cell cycle start gene. In G_2 *cdc2*$^+$ acts at the rate-limiting step in mitotic initiation, and alleles of *cdc2* (*wee* alleles) have been isolated that do not cause cell cycle arrest but instead provoke premature mitotic initiation at an abnormally small cell size (Nurse and Thuriaux, 1980).

The *CDC28* start gene of *Saccharomyces cerevisiae* appears to act in both G_1 and G_2 (Piggott *et al.*, 1982) and is functionally equivalent to *cdc2*$^+$. *CDC28* is capable of rescuing ts *cdc2* mutants (Beach *et al.*, 1982a) or a *cdc2* gene disruption (this study). Furthermore the *cdc2* gene, after removal of its four introns, can rescue a ts *CDC28* mutant strain of budding yeast (Booher and Beach, 1986). The *cdc2* and *CDC28* gene products have a mol. wt of ~34 kd and share 62% overall amino acid homology (Hindley and Phear, 1984; Lorincz and Reed, 1984). Both *CDC28* (Reed *et al.*, 1985) and *cdc2*$^+$ (Simanis and Nurse, 1986) encode protein kinases, but at present no physiologically significant substrates of either kinase have been identified. Recently a human homolog of the *cdc2*$^+$ and *CDC28* gene products has been discovered (Draetta *et al.*, 1987; Lee and Nurse, 1987). Human p34 has also been shown to act *in vitro* as a protein kinase (Draetta *et al.*, 1987).

Since the *cdc2*$^+$ gene plays a central role in the regulation of two separate phases of the fission yeast cell cycle it is important to identify genes that interact with *cdc2*$^+$. Both *cdc25*$^+$ and *wee1*$^+$ cooperate with *cdc2*$^+$ in regulating the initiation of mitosis. *wee1*$^+$ acts as a mitotic inhibitor (Nurse and Thuriaux, 1986; Russell and Nurse, 1987) whereas *cdc25*$^+$ functions as a mitotic activator (Fantes, 1979; Russell and Nurse, 1986). *wee1*$^+$ and *cdc25*$^+$ act antagonistically to control mitotic initiation, perhaps by regulating the activity of *cdc2*$^+$ (Fantes, 1979; Russell and Nurse, 1986). At present there are no biochemical data to indicate direct interaction between the products of any of these genes, and the available genetic evidence does not exclude a variety of other models.

The product of the *suc1*$^+$ gene is presently the only protein that has been demonstrated to interact physically with the *cdc2*$^+$ gene product. *suc1*$^+$ was initially isolated as a gene, carried on a high copy number vector, that could rescue some but not all ts *cdc2* mutants (Hayles *et al.*, 1986a). A null allele of the *suc1* gene causes cell cycle arrest (Hayles *et al.*, 1986b; Hindley *et al.*, 1987). It has recently been demonstrated that ~5% of the *cdc2*$^+$ gene product can be isolated in a complex with the *suc1*$^+$ product, but this protein appears not to be a substrate of the *cdc2*$^+$ protein kinase (Brizuela *et al.*, in press). The available evidence suggests that the *cdc2*$^+$ and *suc1*$^+$ gene products interact throughout the cell cycle.

We have been particularly interested in a question that is not directly addressed by any existing experimental data; does *cdc2*$^+$ play the same role in G_1 and G_2 or can its activities in the control of these two phases of the cell cycle be dissociated? In biochemical terms the question is simply whether the spectrum of substrates of the *cdc2*$^+$ protein kinase is identical in G_1 and G_2 or whether at least some substrates are specific to one or the other phase of the cell cycle. If the latter were the case it would be predicted that it might be possible to isolate mutant alleles of *cdc2* that are defective for either G_1 or G_2 progression but not for both. This paper describes the first such allele of *cdc2* to be identified and also suppression of the mutant phenotype of this allele by a compensating mutation in a gene that acts exclusively in G_2.

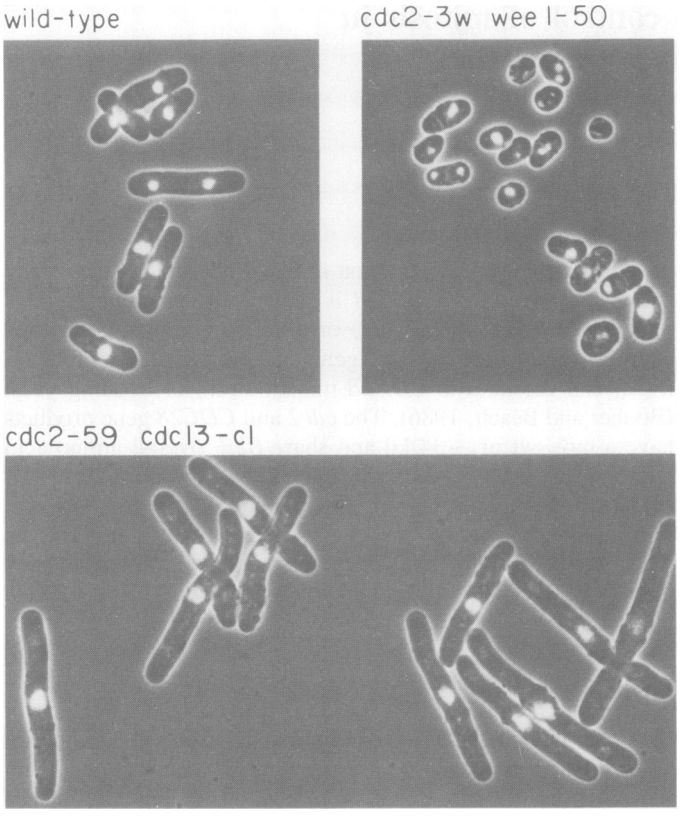

Fig. 1. Photomicrographs of propidium iodide stained wild-type (972), *wee1-50 cdc2-3w* (SP638) and *cdc2-59 cdc13-c1* (SP675) mutant strains. Prior to fixation and staining the cultures had been held at 36°C for 4 h in order to allow expression of the ts phenotypes.

