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The role of proteolysis in cell cycle progression in *Schizosaccharomyces pombe*

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A cell-free system derived from *Xenopus* eggs was used to identify the 'destruction box' of the *Schizosaccharomyces pombe* B-type cyclin, Cdc13, as residues 59–67: RHALDDVSN. Expression of indestructible Cdc13 from a regulated promoter in *S.pombe* blocked cells in anaphase and inhibited septation, showing that destruction of Cdc13 is necessary for exit from mitosis, but not for sister chromatid separation. In contrast, strong expression of a polypeptide comprising the N-terminal 70 residues of Cdc13, which acts as a competitive inhibitor of destruction box-mediated proteolysis, inhibited both sister chromatid separation and the destruction of Cdc13, whereas an equivalent construct with a mutated destruction box did not. Appropriately timed expression of this N-terminal fragment of Cdc13 overcame the G_1 arrest seen in *cdc10* mutant strains, suggesting that proteins required for the initiation of S phase are subject to destruction by the same proteolytic machinery as cyclin.

Keywords: cell cycle/cyclin B/destruction box/*S.pombe*/*Xenopus* egg

Introduction

Cyclins are the activating subunits of the cdc2 family of protein kinases (Hunt, 1991), which are required to promote both the G_1/S and G_2/M cell cycle transitions (Nurse, 1990; King *et al.*, 1994). An important mechanism for regulating the activity of this class of protein kinase is the programmed proteolysis of the cyclin subunit (Murray *et al.*, 1989; Hunt *et al.*, 1992). For example, the proteolysis of mitotic A- and B-type cyclins is a key event in the cell cycle that irreversibly initiates exit from mitosis. The destruction of cyclins involves ubiquitin-dependent proteolysis, which requires the presence of a short amino acid sequence near the N-terminus known as the 'destruction box' (Glotzer *et al.*, 1991; Hershko *et al.*, 1991).

When non-degradable B-type cyclins, carrying deletions of or mutations in their destruction boxes, were expressed in *Xenopus* egg extracts (Murray *et al.*, 1989; Holloway *et al.*, 1993), in budding yeast (Surana *et al.*, 1993) or in *Drosophila* embryos (Rimmington *et al.*, 1994; Sigrist *et al.*, 1995), the cell cycle was blocked in late anaphase, indicating that the onset of anaphase can occur in the presence of active cdc2/CDC28 protein kinase. Final exit from mitosis requires inactivation of the cdc2/CDC28 protein kinase by proteolysis of the cyclin subunits (Holloway *et al.*, 1993; Surana *et al.*, 1993; Sigrist *et al.*, 1995). Irniger *et al.* (1995) recently isolated yeast mutants defective in the proteolysis of Clb2, a B-type cyclin, during anaphase and G_1 phase and identified three genes, *CDC16*, *CDC23* and *CSE1* that are required for this process. At the same time, biochemical analysis of ubiquitination and degradation of a fragment of B-type cyclin containing a destruction box in both clam and frog egg extracts revealed that destruction box-dependent ubiquitination requires three components: E1, E2 and a large E3 complex, referred to as either the 'cyclosome' or 'Anaphase-Promoting Complex' (APC) (King *et al.*, 1995; Sudakin *et al.*, 1995). Whereas E1 and E2 ubiquitin ligase enzymes are equally active in mitotic and interphase extracts, the E3 complex is only active when prepared from mitotic extracts. The APC contains homologues of budding yeast Cdc16 and Cdc27 among its constituents (King *et al.*, 1995). Since these proteins are required for cyclin degradation in budding yeast (Irniger *et al.*, 1995) and for anaphase progression in fungi and mammalian cells (Hirano *et al.*, 1988; O'Donnell *et al.*, 1991; Tugendreich *et al.*, 1995), it seems that ubiquitin-mediated proteolysis is essential for cyclin destruction and the initiation of anaphase in all eukaryotes. However, indestructible cyclins were recently shown to be ubiquitinated in frog egg extract (Klotzbücher *et al.*, 1996) and ubiquitin conjugates of cyclin were found in both cytostatic factor-arrested and interphase extracts, where cyclin is perfectly stable (Mahaffey *et al.*, 1995). These results suggests that while ubiquitination is necessary for programmed cyclin destruction, it is probably not sufficient. Another part of the mechanism to trigger the programmed destruction of cyclins has yet to be identified.

Proteolysis is also important to initiate sister chromatid separation at the onset of anaphase, for as Holloway *et al.* (1993) showed, high concentrations of N-terminal fragments of cyclin B or methylated ubiquitin were able to delay sister chromatid separation in frog egg extract, suggesting that ubiquitin-dependent proteolysis of proteins other than cyclin is required to allow sister chromatid separation. This idea is supported by recent observations in budding yeast and HeLa cells (Irniger *et al.*, 1995; Tugendreich *et al.*, 1995). Very recently, Cut2 in fission yeast has been shown to be a protein whose degradation is required for sister chromatid separation (Funabiki *et al.*, 1996). However, many more such proteins may exist. For example, CENP-E, a mammalian kinetochore motor protein, the protein kinase NIMA, which is involved in chromosome condensation, and the *Drosophila* pimples protein are all abruptly degraded in anaphase (Brown *et al.*, 1994; Ye *et al.*, 1995; Stratmann and Lehner, 1996).

The fission yeast *Schizosaccharomyces pombe* has three B-type cyclins, Cdc13, Cig1 and Cig2, of which *cdc13*$^+$ is the only essential gene and the most fully characterized.

Cdc13/Cdc2 mitotic kinase is required for the onset of mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989) and also inhibits re-replication of chromosomes within a single cell cycle. It seems to play a critical role in determining whether a cell enters S phase or mitosis (Hayles *et al.*, 1994). However, we know little about the mechanism and role of Cdc13 destruction, except that Cdc13 disappears as cells exit mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989; Hayles and Nurse, 1995). Inspection of the sequence of Cdc13 reveals two potential destruction boxes in Cdc13 but it is not clear which of these is genuine or whether either or both are necessary for its destruction.

