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Involvement of *cdc13*$^+$ in mitotic control in *Schizosaccharomyces pombe*: possible interaction of the gene product with microtubules

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Previous genetic studies have shown that the fission yeast *cdc13*$^+$ gene product interacts closely with the *cdc2*$^+$ protein kinase during mitosis. Here, we have cloned the *cdc13*$^+$ gene from a *S.pombe* gene bank by complementation of the temperature-sensitive defect of a *cdc13-117* mutant strain. The complementing activity was localized to a 1.9-kb *Xba*I–*Nsi*I DNA fragment, and nucleotide sequencing revealed a 1446-bp open reading frame. The predicted amino acid sequence contained 482 residues and was not homologous to any protein in a protein database. The *cdc13*$^+$ gene function was confirmed to be essential for cell division since cells carrying a *cdc13* null allele arrested with a cdc phenotype. However, unlike any existing temperature-sensitive *cdc13* mutants, *cdc13* null mutants arrested in G_2 without septa or condensed chromosomes indicating that *cdc13*$^+$ gene function is required at or prior to the initiation of mitotis. *cdc13-117* mutant strains were found to be hypersensitive to the tubulin inhibitor thiabendazole. This observation suggests that the *cdc13*$^+$ gene product, which is required for mitotic initiation, may interact with microtubules.

Key words: Schizosaccharomyces pombe/cdc13$^+$/cell cycle

Introduction

The regulation of the transition from interphase to mitosis is a subject of major interest. It has been approached successfully from a genetic angle in a variety of ascomycete fungi including *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Aspergillus nidulans* (Hartwell *et al.*, 1974; Nurse *et al.*, 1976; Morris, 1976). In fission yeast, the *cdc2*-encoded protein kinase, which is homologous to the *CDC28* protein kinase of *S.cerevisiae* and a similar protein in human cells, plays a central role at the G_2/M transition (Beach *et al.*, 1982a; Hindley and Phear, 1984; Booher and Beach, 1986; Simanis and Nurse, 1986; Draetta *et al.*, 1987; Lee and Nurse, 1987). The *cdc2*$^+$ gene function is required at this point in the cell cycle and it appears to act as a rate-limiting step in mitotic initiation (Nurse *et al.*, 1976; Nurse and Thuriaux, 1980).

In order to understand the role of *cdc2*$^+$ it is necessary to identify those proteins with which it interacts, either as regulators or substrates of the protein kinase. To date, only one protein has been directly demonstrated to physically interact with the *cdc2*$^+$-encoded protein. This is the product of the *suc1*$^+$ gene. *suc1*$^+$ was initially identified as a DNA sequence, carried on a high copy number vector, that could rescue some but not all *cdc2* mutants (Hayles *et al.*, 1986). It encodes a 13-kd polypeptide that is apparently not a substrate for the protein kinase but exists in association with p34^{cdc2} and is necessary for its biological function (Brizuela *et al.*, 1987).

Genetic screens for genes that interact directly with *cdc2*$^+$ in the regulation of mitosis have revealed one particular candidate in addition to *suc1*$^+$. A temperature-sensitive allele of the *cdc13* gene was isolated as an extragenic suppressor of a cold-sensitive allele of *cdc2* (Booher and Beach, 1987). A variety of allele-specific interactions between *cdc2*$^+$ and *cdc13*$^+$ were uncovered, including the observation that the *cdc2*$^+$ gene carried on a high copy number plasmid could rescue both of the known temperature-sensitive alleles of *cdc13*. One of these *cdc13* mutations, *cdc13-117*, causes cells to arrest in mitosis in a state similar to that of mitotic metaphase of higher eukaryotes. These observations led to the suggestion that the *cdc13*$^+$ gene product might be a G_2-specific substrate of the *cdc2*$^+$ protein kinase, phosphorylation of which is essential for mitosis. Here we have isolated the *cdc13*$^+$ gene and have defined its role more precisely. The gene is required for the transition from G_2 to mitosis, and evidence is presented that suggests its products may interact with microtubules.

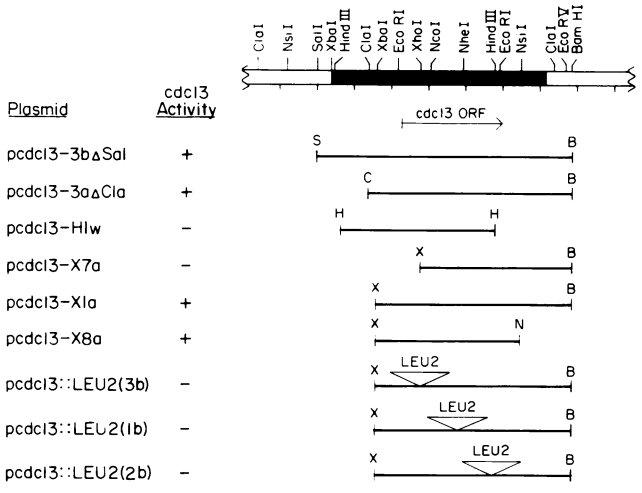

Fig. 1. Restriction map and subcloning of the *cdc13-117* complementing sequence. The restriction map is that of the 5.5 kb of *S.pombe* DNA insert in plasmid pcdc13-1. The wavy line corresponds to the junction of *S.pombe* DNA and shuttle-vector pWH5. The map is marked by 0.5-kb intervals. The nucleotide sequence of the shaded region was determined. Below this restriction map are various DNA fragments that were subcloned into a *S.pombe* replicating vector. Some of these contain an insertion of the *S.cerevisiae LEU2* gene at the indicated restriction sites. See Materials and methods for details of specific plasmid constructions. Each was tested for its ability to complement the *cdc13-117* mutation. ± corresponds to the plasmid's ability or inability to complement a *cdc13-117* mutant.