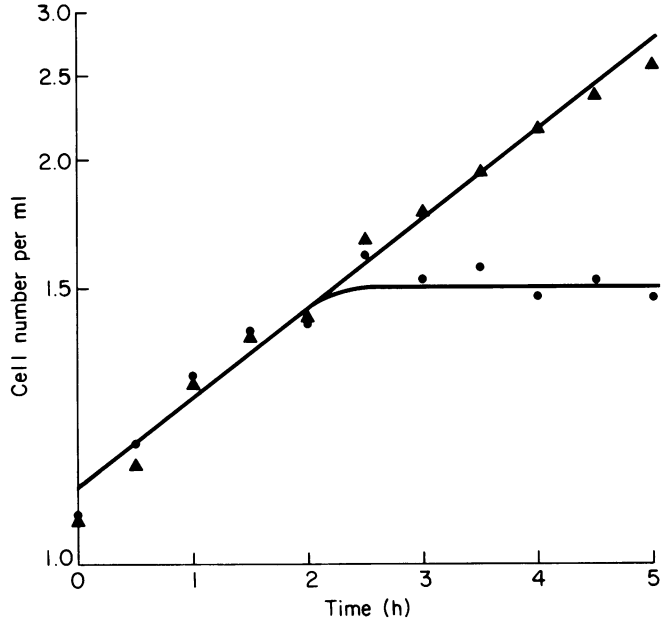

Fig. 2. Cell cycle arrest of a *cdc2-59 cdc13-c1* double mutant. A *cdc2-59 cdc13-c1* strain (SP675) was cultured at 25°C in PM + 0.5% YE medium. At time 0 h, half of the culture was shifted to 36°C (●) while the other half remained at 25°C (▲). The cell number/ml is plotted on a log scale. These values are related to the actual cell number/ml by the factor of 6.7×10^6.

Results

Isolation of cold-sensitive cdc2 alleles

cdc25$^+$ acts in the G_2 phase of the cell cycle as an inducer of mitosis, and *wee1*$^+$ acts as a mitotic inhibitor (Fantes, 1979; Russell and Nurse, 1986, 1987). *S. pombe* strains that overexpress *cdc25*$^+$ while lacking *wee1*$^+$ undergo cell death in a manner that suggests that they are locked in a mitotic state (Russell and Nurse, 1986). Although poorly understood at present this phenomenon has been described as 'mitotic catastrophe' (Russell and Nurse, 1986). The same behavior has been observed also in strains that carry a particular dominant *cdc2* allele (*cdc2-3w*; Fantes, 1981) and also lack *wee1*$^+$ (Russell and Nurse, 1987; and see Figure 1).

A ts lethal strain, *cdc2-3w wee1-50*^{ts} (SP638), was constructed and mutagenized (see Materials and methods) with the initial objective of isolating extragenic suppressors that rescued this double mutant. Ninety-three revertants that were viable at 37°C were obtained. By outcrossing each revertant to a *cdc2*$^+$ *wee1*$^+$ strain, 20 extragenic and 73 intragenic suppressors were defined (the intragenic revertants might carry secondary mutations in either *cdc2-3w* or *wee1-50*). Pair-wise crosses between each of the 20 strains carrying extragenic suppressors allowed assignment of each mutation to one of five linkage groups: *mcs1, 2, 3, 4* or *5* (*mcs*:*m*itotic *c*atastrophe *s*uppressor). The *mcs* mutants are not the primary focus of this paper and are being described in detail elsewhere (R.Booher and D. Beach, in preparation).

Each of the 73 strains that carried intragenic suppressors within either *cdc2-3w* or *wee1-50* was viable at 37°C, but six of these (designated r16, r20, r25, r34, r59 and r60) displayed a cold-sensitive (cs) cdc phenotype at 25°C. One revertant, r59, was picked for further study. Several lines of evidence indicated that the r59 mutation lay within the *cdc2* gene. (i) Outcrossing the original r59 isolate with a wild-type strain revealed that the cs *cdc* mutation segregated independently of the ts *wee1-50* mutation. (ii) In a cross between strains carrying r59 (SP654) and *his3* (SP1), the cs *cdc* mutation mapped within 0.5 centimorgans (cM) of *his3* (three recombinants among 551 spores analyzed). This approximates to the reported linkage distance between *cdc2* and *his3* of <2.3 cM (Kohli *et al.*, 1977). (iii) The cs cdc phenotype of r59 was rescued following introduction of the *cdc2*$^+$ gene on a multi-copy plasmid into this strain (Materials and methods). The r59 mutation thus defines an allele of *cdc2* and is designated *cdc2-59*. It should be noted that this allele presumably contains two mutations: the original *cdc2-3w* mutation which causes a *cdc2-59* strain to be wee at 37°C and the secondary mutation that prevents the lethality in a *wee1-50* background at 37°C and also confers a cdc phenotype at 25°C.

Isolation of a cdc13^{ts} *allele that suppresses cdc2-59*

cdc2-59 is a leaky cell cycle mutation. Mutant cells do not show first-cycle arrest after shift-down from 35°C to 25°C, but they are incapable of forming colonies on solid medium at this temperature. We have made use of this property to isolate revertants. A *cdc2-59* strain (SP654) was mutagenized and plated at 25°C with the objective of identifying extragenic suppressors of *cdc2-59* that themselves confer a ts cdc phenotype (see Materials and methods). Approximately 7000 revertants that were viable at 25°C were obtained. Each revertant was tested for viability at 37°C. Approximately 200 of the revertants displayed a variety of ts phenotypes. The most striking among these were 11 strains that displayed a ts cdc phenotype. Each of these 11 strains was outcrossed with a wild-type strain, and six were found to carry extragenic suppressors of *cdc2-59*. These suppressors (designated c1−6) each conferred a ts cdc phenotype in the absence of

Table I. Tetrad analysis of *cdc2-59 cdc13-c1* outcross[a]

Class	Total	Spore	cdc phenotype[b] 25°C	cdc phenotype[b] 37°C	his$^{+/-}$	Assumed genotype[c]
PD	12	*i*	cdc$^+$	cdc$^-$	+	*cdc2-59 cdc13-c1*
		ii	cdc$^+$	cdc$^-$	+	*cdc2-59 cdc13-c1*
		iii	cdc$^+$	cdc$^+$	−	*cdc2$^+$ cdc13$^+$*
		iv	cdc$^+$	cdc$^+$	−	*cdc2$^+$ cdc13$^+$*
NPD	7	*i*	cdc$^-$	na	na	*cdc2-59 cdc13$^+$*
		ii	cdc$^-$	na	na	*cdc2-59 cdc13$^+$*
		iii	cdc$^+$	cdc^{-L}	−	*cdc2$^+$ cdc13-c1*
		iv	cdc$^+$	cdc^{-L}	−	*cdc2$^+$ cdc13-c1*
T	52	*i*	cdc$^+$	cdc$^-$	+	*cdc2-59 cdc13-c1*
		ii	cdc$^+$	cdc$^+$	−	*cdc2$^+$ cdc13$^+$*
		iii	cdc$^-$	na	na	*cdc2-59 cdc13$^+$*
		iv	cdc$^+$	cdc^{-L}	−	*cdc2$^+$ cdc13-c1*
Others[d]	4					