In this paper we describe the use of a *Xenopus* cell-free destruction assay system to identify the 'real' destruction box(es) of *S.pombe* cyclins by site-directed mutagenesis of the candidate sequences. We investigated the phenotype of yeast cells expressing indestructible Cdc13, and found that as in other cells, this led to anaphase arrest with separation of sister chromatids and inhibition of septation. In contrast, strong expression of the N-terminal 70 or 152 residues of Cdc13 containing a wild-type destruction box inhibited sister chromatid separation and stabilized endogenous Cdc13 *in vivo*, providing evidence that proteolysis of unknown proteins is required for the onset of anaphase in fission yeast. More surprisingly, however, expression of the N-terminal fragment of Cdc13 in G_1-arrested cells promoted DNA replication followed by mitotic arrest, suggesting that proteins required for the initiation of S phase may be regulated by the same proteolytic machinery as Cdc13.

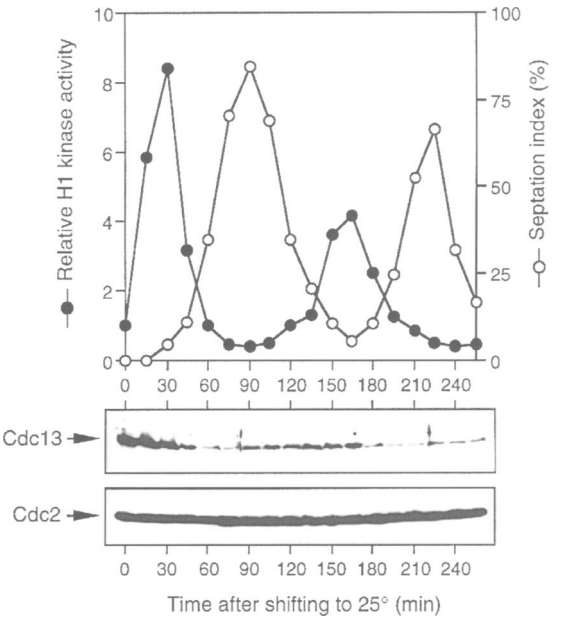

Fig. 1. Oscillation of Cdc13/Cdc2 kinase and Cdc13 protein level during the cell cycle. A synchronous culture of *cdc25-22* mutant cells was prepared by culture at 36°C for 4.25 h followed by shift-down to 25°C. Samples were taken every 15 min. Top panel: relative histone H1 kinase activity of total Cdc2 kinase by $p13^{suc1}$ affinity chromatography (●) and septation index (○). Bottom panel: immunoblotting with anti-Cdc13 polyclonal antiserum (above) or anti-Cdc2 mAb Y100 (below).

Results

Oscillations of Cdc13 protein and Cdc2 kinase activity during cell cycle

In order to study the relationship between Cdc13/Cdc2 kinase activity and the destruction of Cdc13, rabbit polyclonal antibodies were raised against full-length Cdc13 expressed in bacteria. Mouse monoclonal antibodies that recognized the C-terminal 14 residues of Cdc2 were also prepared. Figure 1 shows the levels of Cdc13, Cdc2 and histone H1 kinase activity after release from the G_2 arrest imposed by incubating a *cdc25-22* mutant strain for 4.25 h at 36°C, by returning the temperature to 25°C (Moreno *et al.*, 1989). Histone H1 kinase activity showed sharp peaks at 30 min and 165 min after release. The level of Cdc13 protein showed saw-tooth oscillations during these cell cycles, whereas the Cdc2 protein level was constant. The increase in Cdc2 protein kinase level leading up to mitosis did not precisely correlate with the concentration of either Cdc2 or Cdc13, indicating the existence of other controls of protein kinase activation, whereas the decrease in kinase activity at the end of mitosis coincided with the disappearance of Cdc13.

S.pombe Cdc13 is correctly destroyed in Xenopus egg extracts

To investigate the destruction of *S.pombe* cyclins, we first tested whether the cell-free destruction assay system from unfertilized *Xenopus* eggs could recognize and proteolyse the yeast cyclin in the same way as endogenous frog B-type cyclins. The onset of anaphase in these extracts is controlled by cytostatic factor (CSF), an activity whose physiological role is to maintain unfertilized *Xenopus* eggs in metaphase of meiosis II by preventing cyclin destruction (Masui and Markert, 1971; Murray *et al.*, 1989). In intact eggs, fertilization leads to increased levels of Ca^{2+} in the cytoplasm, which triggers the degradation of cyclin, the inactivation of maturation promoting factor (MPF), and the inactivation of CSF (Sagata *et al.*, 1989; Watanabe *et al.*, 1989, 1991). In these extracts (referred to as CSF extracts) cyclin B destruction and the inactivation of MPF can be triggered by the addition of 0.4 mM Ca^{2+}. Cyclins are otherwise extremely stable in these extracts (Lohka and Maller, 1985; Murray *et al.*, 1989; Lorca *et al.*, 1992; Van der Velden and Lohka, 1993; Stewart *et al.*, 1994). We found that *S.pombe* cyclins were not well translated in pure CSF extract, and we therefore made substrates for proteolysis by translating synthetic mRNAs encoding *S.pombe* cyclins in mixtures containing 90% reticulocyte lysate and 10% CSF extract in the presence of [^{35}S]-methionine. Small aliquots of this 'substrate' were added to fresh CSF extract (giving a final concentration of 80% CSF extract) in the presence of cycloheximide (100 μg/ml) to block further protein synthesis. Cyclin destruction was triggered by the addition of Ca^{2+} to 0.4 mM final concentration. As shown in Figure 2, wild-type Cdc13 was destroyed in this system with similar kinetics as endogenous frog B-type cyclins, which served as internal positive controls, whereas it was essentially stable in the absence of added Ca^{2+}. Thus, *S.pombe* Cdc13 appeared to be correctly destroyed in *Xenopus* egg extract which thus provided a useful *in vitro* system to test whether other *S.pombe* cyclins and mutants were biodegradable.