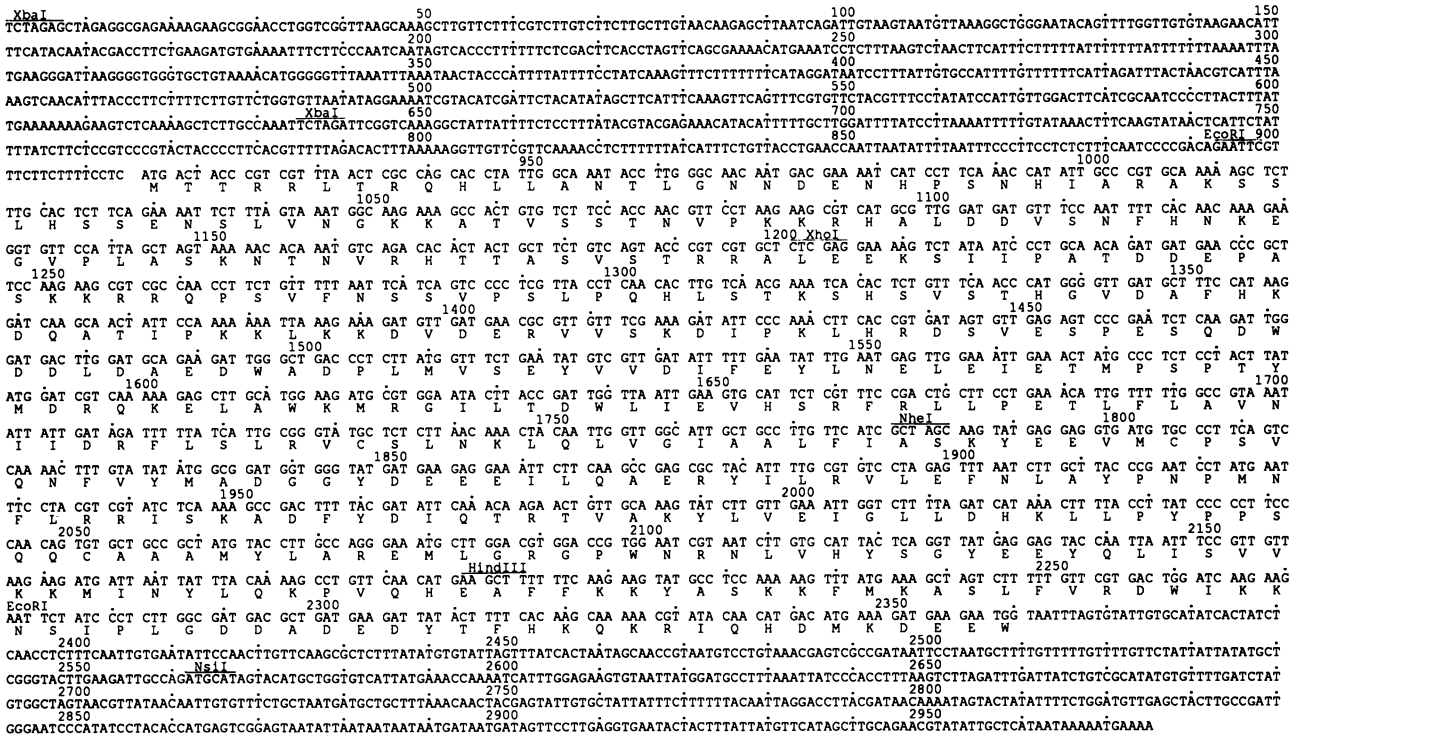

Fig. 2. Nucleotide and predicted amino acid sequence of the *cdc13*$^+$ gene. The nucleotide sequence of a 1930-bp *Xba*I−*Nsi*I DNA fragment plus 5′ and 3′ flanking regions is shown (2975 bases total). This *Xba*I−*Nsi*I DNA fragment is the smallest restriction fragment that retained *cdc13-117* rescuing activity. This region contains a 1446-bp ORF (nucleotides 915−2360) that would encode a protein of 482 amino acids. The predicted amino acid sequence is shown by the single-letter codes below the nucleotide sequence.

Results

Isolation and nucleotide sequence of the cdc13$^+$ *gene*

A genomic library of *S.pombe* DNA was introduced into a temperature-sensitive *cdc13-117 leu1-32* strain (SP672) and leu$^+$ transformants that could grow at the non-permissive temperature of 37°C were obtained (see Materials and methods). In preliminary experiments it was found that the *cdc2*$^+$ gene, which can rescue both known temperature-sensitive alleles of *cdc13* (Booher and Beach, 1987), was repeatedly isolated. To eliminate this unwanted background, advantage was taken of the observation (discussed in detail below) that *cdc13-117* strains are hypersensitive to the anti-microtubule drug thiabenzadole (TBZ) and that this sensitivity is not rescued by the *cdc2*$^+$ gene carried on a multi-copy plasmid. Leu$^+$ transformants were therefore screened both for growth at the non-permissive temperature and also resistance to TBZ.

A plasmid, referred to as pcdc13-1, was recovered from one of these cdc$^+$,leu$^+$,TBZ^r yeast transformants by reintroduction into *Escherichia coli*. Restriction enzyme mapping revealed that it carried a fragment of *S.pombe* DNA of ~5 kb (Figure 1). Various fragments of this yeast DNA were further subcloned into *S.pombe* replicating vectors and were tested for their ability to rescue the *cdc13-117* mutation (Figure 1). The complementing activity was located within a 1.9-kb *Xba*I−*Nsi*I DNA fragment (pcdc13-X8a), which contained internal *Hin*dIII and *Xho*I restriction sites. Interference with either of these sites resulted in loss of activity (pcdc13-H1w and pcdc13-X7a respectively). Also insertion of the *S.cerevisiae LEU2* gene at either the *Xho*I, *Nhe*I or *Hin*dIII restriction sites [pcdc13::LEU2(1), pcdc13::LEU2(3) and pcdc13::LEU2(2), respectively] within this region abolished the *cdc13-117* complementing activity (Figure 1).

To test whether the isolated yeast DNA fragment carried the *cdc13*$^+$ gene rather than an extragenic suppressor sequence, plasmid pcdc13-8 was integrated into the yeast genome by homologous recombination. This plasmid carries a 2.6-kb *Xba*I−*Bam*HI fragment from the *cdc13-117* complementing DNA sequence (Figure 1) in addition to the *S.pombe ura4*$^+$ gene. pcdc13-8 was introduced into a strain carrying a deletion of the *ura4* gene (*ura4-D18*), and stable ura$^+$ integrants were selected. The integration of this plasmid by homologous recombination was confirmed by Southern blotting (data not shown). Tetrad analysis of a cross between one of these integrant strains (SP840) and a *cdc13-117 ura4-D18* strain showed co-segregation of uracil auxotrophy and the *cdc13-117* mutation in all cases (34 tetrads total). The two cdc$^+$ segregants in each tetrad were ura$^+$ in each case. Thus integration of the cloned DNA fragment at the *cdc13* locus was confirmed and this sequence may therefore be taken to be the *cdc13*$^+$ gene.