na, not applicable; PD, parental ditypes; NPD, non-parental ditypes; T, tetratypes.
[a]Cross; *h$^-$ leu1-32 cdc2-59 cdc13-c1* × *h$^+$ leu1-32 his3* (SP670 × SP1). Spores were germinated on YEA plates at 25°C. Segregants that were cdc$^+$ at 25°C were also scored for the *his3* marker and their cdc phenotype at 37°C. Only asci with four spores that germinated were scored.
[b]At 25°C, cdc$^-$ segregants (*cdc2-59*) underwent several rounds of cell division but finally arrested as highly elongated cells. At 37°C, cdc$^-$ and cdc^{-L} refer either to a tight first-cycle arrest (cdc$^-$) or to the formation of poorly growing colonies containing highly elongated cells (cdc^{-L}).
[c]Assignment of genotype was based both upon the observed cdc phenotypes and on the assumption that a *his3$^+$* segregant carried *cdc2-59*.
[d]These represented *cdc2-59 his3$^-$* and *cdc2$^+$ his3$^+$* recombinants (three in total), and a gene conversion event.

cdc2-59. Pair-wise crosses between the six revealed that they defined four linkage groups. One linkage group consisted of three members (c1, c4 and c6) while the other three consisted of a single member each. Subsequently, it was established that the c1 mutation defined a single nuclear gene and was responsible for suppression of *cdc2-59* (see below). The mutants defining the other three linkage groups have not been pursued further.

Approximately 28 *cdc* genes have been described in *S. pombe* (see Kohli, 1987). A strain carrying the *cdc-c1* mutation was crossed against 22 of the 28 available *cdc* mutants. Wild-type recombinants were observed in each cross except that with a strain carrying *cdc13-117* (SP26) . Among 1912 segregants analyzed by random spore analysis, and in the dissection of 20 tetrads, no wild-type recombinants were observed. Furthermore, a *cdc13-117/cdc-c1^{ts}* diploid was found to have a ts cdc phenotype. Thus, by both linkage and complementation analysis the *c1* mutation defines an allele of *cdc13*. It is henceforth designated *cdc13-c1*. At 37°C a *cdc13-c1* strain forms very poorly growing colonies consisting of highly elongated cells. *cdc13-c1* is therefore a leaky allele of the gene. However, at 33°C the *cdc13-c1 cdc2-59* double mutant shows first-cycle arrest (Figure 2). The interaction between these two mutations is described in greater detail below.

These experiments establish that a ts allele of *cdc13* was isolated during a screen for extragenic suppressors of the cs *cdc2-59* mutation. In order to test whether the *cdc13-c1* allele not only causes a ts cell cycle defect but also carries the *cdc2-59* suppressor activity the following cross was made and analyzed by tetrad dissection: *cdc2-59 cdc13-c1 his3$^+$* × *cdc2$^+$ cdc13$^+$ his3$^-$* (SP670 × SP1). The his$^+$ and his$^-$ phenotypes were used as markers for *cdc2-59* and *cdc2$^+$* respectively so that *cdc2-59* (his$^+$) could be scored irrespective of the presence or absence of its suppressor. Tetrad analysis revealed that the segregant classes could be simply interpreted as a two point cross yielding parental ditypes, non-parental ditypes and tetratypes (Table I). This result implies that the *cdc13-c1* mutation confers both the ts cdc phenotype and the *cdc2-59* suppressor activity. Had this not been the case, a third genetic marker would have had to be invoked in order to interpret the cross.

Table II. Interactions between *cdc13* and *cdc2*

Strain	Relevant genotype	Phenotype[a] 25°C	30°C	33°C	37°C
SP654	*cdc2-59*	cdc$^-$	cdc$^+$	cdc$^+$	cdc$^+$
SP671	*cdc13-c1*	cdc$^+$	cdc$^+$	cdc$^+$	cdc^{-L}
SP26	*cdc13-117*	cdc$^+$	cdc$^+$	−	cdc$^-$
SP675	*cdc13-c1 cdc2-59*	cdc$^+$	cdc$^-$	cdc$^-$	cdc$^-$
−	*cdc13-117 cdc2-59*[b]	cdc$^-$	cdc$^-$	cdc$^-$	cdc$^-$
−	*cdc13-117 cdc2-3w*[b]	cdc$^-$	cdc$^-$	cdc$^-$	cdc$^-$
SP735	*cdc13-117 cdc2-1w*	cdc$^+$	cdc$^+$	−	cdc$^-$
SP708	*cdc13-c1 cdc2-3w*	cdc$^+$	cdc$^-$	cdc$^-$	cdc$^-$
SP729	*cdc13-c1 cdc2-3w wee1-50*	cdc$^+$	cdc$^-$	cdc$^-$	cdc$^-$
SP679	*cdc2-59/+*	cdc$^+$	cdc$^+$	cdc$^+$	cdc$^+$
SP737	*cdc13-c1/+*	cdc$^+$	cdc$^+$	cdc$^+$	cdc$^+$
SP738	*cdc2-59/cdc2-59 cdc13-c1/+*	cdc$^-$	cdc$^+$	cdc$^+$	cdc$^+$
SP710	*cdc2-59/+ cdc13-c1/cdc13-c1*	cdc$^+$	cdc$^+$	cdc$^+$	cdc^{-L}
−	*cdc13-c1 cdc2::LEU2*[b]	cdc$^-$	cdc$^-$	cdc$^-$	cdc$^-$
SP672[c]	*cdc13-117* pcdc2$^+$	cdc$^+$	−	−	cdc$^+$
SP672[c]	*cdc2-117* psuc1	cdc$^+$	−	−	cdc$^-$
SP709[c]	*cdc13-c1* pcdc2$^+$	cdc$^+$	−	−	cdc$^+$

[a]cdc^{-L} designates an ability to form a slowly growing colony containing elongated cells at 37°C (see text).
[b]Spores of this genotype germinated but arrested as single elongated cells. These segregants were obtained from crosses: SP655 × SP26 for *cdc13-117 cdc2-59*; SP673 × SP661 for *cdc13-117 cdc2-3w*; and sporulation of diploid SP734 for *cdc13-c1 cdc2::LEU2*.
[c]This strain contains the plasmid indicated.