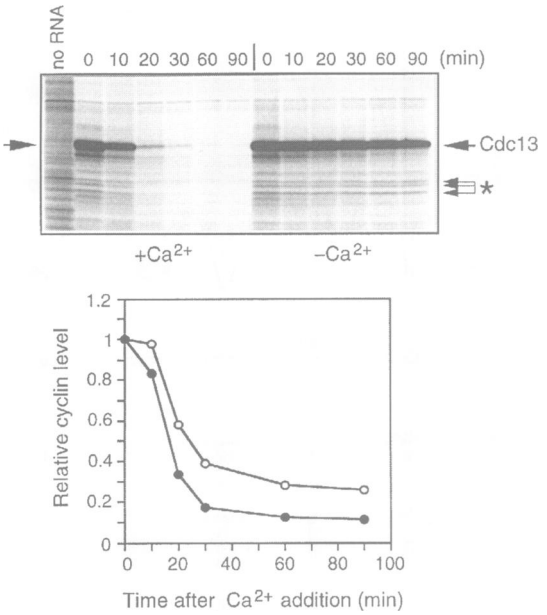

Fig. 2. Destruction of Cdc13 in *Xenopus* egg cell-free system. Top panel: mRNA for *cdc13* was translated and assayed for destruction in *Xenopus* egg extracts. Left side, $CaCl_2$ added at time zero; right side, no Ca^{2+} added. Asterisk at right indicates positions of the endogenous B-type cyclins. The lane marked 'no RNA' denotes no added mRNA. Bottom panel: quantification of the cyclin bands by scanning densitometry; normalized with reference to the intensities found in the absence of Ca^{2+} at each time point. Cdc13 (●), endogenous B-type cyclins (○).

R	39	2	0	0	1	2	0	1	0	4	1
A	0	11	25	0	3	0	0	0	1	1	4
L	0	2	0	38	0	0	7	0	0	0	1
G	0	1	1	0	23	0	0	13	0	3	3
D	0	0	0	0	1	15	1	1	0	0	2
E	0	0	0	0	4	6	0	1	4	0	1
I	0	0	2	0	0	0	18	0	1	2	0
N	0	2	0	0	2	6	0	0	27	6	5
S	0	3	0	0	3	0	0	7	3	0	1
T	0	12	2	0	1	3	0	12	1	2	2
V	0	4	8	0	0	6	11	0	2	4	15
K	0	0	1	0	0	1	2	2	0	10	1
Q	0	1	0	0	1	0	0	1	0	4	0
H	0	1	0	0	0	0	0	0	0	1	2
P	0	0	0	0	0	0	0	1	0	0	1
F	0	0	0	1	0	0	0	0	0	1	0
M	0	0	0	0	0	0	0	0	0	1	0
W	0	0	0	0	0	0	0	0	0	0	0
Y	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0
Consensus	R	A/T	A	L	G	D	I/V	G/T	N	K	V

Fig. 3. Destruction box matrix. Residues found in 39 destruction boxes including A-type, B-type, CLB1-6, Cig2 and Cdc13. The destruction box consensus is R A/T A L G D I/V G/T N. R and L are highly conserved with the exception of cyclin B3, which has F in place of the L. The residues M, W, Y and C have never been found in a destruction box, and Q, H, P and F are rare in this consensus, with the exception noted above.

Identification of the 'destruction box' of S.pombe Cdc13

Cdc13 has two potential destruction boxes in its N-terminus as judged by inspection of its sequence, whose consensus is RxALGxIxN (Glotzer *et al.*, 1991). As Figure 3 shows, the R and L residues are highly conserved in destruction boxes, and their mutation renders the motif inoperative (Glotzer *et al.*, 1991; Lorca *et al.*, 1992; Stewart *et al.*, 1994). In Cdc13, the first potential match

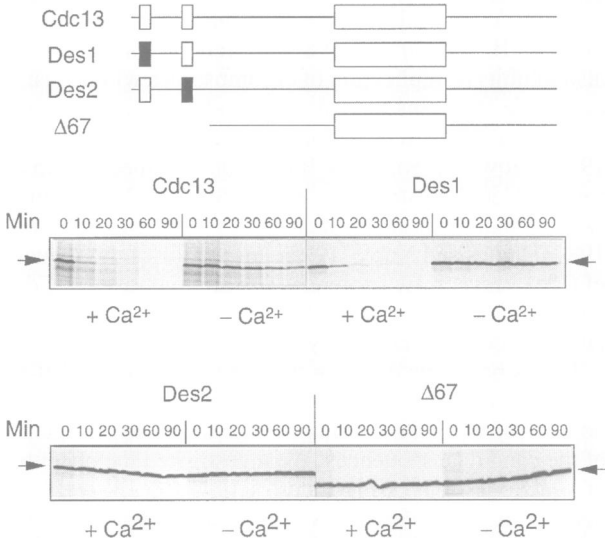

Fig. 4. Identification of the 'destruction box' in Cdc13. Top panel: schematic graph shows that Cdc13 has two potential destruction box in its N-terminus, with the highly conserved cyclin box in the middle of the protein (unfilled boxes). Des1 is the first destruction box mutant, Des2 is the second destruction box mutant (indicated by filled boxes) and Δ67 is N-terminal 67 residues truncated Cdc13. Bottom panel: destruction assay of these constructs in *Xenopus* egg extract.

to the consensus starts at residue 8 (RQHLLANTL) and a second one is found at residue number 59 (RHALDDVSN). Neither of these sequences is a close match to the consensus derived from higher eukaryotes, and to examine whether either serves as a destruction box, we made mutants in which RxxL was mutated to AxxA. A version of Cdc13 lacking the N-terminal 67 residues (Δ67) was also constructed (Figure 4, top panel). We tested the destructibility of these constructs in the *Xenopus* cell-free system described above. The construct with alanines at residues 8 and 11 Cdc13(Des1) proved to be as unstable as wild-type Cdc13, but the second destruction box mutant (alanines at residues 59 and 62), Cdc13(Des2), was quite stable (Figure 4). As expected, the Δ67 construct was completely stable, like the *Arbacia* cyclin BΔ90 (Murray *et al.*, 1989). To make sure that the second destruction box sequence was genuine, we expressed the constructs in *S.pombe* under the control of the thiamine-repressible *nmt1* promoter, pREP41, which has a promoter of intermediate strength. *S.pombe* cells were transformed with wild-type Cdc13 or each of the two destruction box mutants or the empty vector pREP41. Expression of the Cdc13 constructs was regulated by the presence (+T = OFF) or absence (–T = ON) of thiamine in the medium. Figure 5A shows that expression of the second destruction box mutant (Des2) inhibited colony formation on the plate, whereas expression of wild-type or the first destruction box mutant (Des1) did not. Even weak expression of Des2 from the weakest modified *nmt1* promoter (pREP81) strongly inhibited colony formation (data not shown).