The *cdc13*$^+$ locus has been assigned to chromosome II but its precise map position is not known (Kohli *et al.*, 1977). Given the similarity of phenotype between *cdc13.117* and the *nda* (tubulin) mutants (see below) there was a possibility that these genes might be related. It has been reported previously that *cdc13* is not linked to *nda2* (α1-tubulin) or *nda3* (β-tubulin) (Toda *et al.*, 1983). To test whether *cdc13* was allelic with the gene encoding α2-tubulin (mapped to the distal left arm of chromosome II, M.Yanagida, personal communication), we crossed a *cdc13-117* strain with one carrying a marked α2-tubulin

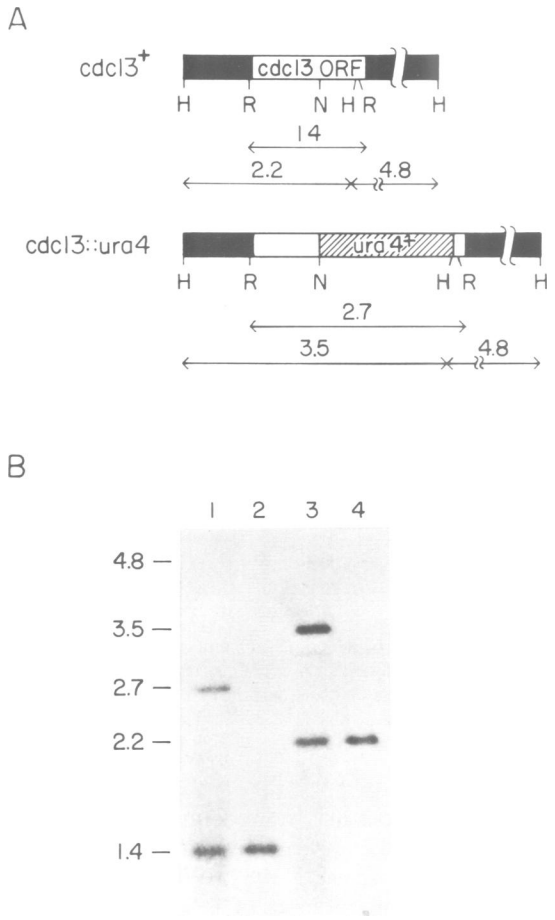

Fig. 3. *cdc13*$^+$ gene disruption. (**A**) Restriction map of the diploid *cdc13* disruption strain (SP827) that is heterozygous for the *cdc13::ura4* insertion–deletion construction. Restriction sites: H, *Hin*dIII; R, *Eco*RI; N, *Nde*I. (**B**) Southern blot hybridization. Total yeast DNA was purified from the diploid disruption strain SP827 (**lanes 1** and **3**) and wild-type strain 972 (**lanes 2** and **4**). The DNA was digested with *Eco*RI (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4), run in a 0.9% agarose gel, transferred to nitrocellulose, and probed with plasmid pcdc13-RI that contains an insert of the *cdc13*$^+$ 1.4-kb *Eco*RI fragment. A faint 4.8-kb *Hin*dIII band is seen due to a 79-bp overlap with the *Eco*RI probe. The faint 3.0-kb *Hin*dIII fragment is a partial *Hin*dIII digestion fragment and was not seen in other gel transfer hybridization experiments.

gene (*a*2-tub::LEU2, Adachi *et al.*, 1986) and found genetic linkage of 34 map units (PD:NPD:TT = 39:1:26), therefore *cdc13*$^+$ and α2-tubulin are not the same gene but are genetically linked.

Having confirmed the identity of the cloned DNA as *cdc13*$^+$, we determined the nucleotide sequence of the *Xba*I–*Nsi*I fragment, in addition to adjacent regions of several hundred nucleotides. The sequence revealed a continuous region of open reading frame (ORF) of 1446 bp that is predicted to encode a polypeptide of 482 amino acids (Figure 2). This is taken to be the full extent of the *cdc13*$^+$-encoded protein because the predicted initiating methionine contains the only ATG sequence within the 5′ flanking region of the biologically active 1.9-kb *Xba*I–*Nsi*I fragment. Also, no intron splicing consensus sequences were found within this fragment. We have obtained antisera prepared against the 482-amino acid *cdc13*$^+$ protein, following expression in *E.coli*, and have used the serum to confirm that the yeast *cdc13*$^+$ gene product displays the predicted mobility in SDS–polyacrylamide gels (Booher and Beach, in preparation). This observation further suggests that the *cdc13*$^+$ gene contains no introns. The predicted *cdc13*$^+$ polypeptide was not found to be significantly homologous to any protein in the Protein Identification Resource database.

cdc13 null allele

The *cdc13*$^+$ gene is not essential for nuclear DNA synthesis, and *cdc13-117* mutants arrest with a 2C DNA content upon shift to the non-permissive temperature (Booher and Beach, 1987). The terminal phenotype of this mutant allele suggests that cells enter mitotis but fail to complete either cell or nuclear division. Cells containing multiple incomplete division septa accumulate and under certain circumstances three condensed mitotic chromosomes can be visualized (Nasmyth and Nurse, 1981; see Figure 4). In order to establish whether this terminal phenotype was also displayed by a strain carrying a null allele of *cdc13*, the genomic *cdc13*$^+$ gene was disrupted by the one-step gene disruption method (Rothstein, 1983).

A homozygous *ura4-D18* diploid (SP817) was transformed to ura$^+$ with the plasmid pcdc13::ura4(2) after it had been digested with *Xba*I and *Bam*HI restriction enzymes. The resulting DNA fragment carries the *cdc13* gene from which the internal coding region, from the *Hin*dIII to *Nhe*I restriction sites, was removed and replaced by a 1.8-kb DNA fragment carrying the *S.pombe ura4*$^+$ gene. Approximately 29% of the *cdc13* coding region is deleted in this construction. One stable ura$^+$ yeast transformant (SP827) was analyzed by Southern hybridization and was shown to be heterozygous for the *cdc13* gene disruption (Figure 3). Tetrad analysis of this diploid confirmed that the *cdc13*$^+$ gene is essential for cell division. In each tetrad (63 total tetrads analyzed) two spores germinated but arrested as single highly elongated cells without observable septa. The two cdc$^+$ segregants in each tetrad were always uracil auxotrophs. These observations confirm that the *cdc13* gene had been disrupted.