In order to further test whether the *cdc13-c1* allele acts as the suppressor of *cdc2-59*, each mutation was individually outcrossed several times against a wild-type strain and then recombined (cross SP655 × SP671). *cdc2-59 cdc13-c1* segregants, which could be unambiguously identified in non-parental ditype tetrads, were always cdc$^+$ at 25°C (data not shown). The preceding two experiments demonstrate that the *cdc2-59* suppressor activity co-segregates with *cdc13-c1* indicating that *cdc13-c1* has two effects: at 37°C it causes cell cycle arrest, whereas at 25°C it suppresses the cs cell cycle defect of *cdc2-59*.

cdc2-59 and cdc13-c1 are recessive mutations

We have tested whether *cdc2-59* and *cdc13-c1* are dominant or recessive mutations with respect either to their individual phenotypes or to their interactions with each each other. *cdc2-59/cdc2$^+$* (SP679) and *cdc13-c1/cdc13$^+$* (SP737) diploids were constructed, and neither displayed a cdc phenotype at 25, 30, 33 or 37°C (Table II). Each mutant allele is therefore recessive to the wild-type allele with respect to their individual cdc phenotypes.

In order to investigate whether both mutant alleles are also recessive to wild-type in their respective interactions, *cdc2-59 cdc13$^+$/cdc2-59 cdc13-c1* (SP738) and *cdc2$^+$ cdc13-c1/cdc2-59 cdc13-c1* (SP710) diploids were constructed (see Materials and

methods). SP738, unlike a *cdc2-59 cdc13-c1* strain, was incapable of growth at 25°C (Table II). Thus the *cdc13-c1* allele is recessive to $cdc13^+$ in its ability to rescue the cs *cdc2-59* defect. Also SP710, unlike a *cdc2-59 cdc13-c1* strain, showed a leaky rather than a tight ts cdc phenotype at 37°C (Table II). Thus with respect to its interaction with *cdc13-c1*, *cdc2-59* is recessive to $cdc2^+$.

Interaction between cdc2-59 and cdc13-c1 is allele specific

The *cdc13-c1* mutation suppresses the cs phenotype of *cdc2-59*, and conversely *cdc2-59* causes a leaky ts *cdc13-c1* strain to show first-cycle arrest at 33° C (Figure 2). Thus the primary phenotype of each allele (cs cdc or leaky ts cdc) is affected by the presence of the other mutation. We have tested whether either of these two interactions is specific for either allele.

A strain carrying a previously isolated non-leaky ts allele of *cdc13*, *cdc13-117* (SP26), was crossed with one carrying *cdc2-59* (SP655) with the intention of constructing a *cdc13-117 cdc2-59* double mutant. It was found, however, that at no temperature between 25 and 37°C were germinating *cdc13-117 cdc2-59* spores able to divide even though at 30°C both individual mutants are capable of cell division (Table II). This experiment demonstrates that the suppression of *cdc2-59* by *cdc13-c1* is specific to that allele and that the combination of *cdc2-59* and *cdc13-117* is incompatible even though a permissive temperature (30°C) would have been anticipated.

In a parallel experiment, we have tested whether the suppression of *cdc2-59* by *cdc13-c1* is specific to the *cdc2-59* allele. Since this experiment cannot be constructively undertaken using the many existing ts alleles of *cdc2* (the *cdc13-c1* allele is also ts), a strain carrying a null allele of *cdc2* (*cdc2::LEU2*) was crossed with a *cdc13-c1* strain in order to obtain a *cdc13-c1 cdc2::LEU2* double mutant (see Materials and methods). Following tetrad dissection spores were allowed to germinate at 25, 30, 33 and 37°C. At each temperature the *cdc2::LEU2 cdc13-c1* recombinant spores, unequivocally assigned in non-parental ditype tetrads, germinated but arrested as single elongated cells that were incapable of cell division (Table II). This result demonstrates that the *cdc13-c1* allele does not suppress a null allele of *cdc2* and therefore suggests that *cdc13-c1* does not act to simply by-pass the requirement for $cdc2^+$.

Allele-specific interactions between cdc2-3w and $cdc13^{ts}$

The *cdc2-59* allele is presumed to carry two mutations: *cdc2-3w* and a secondary mutation that confers a cs cdc phenotype (see above). We have investigated whether the *cdc2-3w* component of *cdc2-59* makes any contribution to the interactions between *cdc2-59* and either *cdc13-c1* or *cdc13-117* (see above).

cdc2-3w cdc13-c1 (SP708) and *cdc2-3w cdc13-117* double mutants were constructed by tetrad dissection of appropriate crosses. Surprisingly, both double mutants displayed exactly the same phenotype as the respective *cdc2-59 cdc13-c1* and *cdc2-59 cdc13-117* strains (Table II). Thus at no temperature between 25 and 37°C was a germinating *cdc2-3w cdc13-117* spore capable of cell division, and a *cdc2-3w cdc13-c1* strain displayed first-cycle arrest at 33°C rather than the leaky cdc phenotype of *cdc13-c1* alone. These data demonstrate that two distinct phenotypes of *cdc2-59*, cold-sensitivity and interaction with $cdc13^{ts}$, are attributable to the two different mutations that this allele contains.

The failure of *cdc2-3w cdc13-117* recombinants to divide at a temperature that is normally fully permissive for *cdc13-117* (25°C) allows a test of whether this effect is specific to the *cdc2-3w* wee allele. A *cdc2-1w cdc13-117* double mutant was constructed (SP735). This strain was viable at 25°C and displayed the characteristic cdc phenotype of *cdc13-117* (Table II). The interaction between *cdc2-3w* and *cdc13-117* is thus shown to be specific to the *cdc2-3w* allele and is not shared by all wee alleles of *cdc2*.

Rescue of $cdc13^{ts}$ *by overexpression of* $cdc2^+$

In light of the range of interactions between *cdc2* and *cdc13* described above, we have tested whether overexpression of $cdc2^+$ might rescue a ts *cdc13* defect. The $cdc2^+$ gene (Beach *et al.*, 1982a) was introduced on a multi-copy vector into *cdc13-c1* and *cdc13-117* strains (SP709 and SP672). Surprisingly, the ts cell cycle defect of both strains was rescued by the $cdc2^+$ plasmid. The $cdc2^+$ plasmid allowed rapidly growing *cdc13-117* and *cdc13-c1* colonies to form at 37°C (Table II).