To examine the effects of the destruction box mutations on the stability of Cdc13 in yeast, we measured the level of wild-type Cdc13 or the destruction box mutant Des2 in cells that had been arrested in G_1 or G_2 by temperature-sensitive mutations in the *cdc10* or *cdc25* genes respectively. Hayles *et al.* (1994) found that the level of Cdc13 was very low in G_1-arrested cells. We wished to investigate

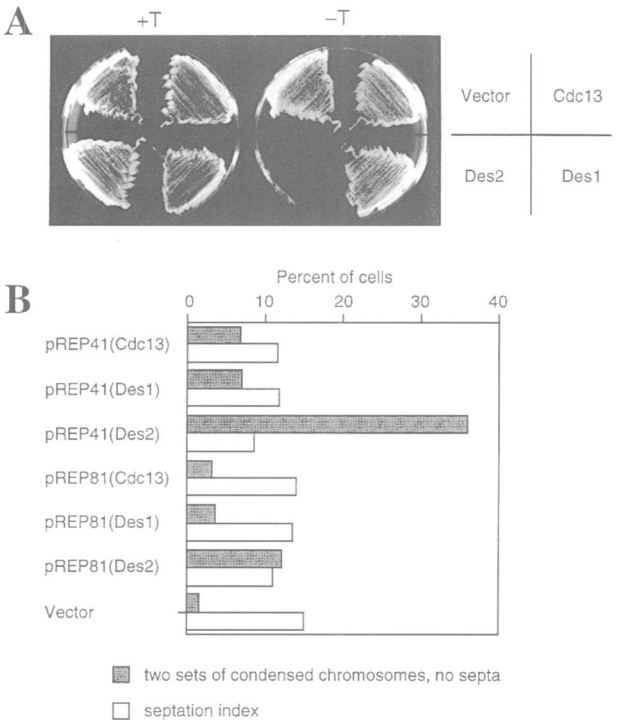

Fig. 5. Phenotype of *S.pombe* expressing indestructible Cdc13. (**A**) Wild-type cells were transformed with vector pREP41, pREP41–Cdc13, pREP41–Des1 or pREP41–Des2 and the transformants were streaked on a minimal plate in the presence (+T = OFF, left panel) or absence (–T = ON, right panel) of thiamine and incubated at 32°C. (**B**) Cells containing the indicated constructs were cultured at 32°C in minimal medium containing thiamine and then thiamine was removed from the medium. At 14 h following thiamine removal, cells were stained with DAPI and Calcofluor, and the fraction of cells having the indicated phenotype was determined by microscopic examination.

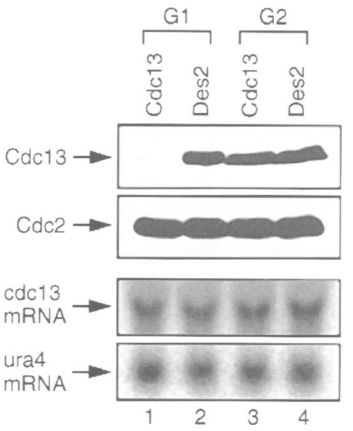

Fig. 6. Levels of wild-type and mutant Cdc13 in G_1- and G_2-arrested cells. *cdc10-V50* or *cdc25-22* mutant cells were transformed with either pREP81(Cdc13) or pREP81(Des2). The transformed cells were grown at 25°C in medium lacking thiamine for 11 h and then shifted to 36°C for 4 h to arrest the *cdc10* mutant cells in G_1 or *cdc25* mutant cells in G_2 phase. The cells were analysed by immunoblotting with anti-Cdc13 polyclonal antiserum or anti-Cdc2 mAb Y100 (upper two panels) or by Northern blotting for *cdc13* transcript with *ura4* transcript as a loading control (lower two panels) as indicated on the figure.

whether the Des2 mutant of Cdc13 could accumulate in these cells. We transformed *cdc10-V50* and *cdc25-22* mutant cells with either pREP81(Cdc13) or pREP81-(Des2). The transformed *cdc10* or *cdc25* mutant cells were incubated at 25°C in medium lacking thiamine for 11 h, and then shifted to 36°C for 4 h. FACScan analysis showed that the DNA content in the transformed *cdc10* mutant or *cdc25* mutant cells were 1C or 2C, respectively, after this protocol (data not shown). Figure 6 shows that the level of Cdc13 was very low in the G_1-arrested cells, whereas Des2 was present at essentially the same level in both G_1- and G_2-arrested cells. To check that this was not due to differences in the level of mRNA, the *cdc13*$^+$ and *Des2* transcript levels in these cells were measured by Northern blotting, and found to be very similar (Figure 6, lower two panels). The simplest interpretation of this result is that mutations in the second destruction box strongly stabilize Cdc13 *in vivo*. Taking these data together, we conclude that the second destruction box (RHAL-DDVSN), which arguably shows a better match to the overall destruction box consensus, is necessary for correct destruction of Cdc13 in *S.pombe*.

Expression of indestructible Cdc13 does not inhibit sister chromatid separation but arrests cells in anaphase

Fission yeast is a good model system to study the cytology of chromosome behaviour during mitosis. In mitosis, diffuse interphase chromatin condenses into well-defined mitotic chromosomes whose behaviour is representative of that in higher eukaryotic systems where centromere DNA sequences congress to form a metaphase plate and separate to the opposing spindle poles in anaphase (Funabiki *et al.*, 1993). The microtubule cytoskeleton also changes from an interphase cytoplasmic array to a mitotic intranuclear spindle, which elongates after the metaphase–anaphase transition (Hagan and Hyams, 1988). We therefore examined the morphology of cells expressing wild-type Cdc13 or the destruction box mutants as described in the previous section, taking samples 14 h after removal of thiamine, the time at which protein expression from the *nmt1* promoter reached a maximum level (data not shown). Figure 5B shows that when the indestructible mutant Cdc13(Des2) was expressed from the intermediate-strength pREP41 thiamine-repressible promoter, ~35% of the cells showed two sets of condensed chromosomes and no septa. The two sets of chromosomes appeared to be on their way to the poles of the cell, suggesting that cells were arrested in the early or middle anaphase. Less than 2% of the cells showed such a phenotype in the same strain carrying empty vector (Figure 5B).