To examine the phenotype of cells carrying a null allele of *cdc13* in greater cytological detail, the heterozygous diploid strain described above was transformed to leu$^+$ with plasmid pcdc13-1. A diploid transformant was allowed to sporulate and germinated on selective medium such that haploid segregants carrying both the genomic *cdc13::ura4* disruption and the autonomous pcdc13-1 plasmid were selected (see Materials and methods). In this situation, the haploid *cdc13* null mutant is viable since the plasmid provides a functional copy of the *cdc13*$^+$ gene. However the plasmid is unstable and cells that do not maintain the plasmid become arrested with a cdc phenotype due to loss of the *cdc13*$^+$ gene product.

To observe cells that had recently lost the *cdc13*$^+$ plasmid, a yeast culture was subjected to elutriation centrifugation and wild-type size cells were selected (see Materials and methods). This was done in order to examine only those cells that had lost the plasmid and thus expressed a cdc phenotype during a defined period of time. Upon inoculation of the elutriated cells into fresh non-selective growth medium (YEA medium at 32°C), samples were removed at various time intervals and examined by fluorescence microscopy. Cells were stained with diamidinophenylindole (DAPI) to visualize the nuclear structure and with rhodamine-conjugated phalloidin to reveal the distri-

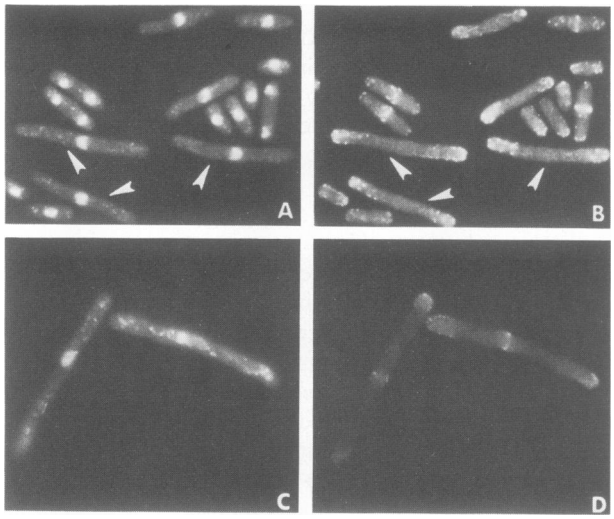

Fig. 4. Fluorescence micrographs of *cdc13* mutant cells double-stained with DAPI (**A,C**) and rhodamine-conjugated phalloidin (**B,D**). A,B contain haploid *cdc13::ura4* mutant cells that are rescued by the plasmid pcdc13-1. Cells that have lost the plasmid, indicated by the arrows, are showing the *cdc13* null phenotype. C,D are *cdc13-117* mutant cells that had been temperature arrested at 37°C for 6 h prior to fixation and staining. Note that both of these mutants show F-actin staining at both cell ends, while only *cdc13-117* cells show a central actin ring.

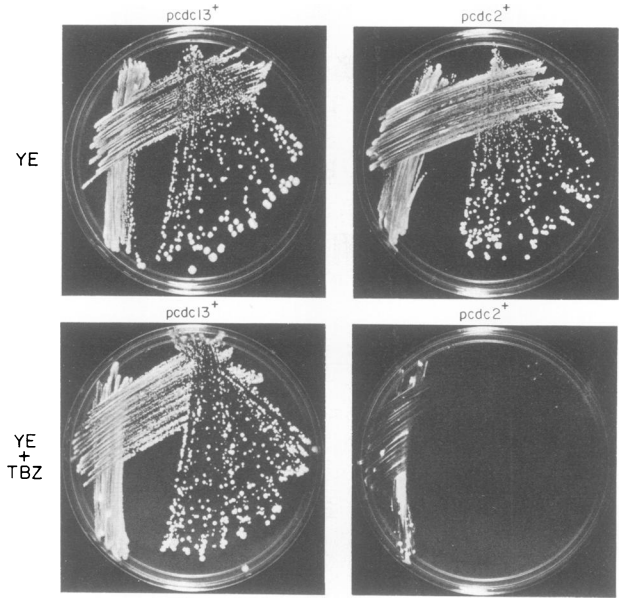

Fig. 5. Plasmid rescue of the *cdc13-117* mutant strain. A *cdc13-117* (SP672) strain containing either a *cdc13*$^+$ plasmid or a *cdc2*$^+$ plasmid was struck out for single colonies on solid YE and YE + TBZ (10 μg/ml) medium and incubated at 37°C. The pcdc13$^+$ plasmid used in this experiment was pcdc13-1.

bution of F-actin. This provides a useful marker of the cell cycle stage in fission yeast (Toda *et al.*, 1981; Marks and Hyams, 1985; Marks *et al.*, 1986).

After 6 h of growth, ~5% of the cells were of a length conspicuously greater than that at normal septation. These cells may be taken to be those that lack the pcdc13-1 plasmid. The phenotype of the cells lacking the plasmid was clearly different from that of *cdc13-117* mutants (Figure 4). No septa were ever observed in the null mutants, even at time intervals up to 12 h after elutriation, and all the nuclei retained a hemispherical shape indicative of interphase cells, rather than condensed chromosomes which could be seen infrequently in certain *cdc13-117* mutant strains (Nasmyth and Nurse, 1981). Likewise the null mutants displayed F-actin staining at both cell tips, indicative of two sites of cell growth. This pattern predominates during G_2. By contrast the *cdc13-117* cells frequently showed a central actin ring that, in wild-type cells, develops after the cessation of tip growth and predicts the position of the septum. This actin ring was never observed in the *cdc13* null mutants. Taken together, these observations demonstrate that *cdc13-117* is a leaky mutant. Although this allele displays first cycle arrest at 36°C, partial progression from G_2 into mitosis occurs. By contrast, the *cdc13* null mutant is unambiguously arrested in G_2 prior to any discernible mitotic event.

Thiabendazole sensitivity of cdc13-117 mutants

Anti-mitotic benzimidazole compounds such as benomyl and TBZ are believed to specifically inhibit microtubule-mediated functions in a variety of ascomycete fungi (Davidse, 1973; Walker, 1982). In *S.pombe*, genetic screens for TBZ-resistant or sensitive mutants have uncovered the genes encoding both α1-tubulin (*nda2*) and β-tubulin (*nda3*) (Yamamoto, 1980; Umesono *et al.*, 1983a). These genes were additionally identified as cold-sensitive nuclear division arrest (*nda*) mutations that showed three condensed mitotic chromosomes at the non-permissive temperature (Umesono *et al.*, 1983b; Hiraoka *et al.*, 1984). Wild-type cells that are exposed to high concentrations of TBZ become arrested in cell division and frequently form a septum across the nucleus (Umesono *et al.*, 1983a). Since the terminal phenotype of the *cdc13-117* mutant shares features in common with cells that have disrupted microtubule function, we tested the sensitivity of strains carrying this allele to TBZ.