The $suc1^+$ gene, when cloned on a multi-copy vector, is capable of rescuing some but not all ts *cdc2* mutants (Hayles *et al.*, 1986a). Recently it has been demonstrated that the $suc1^+$ gene product forms a complex with the $cdc2^+$ protein kinase (Brizuela *et al.*, in press). To test for possible interactions between $suc1^+$ and $cdc13^+$, a plasmid carrying $suc1^+$ was introduced into a strain carrying *cdc13-117*. The ts defect of this strain was not rescued by the $suc1^+$ plasmid (Table II).

cdc13$^+$ *is required for 'mitotic catastrophe'*

$cdc13^+$ is normally essential for mitosis (Nurse *et al.*, 1976). In a *cdc2-3w wee1-50* strain, at the restrictive temperature, mitosis occurs aberrantly and cell death follows (Russell and Nurse, 1987; and see Figure 1). We have investigated whether $cdc13^+$ is required for expression of this 'mitotic catastrophe' phenotype. A triple mutant *cdc13-c1 cdc2-3w wee1-50* (SP729) was constructed. Upon shift to 37°C, this strain displayed an unequivocal cdc phenotype rather than 'mitotic catastrophe'. The aberrant mitosis in a *cdc2-3w wee1-50* strain therefore remains dependent on the normal function of $cdc13^+$.

cdc13$^+$ *acts only in* G_2

Isolation of a ts allele of *cdc13* that suppresses a cs allele of *cdc2* appears paradoxical because $cdc2^+$ function is required during both the G_1 and in G_2 phases of the cell cycle (Nurse and Bissett, 1981) whereas $cdc13^+$ has been thought to act only during mitosis (Nurse *et al.*, 1976). Two possible resolutions are proposed: (i) the *cdc2-59* gene product might be defective only in its G_2 function; or (ii) $cdc13^+$ might in fact be required in G_1 and G_2 and interact with $cdc2^+$ during both phases of the cell cycle. The first possibility could not be approached directly since the cs *cdc2-59* allele does not display first cycle division arrest at 25°C. However, the *cdc2-59 cdc13-c1* double mutant undergoes first cycle arrest at 36°C, a property not shared by either allele individually. We established the point of cell cycle arrest of this double mutant and have investigated the behavior of strains carrying the *cdc13-117* allele more thoroughly than previously.

A *cdc2-59 cdc13-c1* strain (SP675), which has a generation time at 25°C similar to the wild-type, was cultured in liquid medium at 25°C and transferred to a restrictive temperature (36°C). The culture underwent a 43% increase in cell number following the temperature shift (Figure 2). A cell cycle transition point of 0.49 was calculated according to the method of Nurse *et al.* (1976). A transition point of ~0.7 has previously been reported for both *cdc2-33* and *cdc13-117* mutants (Nurse *et al.*, 1976; Fantes, 1982). The earlier transition point of the *cdc2-59 cdc13-c1* strain may be due to the presence of the *cdc2-3w* wee mutation in *cdc2-59* (see Fantes, 1983, for discussion).

In order to test whether either *cdc13-117* (SP26) or *cdc13-c1*

cdc2-59 (SP675) strains display any defect in progression through G_1, cultures of each strain were shifted at a permissive temperature from a complete medium to one lacking a nitrogen source. Under these conditions the majority, but not all, of the cells accumulated at stationary phase in the G_1 phase of the cell cycle (Nurse and Thuriaux, 1977; Costello *et al.*, 1986). After 12 h the starved cultures were reinoculated into fresh complete medium at a restrictive temperature (36°C). The ability of each strain to undergo DNA replication during the following hours was assayed by flow cytometry (see Materials and methods). Both the *cdc13-117* and *cdc2-59 cdc13-c1* strains underwent DNA replication but not cell division. They therefore accumulated with a 2C DNA content (Figure 3). As a control experiment a ts *cdc2-33* strain, previously shown to be defective for G_1 and G_2 progression (Nurse and Bissett, 1981), was shown not to undergo DNA replication or cell division under similar conditions (Figure 3). This experiment confirms that *cdc13*$^+$ plays no apparent role in G_1 and that the *cdc2-59 cdc13- c1* double mutant arrests only in the G_2 phase of the cell cycle.

The *cdc13*$^+$ gene has previously been assumed to act later than *cdc2*$^+$, not during mitotic initiation but during mitotic chromosome segregation (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981; Fantes, 1982). At prolonged time intervals after transfer to a restrictive temperature, a *cdc13-117* mutant strain forms multiple abortive fission plates and a small proportion of cells display three microscopically visible nuclear structures that appear to be chromosome pairs arrested at metaphase (Nasmyth and Nurse, 1981). By contrast, cells arrested in G_2 by a *cdc2*^{ts} defect retain an interphase nuclear structure (Nurse *et al.*, 1976). The *cdc2-59 cdc13-c1* double mutant was investigated 4 h after transfer to a nonpermissive temperature (36°C). The nuclear structure in this strain was similar, at the light microscopic level, to *cdc2-33* rather than to *cdc13-117* mutants (Figure 3), and no division plates were observed even after prolonged incubation at the restrictive temperature. These results, in combination with the preceding genetic analysis, indicate that *cdc13*$^+$ does not act late during nuclear division but rather early during mitotic initiation (see Discussion).