To avoid possible confusion from variations due to variable copy number and plasmid loss, Cdc13 was stably integrated into the fission yeast genome under the control of the intermediate *nmt 1* promoter (pREP41). The amount of indestructible Cdc13 in the integrant strain was ~7-fold less than the protein level expressed by the plasmid pREP41cdc13(Des2) but removal of thiamine completely inhibited colony formation (data not shown). We determined the intracellular distribution of microtubules and chromosomes in these cells by immunofluorescence microscopy using the anti-tubulin antibody TAT1 (Woods *et al.*, 1989) and DAPI for DNA staining. At 14 h after the removal of thiamine, ~43% of cells displayed anaphase in which sister chromatids were separated with no septum formation (Figure 7). These cells also showed ~8-fold

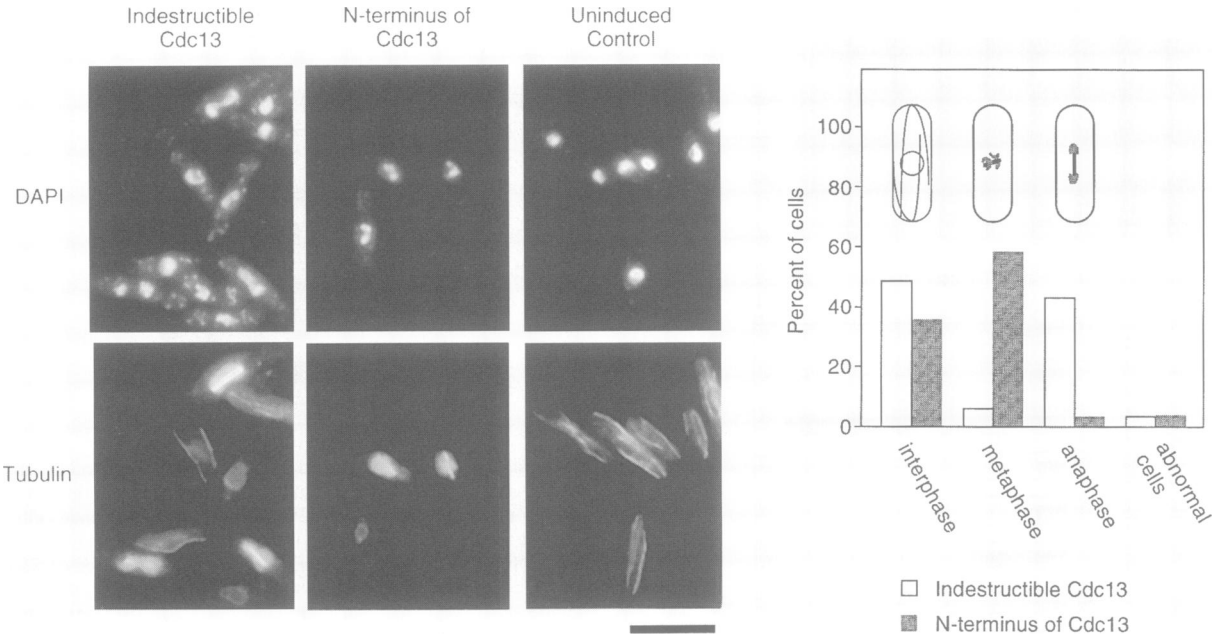

Fig. 7. Comparison between phenotypes of cells expressing indestructible Cdc13 and N-terminus of Cdc13. Left panel (micrographs): at 14 h after removal of thiamine, cells expressing indestructible Cdc13, the N-terminus of Cdc13 or uninduced control cells as indicated were stained with DAPI and antibody against anti-tubulin. Scale bar is 10 μm. Right panel: the fraction of cells showing interphase, metaphase, anaphase or abnormal phenotypes were scored after cytological analysis.

higher histone H1 kinase activity than that of uninduced cells. FACScan analysis indicated that the DNA content of these cells was 2C (data not shown). These results demonstrate that sister chromatid separation does not require the destruction of Cdc13 or inactivation of Cdc13/Cdc2 kinase in *S.pombe*, as has previously been noted in *Xenopus* egg extracts, *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Holloway *et al.*, 1993; Surana *et al.*, 1993; Sigrist *et al.*, 1995). Cyclin destruction does appear to be required to complete anaphase and to initiate septation. It may be worth noting that the tubulin staining of these cells gave unusual patterns. About half of the anaphase cells contained an elongated spindle, but the other half did not, even though sister chromatids were clearly separated.

Strong expression of N-terminal fragments of Cdc13 stabilizes endogenous Cdc13 and inhibits sister chromatid separation

N-terminal fragments of sea urchin cyclin B containing its destruction box are very effective inhibitors of cyclin proteolysis in *Xenopus* egg extract (Holloway *et al.*, 1993; D.Harrison and T.Hunt, unpublished data). To test this *in vivo*, we transformed *S.pombe* with various N-terminal fragments of Cdc13 that were expressed under the control of the strongest thiamine-repressible promoter (pREP1). Figure 8 shows that strong expression of the N-terminal 70 (N-70) or 152 (N-152) residues of wild-type Cdc13 inhibited colony formation on the plates. By contrast, expression of the equivalent constructs with mutated destruction boxes (RHALDDVSN to AHAADDVSN) had no effect on growth (N-70/Des2 or N-152/Des2).

We tested if high levels of the destruction box-containing fragments of Cdc13 inhibited normal cyclin B destruction at the end of mitosis, and also examined the phenotype of N-70 expressing cells by immunofluorescence using anti-tubulin antibodies and DAPI staining for DNA. Wild-type cells transformed with either pREP1(N-70) or pREP1(N-70/Des2) were analysed at the indicated times after removal of thiamine (expression = ON). Endogenous Cdc13 and the induced N-70 polypeptides (top panel) and Cdc2 (lower panel) were detected by immunoblotting (Figure 8B) and total histone H1 kinase activity was determined (Figure 8C). Strong expression of N-70 caused Cdc13 to accumulate and cell extracts had ~9-fold elevated histone H1 kinase activity, whereas the expression of the equivalent constructs with mutated destruction box (N-70/Des2) neither stabilized the endogenous Cdc13 nor increased histone H1 kinase activity. The morphology of cells expressing N-70 was consistent with these data. About 60% of the cells were arrested in metaphase, with three condensed chromosome aligned on a metaphase plate (Figure 7). Less than 3% of the cells appeared to be in anaphase, which is strikingly different from the phenotype of cells expressing indestructible Cdc13 (Figure 7). In contrast, the phenotype of cells expressing N-70/Des2 was indistinguishable from wild-type cells. It is noteworthy that the short mitotic spindle seen in cells expressing high levels of N-70 was only observed in one-third of the cells showing typical metaphase chromosomes, and the remaining two-thirds had no clear tubulin staining despite their similar chromosome staining. This may indicate that the spindles are somewhat unstable in these conditions, similar to the apparent instability of spindles in cells expressing indestructible Cdc13 noted above.