Assayed at 25°C, *cdc13* mutants were indeed found to be hypersensitive to TBZ. At 15 μg/ml TBZ, wild-type cells are capable of forming colonies on agar plates whereas a *cdc13-117* strain (SP672) was not. Using a range of TBZ concentrations, the wild-type strain was shown to be severely inhibited in colony formation at 20 μg/ml, whereas equivalent inhibition of the *cdc13-117* strain required only 12.5 μg/ml. In order to confirm that TBZ sensitivity was due to the *cdc13-117* mutation itself, a cross between wild-type and *cdc13-117* strains was analyzed by tetrad dissection. Among 13 tetrads analyzed, the *cdc13-117* mutation co-segregated in every case with hypersensitivity to TBZ.

This observation was extended further by separately introducing either the *cdc13*$^+$ and *cdc2*$^+$ genes into a *cdc13-117* strain on a multi-copy plasmid. Both genes rescued the cell cycle defect at 37°C, but only the *cdc13*$^+$ gene, and not *cdc2*$^+$, allowed transformants to form colonies at 37°C on plates containing 10 μg/ml TBZ (Figure 5). Finally, the *cdc13-117* allele was recovered from a mutant yeast strain by plasmid gap repair (see Materials and methods; Orr-Weaver *et al.*, 1981). On a multicopy plasmid, the *cdc13-117* mutant allele was able to rescue a *cdc13-117* strain at 37°C. However, transformants were incapable of colony formation at this temperature on plates containing 15 μg/ml TBZ (data not shown). These results demonstrate that the *cdc13-117* allele specifically confers a mild hypersensitivity to TBZ.

cdc13 genetic interactions

Since the *cdc2*$^+$ gene carried on a multi-copy plasmid rescues both existing temperature-sensitive alleles of *cdc13* (*cdc13-117* and *cdc13-c1*; Booher and Beach, 1987) we tested whether *cdc2*$^+$ overexpression might also rescue a null allele of *cdc13*. A replicating plasmid carrying *cdc2*$^+$ was introduced into a diploid strain that was heterozygous for the *cdc13* gene disruption (SP828). Sporulation was allowed to occur and spores were germinated on medium that would permit growth of only those haploid meiotic products that contained both the *cdc13* null mutation and the *cdc2*$^+$ carrying plasmid. However, no such haploid colonies were obtained. This contrasted with the situation described above in which the diploid strain had been transformed with a plasmid carrying *cdc13*$^+$. Following sporulation, numerous haploid segregants of the appropriate class were obtained. Thus, it appears that overexpression of the *cdc2*$^+$ gene can not rescue a null allele of *cdc13*.

Overexpression of the *cdc13*$^+$ gene on a multicopy plasmid had no obvious effect on wild-type yeast strains. The effect of introducing pcdc13-X1b into strains having the following mutant background was also tested: cdc25-22, cdc2-33, cdc2-59, cdc2-3w, wee1-50 and *cdc2-3w wee1-50*. Transformants were examined at their respective non-permissive temperature, except in the case of *cdc2-3w* which is non-conditional. In each case, the strain carrying the *cdc13*$^+$ plasmid showed a phenotype, after shift to the non-permissive temperature, that was identical to the parental phenotype. Overexpressing the *cdc13*$^+$ gene in the *cdc2-3w* strain had no effect at 25, 33 or 37°C.

Discussion

We have previously reported a variety of allele-specific interactions between the *cdc2*$^+$ and *cdc13*$^+$ genes which suggest that the protein products of these two genes are likely to interact physically. Specifically it was proposed that the *cdc13*$^+$ gene product might be a G_2-specific substrate of the *cdc2*$^+$ protein kinase (Booher and Beach, 1987). In this paper we have described the isolation and characterization of the *cdc13*$^+$ gene and have demonstrated that yeast strains carrying a *cdc13* null allele display a terminal phenotype distinctly different from that of any existing temperature-sensitive alleles of this gene. The data indicate that the *cdc13*$^+$ gene is required for the G_2/M transition, rather than only being essential for a late mitotic function. The *cdc13-117* mutant allele product probably retains a low residual activity at the non-permissive temperature since, with respect to the null allele, the phenotype is leaky and multiple copies of this allele can rescue a *cdc13-117* mutant strain.

The *cdc13*$^+$ gene was cloned by its ability to complement both the temperature-sensitive and TBZ-sensitive defects of a *cdc13-117* mutant. The TBZ sensitivity of *cdc13-117* is a provocative observation that suggests that the *cdc13*$^+$ gene product interacts with microtubules. In previous genetic screens, the major TBZ-sensitive mutations were found to be alleles of *nda2* (α_1-tubulin) and *nda3* (β-tubulin) (Umesono *et al.*, 1983a). In fission yeast, as in most eukaryotes, microtubule arrays are present in the cytoplasm during interphase (Hagan and Hyams, 1988). The initiation of mitotis is signalled by the dissolution of cytoplasmic microtubules and the appearance of the mitotic spindle. In the *cdc13-117* mutant, cytoplasmic interphase microtubules appear cytologically normal whereas the mitotic spindle fails to form (Hagan, 1988). Thus, if the *cdc13*$^+$ gene product has an essential function that requires interaction with microtubules this is likely to be restricted to mitotic rather than cytoplasmic microtubules. An attractive hypothesis, that encompasses all that is presently known about the *cdc13*$^+$ gene, is that the gene product is a substrate for the *cdc2*$^+$ protein kinase. Phosphorylation of the *cdc13*$^+$ protein might play a regulatory role in formation of the mitotic spindle. This hypothesis is now amenable to experimental investigation.