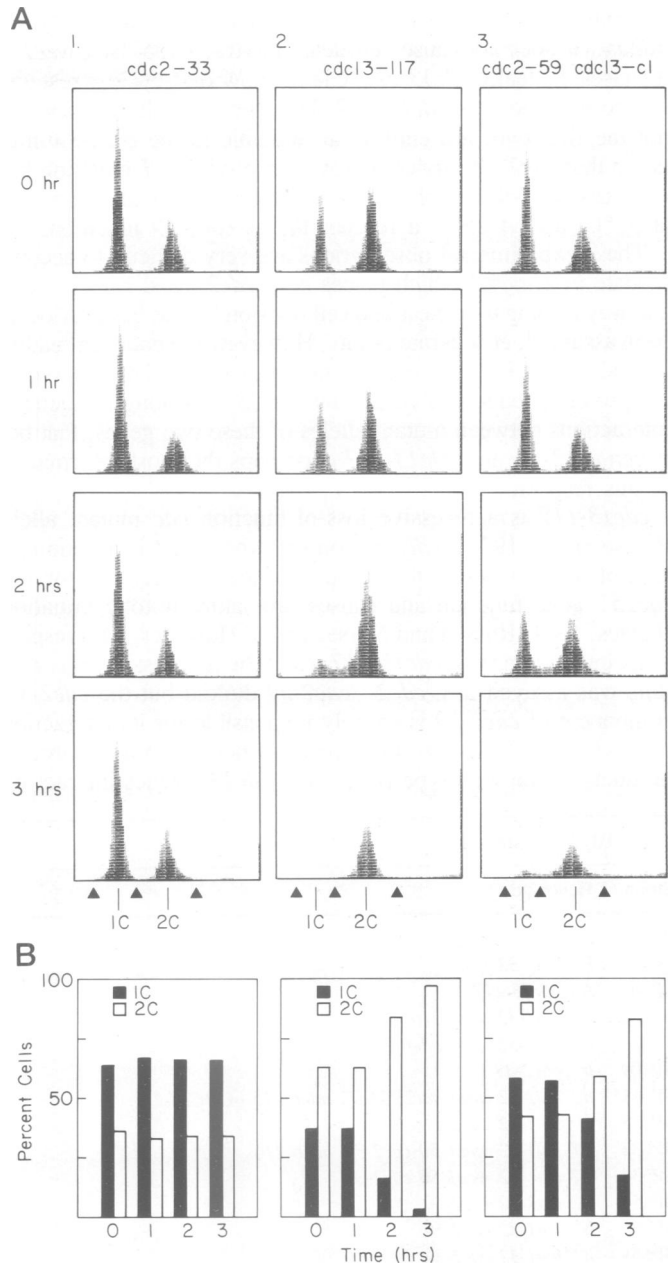

Fig. 3. Cell cycle arrest in *cdc2* and *cdc13* mutants. Mutant strains *cdc2-33* (SP409), *cdc13-117* (SP26) and *cdc2-59 cdc13-c1* (SP675) were held in nitrogen starvation medium for 12 h at 25°C (see Materials and methods). At time 0 h the cells were transferred to complete minimal medium at 36°C after which samples were analyzed at 0, 1, 2 and 3 h. (**A**) Flow-cytometric analysis of distribution of DNA content per cell. The DNA content of the cell (fluorescent intensity) is represented by the abscissa and the number of cells falling into each category by the ordinate. The first peak corresponds to a 1C DNA content per cell, and the second peak corresponds to a 2C DNA content per cell. (**B**) Histogram representation of data in (A). The percentage of cells having a 1C and 2C DNA content was calculated from the area between the set marks (▲). The total percentage (1C + 2C) was set at 100%. Note that the percentage of cells classified as having a 2C DNA content is actually the sum of cells in S phase and G_2.

Discussion

In this study the following experimental results have been obtained. (i) A cs mutant of *cdc2* has been isolated. This allele, *cdc2-59*, was derived from *cdc2-3w* and unlike the parental allele does not confer lethality in the absence of *wee1*$^+$. (ii) An extragenic suppressor of *cdc2-59*, that itself conferred a ts cdc phenotype, was found by linkage and complementation analysis to be an allele of *cdc13* (*cdc13-c1*), a previously identified gene. The interactions between *cdc2-59* and *cdc13-c1* were shown to be specific to both of these alleles. (iii) Both mutations were found to be recessive to the wild-type. (iv) *cdc2-59* and *cdc13-c1* are individually both leaky mutations. In combination they cause first-cycle arrest at 33°C. (v) The *cdc2-3w* mutation carried by the *cdc2-59* allele is responsible for this interaction with *cdc13-c1*. (vi) The combination of *cdc2-3w* and *cdc13-117* is lethal even at a temperature normally permissive for *cdc13-117*. Another *wee* allele of *cdc2*, *cdc2-1w*, does not share this property. (vii) The *cdc2*$^+$ gene, on a multi-copy plasmid vector, rescues both *cdc13-117* and *cdc13-c1* mutant strains. (viii) *cdc13*$^+$ was shown to be required for expression of mitotic catastrophe in a *cdc2-3w wee1-50* strain. (ix) A *cdc13-117* strain was also shown to have no defect during G_1 and to arrest only in G_2 at the nonpermissive temperature. The *cdc2-59 cdc13-c1* double mutant shared these same properties. At a nonpermissive temperature this strain displayed an interphase rather than mitotic nuclear structure.

The genetic interactions between *cdc2*$^+$ and *cdc13*$^+$ described above were unexpected and are unusually extensive. This stems in part from the fact that the *cdc2-59* allele presumably carries

two mutations and has at least four phenotypes. It is a cs *cdc* mutant, it does not cause cell death in strains that lack *wee1*$^+$, it causes the leaky *cdc13-c1* allele to show first-cycle arrest and is incompatible with *cdc13-117*. The latter two phenotypes, but not the first two, are entirely attributable to the *cdc2-3w* mutation that *cdc2-59* carries. Likewise the *cdc13-c1* mutation has more than one distinct phenotype. It causes a leaky cdc phenotype at 37°C, and at 25°C it rescues the cs *cdc2-59* mutation.

These experimental observations are very difficult to accommodate in a model which places both *cdc2*$^+$ and *cdc13*$^+$ on a pathway leading to nuclear and cell division but, as has previously been assumed, at separate points. However, the data can readily be understood in terms of a direct physical association between the protein products of *cdc2*$^+$ and *cdc13*$^+$. Among the genetic interactions between mutant alleles of these two genes, that between *cdc2-3w* and *cdc13-117* is perhaps the most informative in this respect.

cdc13-117 is a recessive loss-of-function *cdc* mutant allele (Nurse *et al.*, 1976). *cdc2-3w* on the other hand is a dominant gain-of-function allele that by-passes the requirement for the *cdc25*$^+$ gene function and causes premature mitotic initiation (Fantes, 1981; Russell and Nurse, 1986). However, with respect to its interaction with *cdc13-117, cdc2-3w* is recessive to *cdc2*$^+$ (this was assayed in a *cdc2*$^+$/*cdc2-59* diploid but the *cdc2-3w* component of *cdc2-59* is entirely responsible for its interaction with *cdc13-117*). This result is readily understood if the protein products of the wild-type *cdc2*$^+$ and *cdc13*$^+$ genes interact by direct, but not necessarily stable, physical association and if the *cdc2-3w* and *cdc13-117* products are incapable of physical interaction irrespective of any other biological activity that either protein may possess.

cdc2$^+$ has previously been shown to play a role in both G_1 control and during mitotic initiation (Nurse *et al.*, 1976; Nurse and Bissett, 1981). On the other hand *cdc13*$^+$ has been presumed to be involved only during mitotis (Nasmyth and Nurse, 1981; Fantes, 1982). We have specifically tested whether *cdc13*$^+$ plays a role in G_1 and found that its activity is not required for DNA replication. Its function is required in G_2 during which it interacts with *cdc2*$^+$ in the regulation of mitotic initiation rather than, as had previously been supposed, acting only late during mitotic chromosome separation.