Strong expression of N-70 promotes entry into S phase in G_1-arrested cells

As described above, and as previously noted by Hayles *et al.* (1994), the level of Cdc13 is very low in G_1-arrested cells. We wished to test if expression of N-70 would allow the accumulation of Cdc13 in these cells, and if so, what

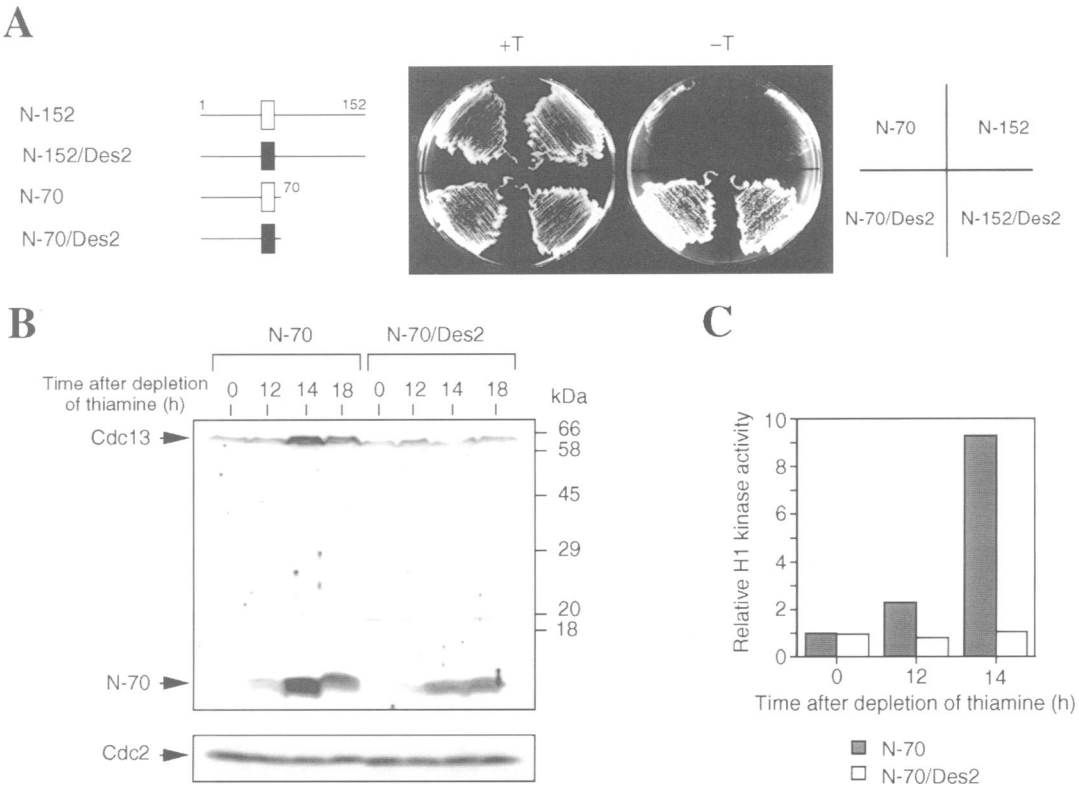

Fig. 8. Strong expression of N-70 inhibits colony formation and stabilizes Cdc13 with high histone H1 kinase activity in cell extracts. (**A**) Wild-type cells were transformed with either pREP1(N-70), pREP1(N-152), pREP1(N-70/Des2) or pREP1(N-152/Des2), and the transformants streaked on minimal plates in the presence (+T = OFF, left panel) or absence (–T = ON, right panel) of thiamine and incubated at 32°C. (**B**) Samples of the transformed cells expressing N-70 or N-70/Des2 were taken at the indicated time, analysed by immunoblotting with anti-Cdc13 polyclonal antiserum (above) or anti-Cdc2 mAb Y100 (below). (**C**) Histone H1 kinase activity in extracts of these cells.

(if anything) would result from such abnormal expression. We transformed *cdc10-V50* cells with either N-70, N-70/Des2 or empty vector. To exclude the possibility that strong expression of N-70 would arrest cells at metaphase of the previous cell cycle, the transformed *cdc10* mutant cells were incubated at 25°C in medium lacking thiamine for 12 h (just before expression of N-70 started), and then shifted to 36°C. FACScan analysis showed that by 2 h after shift-up, the *cdc10* mutant cells had mainly a 1C (G_1) DNA content (Figure 9C), irrespective of what construct they contained. Figure 9A shows that strong expression of N-70 led to the accumulation of high levels of Cdc13 in these G_1-arrested cells, whereas expression of the same level of N-70/Des2 with the mutated destruction box (AxxA) did not. Cdc2 levels were not affected by these manipulations. We also found that *cdc10-V50* strain expressing high levels of the N-70 displayed high histone H1 kinase activity, whereas the same strains expressing N-70/Des2 or empty vector showed very low histone H1 kinase activity (Figure 9B), as might be expected from the high levels of Cdc13 in the former case.