Table I. Yeast strains

Strain	Genotype
972	*h*$^-$
SP23	*h*$^-$ *cdc2-33 leu1-32*
SP202	*h*$^+$ *leu1-32 ade6-210*
SP614	*h*$^+$ *wee1-50 leu1-32*
SP628	*h*$^+$ *cdc25-22 leu1-32*
SP636	*h*$^+$ *cdc2-3w wee1-50 leu1-32*
SP661	*h*$^-$ *cdc2-3w leu1-32*
SP672	*h*$^+$ *cdc13-117 leu1-32*
SP683	*h*$^-$ *cdc13-c1 leu1-32*
SP806	*h*$^-$ *ura4-D18*
SP817	*h*$^-$/*h*$^+$ *ura4-D18/ura4-D18 ade6-210/ade6-216*
SP823[a]	*h*$^+$/*h*$^+$ *cdc13::ura4/cdc13*$^+$ *ura4-D18/ura4-D18 ade6-210/ ade6-216*
SP824	*h*$^-$ *cdc13-117 ura4-D18*
SP825	*h*$^-$/*h*$^-$ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-210/ ade6-216*
SP827[a]	*h*$^-$/*h*$^+$ *cdc13::ura4/cdc13*$^+$ *ura4-D18/ura4-D18 ade6-210/ ade6-216*
SP828[a]	*h*$^-$/*h*$^+$ *cdc13::ura4/cdc13*$^+$ *leu1-32/leu1-32 ura4-D18/ ura4-D18 ade6-210/ade6-216*
SP840[a]	*h*$^-$ *cdc13int::pcdc13-8 ura4-D18*
SP842[a]	*h*$^-$ *cdc13-117int::pcdc13-11 ura4-D18*
SP852[a]	*h*$^+$ *cdc13::ura4 leu1-32 ura4-D18 ade6-210 pcdc13-1*

[a]See Materials and methods for strain constructions.

Materials and methods

Strains and genetic techniques

Table I contains a complete list of strains used in this study. Standard *S.pombe* genetic nomenclature (Kohli, 1987) and genetical procedures (Gutz *et al.* 1974) were followed. *S.pombe* mutations *cdc13-117, cdc25-22* (Nurse *et al.*, 1976), *cdc2-59,cdc13-c1* (Booher and Beach, 1987), *cdc2-1w,wee1-50* (Nurse and Thuriaux, 1980), and *cdc2-3w* (Fantes, 1981) have been described previously. The *S.cerevisiae LEU2* gene can rescue *S.pombe leu1* mutants. A strain containing α_2-*tub::LEU2* (Adachi *et al.*, 1986) was kindly provided by Y.Adachi and M.Yanagida (Kyoto University). A strain in which the *ura4*$^+$ gene had been deleted, *ura4-D18*, was provided by C.Grimm (University of Bern). The *cdc13-117* allele is osmotically remedial and was therefore always analyzed on solid medium that lacked sorbitol.

Media and drugs

S.pombe strains were grown in standard YE, YEA, PM and PMA medium (Beach *et al.*, 1985). Amino acids were added to minimal media at 75 μg/ml. TBZ (Sigma) was prepared in dimethylsulfoxide as a 20 mg/ml stock solution and stored at 4°C. TBZ was added to autoclaved YE medium after cooling to 55°C. Phloxin B (Sigma) was added to solid medium at 20 mg/l in some cases, as an indicator of cell viability, as described by Gutz *et al.* (1974).

S.pombe transformation and plasmid recovery

S.pombe strains were transformed as described by Beach *et al.* (1982b) with the modifications of Booher and Beach (1987). Plasmids were introduced

into *cdc2-59* mutants as previously described (Booher and Beach, 1987). The *cdc13*$^+$ gene was cloned from the *S.pombe* gene bank SP5, a generous gift from P.Schuchert and J.Kohli (University of Bern). SP5 consists of wild-type *S.pombe* DNA that was partially digested with *Sau3A* and ligated into the *Bcl*I site of the yeast vector pWH5 (Wright *et al.*, 1986). Plasmids were recovered from *S.pombe* transformants in *E.coli* strain JA226 as described by Beach *et al.* (1982b).

cdc13 cloning and gene disruption

S.pombe strain SP672 (*h*$^-$ *leu1-32 cdc13-117*) was transformed with the plasmid gene bank SP5 and plated onto PMA + sorbitol plates and incubated at 25°C for 2 days. The plates were then shifted to 37°C. This is normally the non-permissive temperature, however, on solid sorbitol containing medium the *cdc13-117* mutation is osmotically remedial, i.e. the mutation is suppressed. Therefore the transformants that grew at 37°C were further replica plated to YEA + phloxin B plates and incubated at 37°C. Approximately 100 colonies were isolated that could grow at 37°C. These transformants were subsequently patched onto a YEA plate that contained TBZ at a concentration of 10 μg/ml and incubated at 37°C. Microscopic examination showed that ~50 of these isolates were non-viable. Plasmid stability analysis indicated that in 24 of these transformants the plasmid's *LEU2* auxotrophic marker co-segregated with the *cdc13-117* complementing activity. The plasmid DNA was recovered from one of these transformants and designated pcdc13-1.

The *cdc13-117* allele was cloned by transforming strain SP824 (*h*$^-$ *cdc13-117 ura4-D18*) with pcdc13-11 that had been linearized by *Eco*RI digestion. Stable ura$^+$ transformants were selected and the structure of the integrated plasmid in several of these strains was determined by Southern blot analysis. This showed that the majority of the plasmids had faithfully repaired the *Eco*RI gap upon integration at the *cdc13-117* locus (data not shown). To recover the *cdc13-117* allele, yeast genomic DNA was prepared from one of these strains (SP842) and digested with *Bam*HI. This liberated a *Bam*HI DNA fragment that carried the entire pUC119-dR vector, *ura4*$^+$ gene, and the *cdc13-117* allele. The restriction digest was diluted and religated. The gap-repaired plasmid was recovered by transforming the ligation reaction into *E.coli* strain TG1 and selecting for ampicillin-resistant transformants. Plasmid DNA was isolated from a representative Amp^R colony and it was confirmed to contain the entire *cdc13-117* allele by restriction enzyme analysis. This plasmid is referred to as pcdc13-117a.

The *cdc13* gene disruption was performed by transforming strain SP817 with 0.5−1.0 μg of *Xba*I/*Bam*HI-digested pcdc13::ura4(2) plasmid DNA and selecting for uracil auxotrophs on PM + sorbitol plates. Approximately 200 transformants were patched on PM plates and replica plated onto PM + ura plates. After 1 day, the plates were replica plated again onto fresh PM + ura plates; this was repeated five more times. This was done to permit dilution of autonomously replicating DNA fragments that carried the *ura4*$^+$ gene. The transformants were then replica plated once again onto PM medium. Seventeen of these transformants were found to still be uracil auxotrophs. Each of these was sporulated and the meiotic products were examined by a mini-random spore analysis in which a single drop of the spore mixture was placed on a YEA plate. After 1 day it was apparent, by microscopic examination, that among four of the diploids ~50% of the spores were germinating and arresting as single cdc cells. Southern blot hybridization showed that one of these strains (SP827) contained a single copy of the *cdc13::ura4* disruption construction.