Since *cdc2-59* fails to show first-cycle arrest at a nonpermissive temperature, we have been unable to test directly whether this allele causes cells to arrest both in G_1 and G_2, the characteristic feature of all previously described recessive alleles of this gene. However, there are two independent lines of evidence suggesting that *cdc2-59* has no G_1 defect. Firstly, *cdc2-59* and *cdc13-c1* are both leaky cell cycle mutations, but in combination they cause first-cycle arrest in G_2. Secondly, a mutation within *cdc13* can rescue *cdc2-59* mutants at 25°C. This result implies that at 25°C the deficiency of *cdc2-59* can be overcome by a single compensatory mutation in a gene that acts only in G_2. *cdc2-59* is the first described recessive loss-of-function allele of *cdc2* that is defective for G_2 function but not also in its G_1 activity.

The biochemical function of the *cdc13*$^+$ gene product and its precise role in mitosis is presently unknown. However, since *cdc2*$^+$ encodes a protein kinase it is possible that the *cdc13*$^+$ product is a G_2-specific substrate, phosphorylation of which leads to mitotic initition. This hypothesis will be investigated in future experiments.

Table III. Yeast strains

Strain	Genotype
972	*h*$^-$
SP1	*h*$^+$ *leu1-32 his3*
SP26	*h*$^-$ *cdc13-117*
SP202	*h*$^+$ *leu1-32 ade6-210*
SP223	*h*$^-$ *leu1-32 ura4 ade6-216*
SP409	*h*$^-$ *cdc2-33*
SP611[a]	*h*$^-$ *leu1-32 ura4 cdc2::LEU2 ade6-216 pCDC28-2*
SP623	*h*$^-$ *leu1-32 cdc2-1w*
SP635	*h*$^+$ *leu1-32 wee1-50 cdc2-3w ade6-216*
SP638	*h*$^+$ *leu1-32 wee1-50 cdc2-3w*
SP654	*h*$^-$ *leu1-32 cdc2-59*
SP655	*h*$^+$ *leu1-32 cdc2-59*
SP661	*h*$^-$ *leu1-32 cdc2-3w*
SP667	*h*$^-$ *leu1-32 cdc2-59 ade6-210*
SP670	*h*$^-$ *leu1-32 cdc2-59 cdc13-c1*
SP671	*h*$^+$ *cdc13-c1*
SP672	*h*$^+$ *leu1-32 cdc13-117*
SP673	*h*$^+$ *leu1-32 cdc13-117 ade6-216*
SP675	*h*$^-$ *cdc2-59 cdc13-c1*
SP679	*h*$^-$/*h*$^+$ *leu1-32/leu1-32 cdc2-59/+ ade6-210/ade6-216*
SP701	*h*$^+$ *leu1-32 cdc2-59 cdc13-c1 ade6-216*
SP708	*h*$^-$ *cdc2-3w cdc13-c1*
SP709	*h*$^+$ *leu1-32 ura4 cdc13-c1*
SP710	*h*$^+$/*h*$^-$ *leu1-32/leu1-32 cdc2-59/+ cdc13-c1/cdc13-c1 ade6-216/ade6-210*
SP724	*h*$^+$ *leu1-32 ura4 cdc13-c1 ade6-210*
SP729	*h*$^-$ *leu1-32 cdc2-3w wee1-50 cdc13-c1*
SP734[a]	*h*$^+$/*h*$^-$ *leu1-32/leu1-32 ura4/ura4 cdc13-c1/+ +/cdc2::LEU2 ade6-210/ade6-216*
SP735	*h*$^-$ *leu1-32 cdc2-1w cdc13-117*
SP737	*h*$^+$/*h*$^-$ *leu1-32/leu1-32 cdc13-c1/+ ade6-216/ade6-210*
SP738[a]	*h*$^+$/*h*$^-$ *leu1-32/leu1-32 cdc2-59/cdc2-59 cdc13-c1/+ ade6-216/ade6-210*

[a]See Materials and methods for strain constructions.

Materials and methods

Media

Standard *S. pombe* media (YE, YEA, PM and PMA) have been described (Beach *et al.*, 1985). PM−NH_4 medium is PM medium that lacks NH_4Cl. PM + 0.5% YE is PM medium supplemented with 0.5% yeast extract (w/v, Difco). Phloxin B (Sigma) was added to some agar plates at 20 mg/l as an indicator of cell viability a described by Gutz *et al.* (1974). In certain genetic backgrounds, for example *his3*$^-$, Phloxin B has an adverse effect on cell growth and was therefore omitted.

Strain constructions

All strains were derived from wild-type *S. pombe* strains 972 (*h*$^{-S}$), 975 (*h*$^{+N}$), or 968 (*h*^{90}) introduced by U.Leupold. Conventions for the genetic nomenclature of *S. pombe* are discussed by Kohli (1987). The isolation and characterization of the *cdc13-117, cdc25-22* (Nurse *et al.*, 1976), *cdc2-1w, wee1-50* (Nurse and Thuriaux, 1980) and *cdc2-3w* (Fantes, 1981) mutations have been previously described. Isolation of the *cdc2-59* and *cdc13-c1* alleles are described in this study. Table III contains a complete list of yeast strains used in this study. Heterothallic strains listed as *h*$^-$ and *h*$^+$ refer to the mating types *h*$^{-S}$ and *h*$^{+N}$ respectively (Beach and Klar, 1984). All diploid strains used in this study were constructed by using the complementing *ade6-210* and *ade6-216* alleles as described by Gutz *et al.* (1974). Standard genetical procedures used for *S. pombe* were performed as described by Gutz *et al.* (1974) except that matings were done on complete minimal medium (PMA) plates rather than on malt extract plates.