The DNA content of *cdc10-V50* cells expressing N-70 was measured at 0, 2 and 4 h after shifting to 36°C (Figure 9C). As mentioned above, the majority of the *cdc10* mutant cells showed 1C DNA content by 2 h, and even 4 h after shift-up, most of the *cdc10* mutant cells containing empty pREP1 vector or expressing N-70/Des2 arrested with a G_1 DNA content. In contrast, *cdc10* mutant cells making high amounts of N-70 did not arrest in G_1, and by 4 h after shift-up, ~80% showed a 2C (G_2) DNA content (Figure 9C), indicating that strong expression of N-70 could overcome the *cdc10* arrest of DNA replication. DAPI-staining images of these cells were consistent with the FACScan data. After 4 h at the non-permissive temperature in the presence of N-70, a significant number of the cells displayed a mitotic phenotype despite the *cdc10* mutation. This phenotype was similar to that observed in wild-type cells expressing N-70 (Figure 7). By contrast, *cdc10* mutant cells expressing N-70/Des2 or transformed with empty vector showed a typical G_1-arrested phenotype (data not shown). When the same *cdc10* strain was transformed with a plasmid expressing indestructible Cdc13 under the control of the strongest or the intermediate *nmt1* promoter (pREP1, pREP41), a high fraction of cells appeared to be in mitosis 4 h after shift up; however, unlike what was found with N-70 overexpression, these cells had a G_1 DNA content, so that merely overexpressing stable Cdc13 did not overcome the *cdc10* arrest of DNA replication (data not shown). Immunoblotting showed no increase in the level of Cdc10 protein in the presence of high levels of N-70 (data not shown).

Hayles *et al.* (1994) previously reported that *cdc10-129* mutant cells overexpressing Cdc2 and Cdc13 entered mitosis even though they were unable to enter S phase, and had a 1C DNA content. Taken together, the results indicate that N-70 must stabilize not only Cdc13, but also other protein or proteins that are normally produced as a result of transcription promoted by Cdc10. It would thus

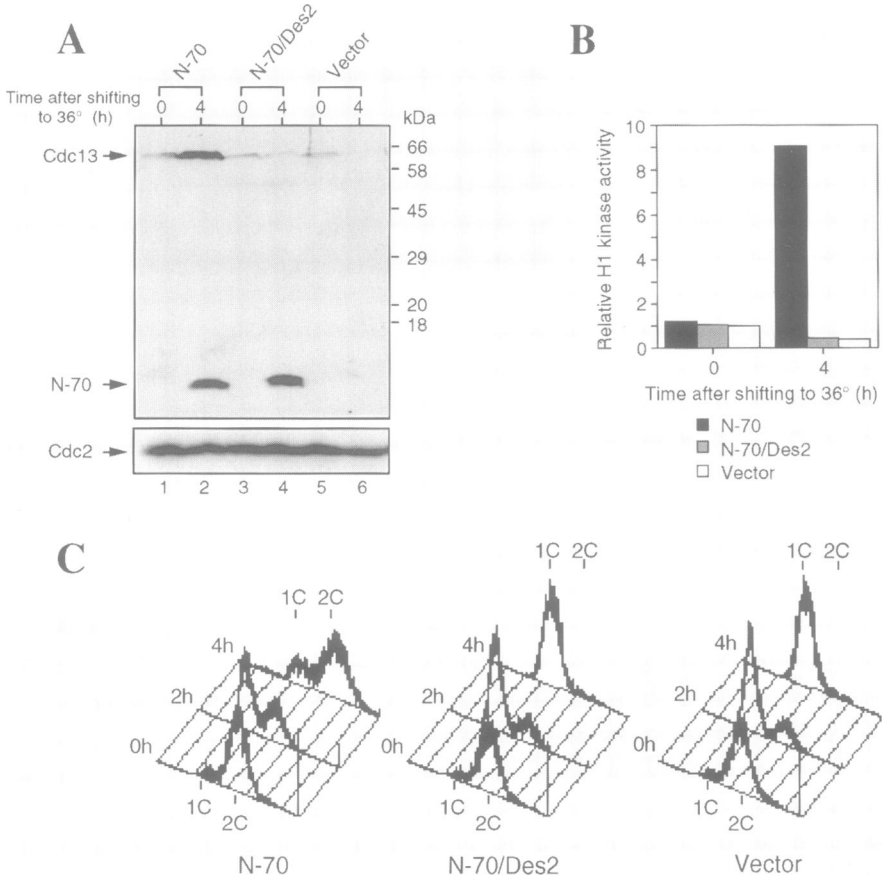

Fig. 9. Strong expression of N-70 promotes entry into S phase in *cdc10* G_1-arrested cells. *S.pombe* strain *cdc10-V50* was transformed with empty vector pREP1, pREP1(N-70) or pREP1(N-70/Des2). The transformed cells were grown at 25°C in medium lacking thiamine for 12 h (just before expression of N-70 could be detected) and then shifted to 36°C (lane 1, time zero). Samples were taken 4 h after shift-up and analysed (A) by immunoblotting with anti-Cdc13 polyclonal antiserum (above) or anti-Cdc2 mAb Y100 (below), or (**B**) by histone H1 kinase assay. In (**C**), DNA content was analysed by FACScan every 2 h after shift-up.

appear that one reason why the activity of Cdc10 is required to initiate S phase is that it replenishes proteins that are normally subjected to destruction by the cyclin proteolysis machinery at the end of mitosis. If such proteins are stabilized by some means, the requirement for Cdc10 is bypassed.

The first 70 residues of Cdc13 can act as a mitotic destruction module for other proteins

Finally, we investigated whether N-70 could act as a mitotic destruction module for other proteins. To do this, we constructed N-70 or N-70 with a mutated destruction box fused to Gal4 DNA binding domain (Gal4bd) at either the N- or C-terminus, and tested the stability of the resulting constructs in *Xenopus* cell-free destruction assay. Figure 10 shows that (N-70)-Gal4bd was destroyed with essentially the same kinetics as *Xenopus* B-type cyclins in a Ca^{2+}-dependent manner, whereas the equivalent constructs with mutated destruction box were stable. The construct with the Gal4 DNA binding domain with N-70 fused to its C-terminus was equally well destroyed in the *Xenopus* assay, and the equivalent constructs with mutated destruction box were stable (Figure 10, lower panel). It is thus apparently not important whether N-70 is at the N- or C-terminus of this fusion protein, and indicates that the

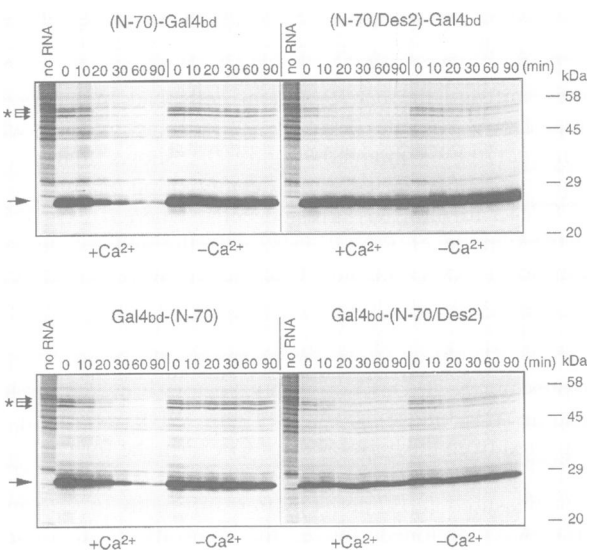

Fig. 10. N-70 can act as a mitotic destruction module for other proteins. Destruction assay of (N-70)–Gal4bd, (N-70/Des2)–Gal4bd, Gal4bd–(N-70) or Gal4bd–(N-70/Des2) in the *Xenopus* egg cell-free system as described in Figure 2. Asterisks on left indicate position of the endogenous B-type cyclins.