To introduce the *leu1-32* mutation into the diploid *cdc13* disruption strain, a derivative of SP827 that had become homozygous at the *mat* locus was first obtained. This strain, SP823, was mated with SP825, sporulated, and then germinated on PM + leu plates. This selected for diploids that were heterozygous at the *cdc13* and *ade6* loci. A homozygous *leu1-32* and heterozygous *mat* strain (SP828) was isolated from among these segregants. The diploid strain SP828 was transformed separately with pcdc13-1 and pcdc2$^+$. Each of these transformants was sporulated, and the spores were permitted to germinate on PMA + uracil medium. Haploid *cdc13::ura4* segregants were obtained at a high rate from the pcdc13-1 transformant, but none were obtained from the pcdc2$^+$ transformant. A strain (SP852) carrying both the genomic *cdc13::ura4* disruption and the pcdc13-1 plasmid was obtained from these haploid segregants.

The cells from disruption strain SP852 that carried the pcdc13-1 plasmid (wild-type size) were separated from those that had lost the plasmid (elongated) by centrifugal elutriation. Strain SP852 was cultured in 1 l of YEA medium at 33°C to a density of 5 × 10^6 cells/ml and loaded into a Beckman JE-10X elutriator rotor (73 ml chamber) spinning at 2000 r.p.m., 33°C. The wild-type size cells were separated by increasing the flow rate while maintaining a constant rotor speed. This fraction of cells was pelleted, resuspended in fresh YEA medium and incubated at 33°C. Samples were removed at various times and examined by fluorescence microscopy.

Plasmid constructions

The source of *LEU2* constructs used in this study is described here. *Hin*dIII linkers were blunt-end ligated onto a 2.2-kb *Sal*I−*Xho*I DNA fragment containing the *LEU2* gene whose 5′ overhangs had been filled in with Klenow enzyme. In this case, the parental *Sal*I restriction site is regenerated. The resulting 2.2-kb *Hin*dIII fragment was inserted into the *Hin*dIII site of pTR262 (Roberts *et al.*, 1980) to produce plasmid pTR-LEU2. This 2.2-kb *Hin*dIII fragment was blunt-end ligated, using Klenow enzyme for filling in the 5′ overhangs, into the *Xba*I site of pUC18 (Yanisch-Perron *et al.*, 1985) resulting in plasmid pLEU2-XbaI. In this case a *Xba*I restriction site is regenerated at both ends of the inserted *LEU2* fragment. The orientation of the *LEU2* gene is such that digestion with *Sal*I will produce a 2.2-kb *Sal*I fragment bearing *LEU2*.

The *S.pombe* replicating vector pIRT3 is pUC118 that contains a 1.2-kb *Eco*RI fragment of *S.pombe* DNA carrying *ars1* (Losson and Lacroute, 1983) and a 2.2-kb *Hin*dIII fragment bearing *LEU2* from plasmid pTR-LEU2. Plasmid pcdc2$^+$ used in this study is the vector pDB248x (Beach *et al.*, 1982b) that contains a 3.4-kb *Pst*I fragment bearing the *cdc2*$^+$ gene. Cloning of the *S.pombe ura4*$^+$ gene to a 1.76-kb *Hin*dIII fragment has been described by Bach (1987), and a derivative of this fragment in which *Sph*I linkers were added and inserted into pUC19, plasmid pUC19-SU4, was constructed by T.Carr. Plasmids pUC118/pUC119 are pUC18/pUC19 derivatives that carry an M13 intragenic region (Vieira and Messing, 1987).

Various restriction fragments from pcdc13-1 were subcloned into pIRT3 to create plasmids pcdc13-3bΔSal, pcdc13-3aΔCla, pcdc13-X7a, pcdc13-X1a and pcdc13-X8a (see Figure 1). Plasmid pcdc13-H1w is vector pWH5 with a 2.2-kb *Hin*dIII insert. The 1.4-kb *Eco*RI DNA fragment that carries almost the entire *cdc13*$^+$ coding region was inserted at the *Eco*RI site of pUC119 to produce plasmid pcdc13-R1a. The 3.4-kb *Sal*I−*Bam*HI DNA fragment bearing the *cdc13*$^+$ gene was cut from pcdc13-1 and inserted between the *Sal*I−*Bam*HI sites of pUC119 to create pcdc13-3d. Plasmid pcdc13-1 contains a *Sma*I site within the pWH5 vector that is immediately adjacent to the inserted *S.pombe* DNA. Digestion with *Sma*I and *Bam*HI produced a 4.5-kb fragment of the *cdc13*$^+$ gene that was inserted between the *Sma*I and *Bam*HI sites of pUC119 to produce pcdc13-3bΔSma. Plasmid pcdc13-X1b is identical to pcdc13-X1a except that the *cdc13* DNA fragment is inserted into pIRT3 in the inverse orientation.

Plasmid pUC119-dH is a pUC119 derivative in which the *Hin*dIII site was eliminated by digesting pUC119 with *Hin*dIII, filling in the 5′ overhangs with Klenow enzyme, and then blunt-end ligation. Plasmid pUC119-dR was constructed in a similar manner, but in this case the *Eco*RI site was eliminated. The 3.5-kb *Bam*HI−*Sal*I fragment from plasmid pcdc13-3bΔSal was ligated into pUC119-dH and pUC119-dR to create plasmids pcdc13-5 and pcdc13-7 respectively. pcdc13-6 is a derivative of pcdc13-5 in which an internal 0.64-kb *Xba*I fragment was removed. The *cdc13* gene disruption construction was made by inserting the ~1.76-kb *Hin*dIII−*Xba*I fragment containing the *ura4*$^+$ gene from plasmid pUC19-SU4 between the *Hin*dIII and *Nhe*I sites of pcdc13-6. The resulting plasmid, pcdc13::ura4(2), was digested with *Bam*HI and *Xba*I to direct integration to the *cdc13* locus.