The *cdc2-59/cdc2-59 cdc13-c1/+* diploid strain (SP738) was constructed by mating the ts *cdc2-59 cdc13-c1* strain (SP701) with the cs *cdc2-59* strain (SP667), which had been previously grown on PMA + Leu + Ura plates for 2 days at 25°C and 37°C respectively, on PMA + Leu + Ura plates at 30°C. These conditions permitted some of the cells to undergo conjugation and survive as diploids before the terminal cdc phenotype was expressed.

Strain SP654 could not be transformed by the standard transformation procedure. To introduce a plasmid into this strain, strain SP1 was initially transformed with pcdc2$^+$ or psuc1 plasmids that carried a leu$^+$ marker. This transformant was then crossed with SP654 and leu$^+$ his$^+$ meiotic products that could grow at 33°C were selected. Since *his3* is closely linked to *cdc2-59* (see Results) most of these colonies should contain the *cdc2-59* mutation. Instability of the leu$^+$

marker confirmed the presence of the plamid. All colonies were examined microscopically to ensure that haploid rather than diploid colonies were present.

The haploid *cdc2* gene disruption strain (SP611) was constructed by transforming strain SP223 that already carried pCDC28-2 with a plasmid, pcdc2::LEU2, linearized by *Pst*I digestion. The *S. cerevisiae CDC28* and *S. pombe cdc2* gene products are functionally equivalent (Beach *et al.*, 1982a; Booher and Beach, 1986). Transformants that carried the *cdc2::LEU2* gene replacement were identifiable as stable leu$^+$ colonies. A minority of cells in these transformant colonies exhibited a cdc phenotype due to the instability of the pCDC28-2 plasmid. These transformants also have a wee phenotype similar to a ts *cdc2* mutant that was rescued by the *CDC28* gene (Beach *et al.*, 1982a). Strain SP611 was mated to SP724 to form the diploid SP611/SP724. The plasmid, pCDC28, was purged from this diploid by passaging it through nonselective growth conditions. This resulting diploid strain, SP734, was induced to undergo meiosis and sporulation on PMA + Ura plates at 33°C. Spores from these azygotic asci were germinated on YEA plates at 25, 30, 33 and 37°C to analyze the phenotype of *cdc2::LEU2 cdc13-c1* recombinant cells.

Plasmids and yeast transformation

The isolation of the *cdc2*$^+$ and *suc1*$^+$ genes has been described previously (Beach *et al.*, 1982a; Hayles *et al.*, 1986a). The *S. pombe* replicating vectors pDB248x (Beach and Nurse, 1981) and pIRT1 were used. pIRT1 is pUC18 (Norrander *et al.*, 1983) that contains a 1.2-kb *Eco*RI restriction fragment of *S. pombe* DNA carrying *ars1* (Losson and Lacroute, 1983), and a 1.1-kb *Hin*dIII fragment bearing the *S. cerevisiae URA3* gene (Rose *et al.*, 1984). The *URA3* and *LEU2* genes of *S. cerevisiae* can rescue, respectively, *ura4* and *leu1* mutants of fission yeast (Beach and Nurse, 1981; Losson and Lacroute, 1983). pcdc2$^+$ used in this study is pDB248x with a 3.4-kb *Pst*I fragment insert bearing the *cdc2*$^+$ gene (Durkacz *et al.*, 1985). psuc1$^+$ has been previously described (Hayles *et al.*, 1986a) Plasmid pcdc2::LEU2 is pcdc2::leu as previously described (Booher and Beach, 1986). pCDC28-2 is pIRT-1 that carries a 4.6-kb *Sph*I–*Xba*I fragment bearing *CDC28* from pBR322 (CDC28-1) (Reed *et al.*, 1982). Transformation of *S. pombe* was performed by the protocol of Beach *et al.* (1982a). The addition of salmon sperm DNA to the transformation reaction was omitted. Also, after the 15-min PEG 4000 incubation, the cells were pelleted and resuspended in 200 μl 1.2 M sorbitol, 10 mM Tris pH 7.6, 10 mM $CaCl_2$ and plated directly without waiting 20–60 min as previously described.

Physiological studies

Cell number was determined either by means of a hemacytometer (Scientific Instruments) or with a Coulter Counter (Coulter Electronics). The transition point for the *cdc2-59 cdc13-c1* mutant was determined from cell number increase after an asynchronous culture was shifted from 25 to 36°C (Nurse *et al.*, 1976). The medium used was PM + 0.5% yeast extract (Difco Laboratory) for the reason discussed by Thuriaux *et al.* (1978).

In order to accumulate ts *cdc* mutants in the G_1 phase, asynchronous cultures were grown in PM medium at 25°C to a density of 5×10^6 cells/ml. At this point the cells were pelleted and washed three times with PM– NH_4 medium. The final pellet was resuspended in PM–NH_4 medium to a final density of 5×10^6 cells/ml and placed in a 25°C shaking water bath for 12 h. The cells were then pelleted and resuspended in PM medium, prewarmed to 36°C, to a final denstiy of 5×10^6 cells/ml (time 0 h). This culture was placed in a shaking water bath at 36°C and 10-ml samples were removed at various times for flow-cytometric analysis. These samples were fixed in 70% EtOH (see below) within 10 min after removal from the 36°C culture.

For flow cytometry, cells were fixed in 70% EtOH, exposed to RNase, stained with propidium iodide and analyzed exactly as described in Beach *et al.* (1985). To examine nuclei, cells were stained with propidium iodide as above and observed with a Zeiss fluorescence microscope.

Mutagenesis

Strains SP635 and SP654 were cultured in 100 ml minimal medium to a density of 10^6 cells/ml at 25 and 36°C respectively. The cells were pelleted, resuspended in 50 ml minimal medium and dispensed into 5-ml aliquots. Ethyl methanesulfonate (Kodak) was added to a final concentration of 2%. Strains SP635 and SP654 were incubated with shaking for 4 h, 25°C and 1.5 h, 36°C respectively. Each aliquot of cells was pelleted and washed four times with YEA medium. The final cell pellet was resuspended in YEA medium and $\sim 2 \times 10^6$ cells were spread on each plate. Strain SP635 was incubated at 37°C whereas strain SP654 was incubated at 25°C. Ninety-three revertants of strain SP635 were collected after 3–5 days at 37°C. Approximately 7000 revertants of strain SP654 were obtained after 7 days at 25°C. These colonies were replica plated to YEA + Phloxin B and incubated at 37°C for 2–3 days. Cells with a ts defect, initially identified a dark red colonies at 37°C, were examined microscopically and retained for further analysis.

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