The *S.cerevisiae LEU2* gene was inserted within the *cdc13*$^+$ gene at various restriction sites using the plasmids and restriction enzymes as follows: pcdc13-X1a/*Nhe*I, pLEU2-XbaI/*Xba*I; pcdc13-6/*Hin*dIII, pTR-LEU2/*Hin*dIII; pcdc13-6/*Xho*I, pLEU2-XbaI/*Sal*I. This resulted in plasmids pcdc13::LEU2(1a), pcdc13::LEU2(2a) and pcdc13::LEU2(3a), respectively. The *Xba*I−*Bam*HI fragment carrying the *cdc13::LEU2* construction from each of these plasmids was inserted into a pUC18 derivative, pars1, that contained the *S.pombe* 1.2-kb *Eco*RI *ars1* fragment. The final plasmid constructs were designated pcdc13-LEU2(1b), pcdc13::LEU2(2b) and pcdc13::LEU2(3b), respectively.

A 1.76-kb *Sph*I fragment containing the *ura4*$^+$ gene from plasmid pUC19-SU4 was inserted into the unique *Sph*I site of plasmids pcdc13-6 and pcdc13-7 to produce pcdc13-8 and pcdc13-10 respectively. Plasmid pcdc13-10 was then digested with *Eco*RI and religated to produce pcdc13-11. Plasmid pcdc13-11 thus contains a gap of an internal 1.38-kb *Eco*RI fragment.

Sequencing

A series of overlapping deletions was created in plasmids pcdc13-3aΔSma and pcdc13-3d by the unidirectional exonuclease III deletion method of Henikoff (1987). These two plasmids are pUC119 derivatives that contain the *cdc13*$^+$ gene in opposite orientations with respect to the universal priming site. Using this deletion series, a stretch of 2975 bp of continuous nucleotide sequence from both DNA strands was obtained. Production of single-stranded plasmid DNA was accomplished by infection with the helper virus M13KO7 (Vieira and Messing, 1987). DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase (United States Biochemical Corp.).

Cytology

S.pombe strains were stained with rhodamine-conjugated phalloidin, kindly provided by J.Hyams, and diamidinophenylindole (DAPI) according to the method of Marks and Hyams (1985).

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References

Adachi,Y., Toda,T., Niwa,O. and Yanagida,M. (1986) *Mol. Cell. Biol.*, **6**, 2168–2178.
Bach,M.-L. (1987) *Curr. Genet.*, **12**, 527–534.
Beach,D., Durkacz,B. and Nurse,P. (1982a) *Nature*, **300**, 706–709.
Beach,D., Piper,M. and Nurse,P. (1982b) *Mol. Gen. Genet.*, **187**, 326–329.
Beach,D., Rodgers,L. and Gould,J. (1985) *Curr. Genet.*, **10**, 297–311.
Booher,R. and Beach,D. (1986) *Mol. Cell. Biol.*, **6**, 3523–3530.
Booher,R. and Beach,D. (1987) *EMBO J.*, **6**, 3441–3447.
Brizuela,L., Draetta,G. and Beach,D. (1987) *EMBO J.*, **6**, 3507–3514.
Davidse,L.C. (1973) *Pest. Biochem. Physiol.*, **3**, 317–325.
Draetta,G., Brizuela,L., Potashkin,J. and Beach,D. (1987) *Cell*, **50**, 319–325.
Fantes,P.A. (1981) *J. Bacteriol.*, **146**, 746–754.
Gutz,H., Heslot,H., Leupold,U. and Loprieno,N. (1974) In King,R.C. (ed.), *Handbook of Genetics*. Plenum Press, New York, Chapter 25.
Hagan,I.M. (1988) Ph.D Thesis, University of London.
Hagan,I.M. and Hyams,J.S. (1988) *J. Cell Sci.*, **89**, 343–357.
Hartwell,L.H., Cullotti,J., Pringle,J.R. and Reid,B.J. (1974) *Science*, **183**, 46–51.
Hayles,J., Beach,D., Durkacz,B. and Nurse,P. (1986) *Mol. Gen. Genet.*, **202**, 291–293.
Henikoff,S. (1987) *Methods Enzymol.*, **155**, 156–165.
Hiraoka,Y., Toda,T. and Yanagida,M. (1984) *Cell*, **39**, 349–358.
Kohli,J. (1987) *Curr. Genet.*, **11**, 575–589.
Kohli,J., Hoptinger,H., Munz,P., Strauss,A. and Thuriaux,P. (1987) *Genetics*, **87**, 471–489.
Lee,M. and Nurse,P. (1987) *Nature*, **327**, 183–185.
Losson,R. and Lacroute,F. (1983) *Cell*, **32**, 371–377.
Marks,J. and Hyams,J.S. (1985) *Eur. J. Cell Biol.*, **39**, 27–32.
Marks,J., Hagan,I.M. and Hyams,J.S. (1986) *J. Cell Sci. Suppl.*, **5**, 229–241.
Morris,N.R. (1976) *Genet. Res.*, **26**, 237–254.
Nasmyth,K. and Nurse,P. (1981) *Mol. Gen. Genet.*, **182**, 119–124.
Nurse,P. and Thuriaux,P. (1980) *Genetics*, **96**, 627–637.
Nurse,P., Thuriaux,P. and Nasmyth,K. (1976) *Mol. Gen. Genet.*, **146**, 167–178.
Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6354–6358.
Roberts,T.M., Swanberg,S.L., Poteete,A., Riedel,G. and Backman,K. (1980) *Gene*, **12**, 123–127.
Rothstein,R. (1983) *Methods Enzymol.*, **101**, 202–211.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Simanis,V. and Nurse,P. (1986) *Cell*, **45**, 261–268.
Toda,T., Yamamoto,M. and Yanagida,M. (1981) *J. Cell. Sci.*, **52**, 271–287.
Toda,T., Umesono,K., Hirata,A. and Yanagida,M. (1983) *J. Mol. Biol.*, **168**, 251–270.
Umesono,K., Toda,T., Hayashi,S. and Yanagida,M. (1983a) *J. Mol. Biol.*, **168**, 271–284.
Umesono,K., Hiraoka,Y., Toda,T. and Yanagida,M. (1983b) *Curr. Genet.*, **7**, 123–128.
Vieira,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.
Walker,G.M. (1982) *J. Gen. Microbiol.*, **128**, 61–71.
Wright,A., Maundrell,K., Heyer,W.-D., Beach,D. and Nurse,P. (1986) *Plasmid*, **15**, 156–158.
Yamamoto,M. (1980) *Mol. Gen. Genet.*, **180**, 231–234.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.

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