N-terminal 70 residues of Cdc13 can function at anaphase as a destruction module for other proteins.

Discussion

The separation of sister chromatids at anaphase is arguably the most important irrevocable event during the cell cycle. In this paper, we describe the use of a cell-free destruction assay system to investigate whether *S.pombe* cyclin or cyclin mutants are destructible. Using this system, we identified the destruction box of Cdc13 and constructed indestructible versions of the protein, which were integrated into the chromosome under the control of inducible promoters. When we compared the effect of expression of indestructible Cdc13 with that of high-level expression of the N-terminal fragment of Cdc13, we found a striking difference. In fission yeast, indestructible cyclin B arrested cells in anaphase, after sister chromatid separation, whereas high levels of N-70 gave a metaphase arrest. Moreover, we found that the N-terminal fragment with an intact destruction box can promote entry into S phase in *cdc10* G_1-arrested cells, presumably by stabilizing protein(s) that are necessary for the G_1/S transition.

The role of Cdc13 destruction in mitosis

Though the amount of Cdc2 is constant during the cell cycle, the kinase activity of Cdc13/Cdc2 increases roughly in parallel with the increase in Cdc13 during G_2 phase and M phase, both of which drop abruptly at the end of mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989; Hayles and Nurse, 1995). We confirmed that the timing of inactivation of total Cdc2 kinase activity was well correlated with the timing of destruction of Cdc13 at the end of mitosis. What is the role of the destruction of Cdc13? Expression of indestructible Cdc13 caused anaphase arrest, rather than metaphase arrest in fission yeast. Sister chromatids were separated, tubulin staining showed an anaphase spindle, and septum formation did not occur in the presence of high levels of Cdc13/Cdc2 kinase, indicating that progression from metaphase to anaphase does not necessarily require the inactivation of Cdc13/Cdc2 kinase. Similar observations were previously found in frog egg extracts, budding yeast and fruit fly; thus, adding non-degradable cyclin B to frog egg extracts prevented MPF inactivation but did not block sister chromatid separation (Holloway *et al.*, 1993) and expression of indestructible B-type cyclins arrested cells at anaphase with separated sister chromosomes (Surana *et al.*, 1993). In *Drosophila*, indestructible cyclins block exit from mitosis (Rimmington *et al.*, 1994) and recent evidence suggests that the sequential destruction of cyclin A, B and B3 occurs (Sigrist *et al.*, 1995), implying that multiple steps in anaphase are committed by the inactivation of the corresponding cyclin/cdc2 kinase. Destruction of Cdc13 may be required to commit exit from mitosis which is also prerequisite to initiate S phase in the succeeding cell cycle.

We do not fully understand the frequent loss of tubulin staining seen when cells arrest for long periods with high levels of Cdc13, but that *cdc13-117* mutant allele is sensitive to thiabendazole (TBZ), an inhibitor of microtubule assembly in *S.pombe* (Toda *et al.*, 1983), previously suggested some kind of interaction between Cdc13 and microtubules (Booher and Beach, 1988).

The role of programmed proteolysis in sister chromatid separation

Strong expression of the N-terminal fragment of Cdc13 (N-70) clearly arrested cells at metaphase, rather than anaphase, although high levels of N-70 stabilized Cdc13 and led to high Cdc13/Cdc2 kinase. Expression of N-70 with a mutated destruction box (N-70/Des2) showed no phenotype and caused neither stabilization of Cdc13 nor increase of Cdc13/Cdc2 kinase. We assume that some proteins whose proteolysis is required for the metaphase to anaphase transition must be stabilized in the presence of N-70, presumably owing to competition for some component of the proteolysis system.

A possible candidate for stabilization has recently been identified in the Cut2 protein, whose proteolysis is required for sister chromatid separation (Funabiki *et al.*, 1996) and we have found that Cut2 is stabilized by N-70 (H.Yamano, unpublished data). Pds1 of *S.cerevisiae* is another such protein, which is required for the proper execution of anaphase and contains a putative destruction box (Yamamoto *et al.*, 1996a,b). It is unclear how many other proteins need to be degraded to initiate sister chromatid separation. Our data suggest that proteolysis of a set of proteins trigger the onset of anaphase, whose programmed proteolysis is regulated by the same pathway as that of the B-type cyclins. Similar observations were reported in frog egg extracts, where the N-terminal fragment of sea urchin B-type cyclin delayed sister chromatid separation in a dose-dependent manner (Holloway *et al.*, 1993), but ours is the first investigation into the effects of high-level expression of an N-terminal fragment of B-type cyclin *in vivo*. This is a useful way to study the mechanism of cyclin destruction, because N-70 seems specifically to inhibit the mitotic ubiquitin-mediated and destruction box-dependent proteolytic machinery, while similar processes which may be present during interphase are unaffected. No other specific inhibitors of cyclin proteolysis have yet been reported.

On the other hand, five mutants that display a clear metaphase arrest have been isolated in fission yeast: *mts2*, *mts3*, *nuc2*, *sds22*, and the *dis2–sds21* double mutant (Hirano *et al.*, 1988; Gordon *et al.*, 1993, 1996; Stone *et al.*, 1993; H.Yamano, K.Ishii and M.Yanagida, unpublished observations). The $mts2^+$ and $mts3^+$ genes encode subunits of the 26S proteasome, while $nuc2^+$ encodes a CDC27 homologue which is a subunit of the large E3 ubiquitin ligase complex—the Cyclosome or APC—indicating that defects in the degradation of ubiquitinated proteins or in ligation of ubiquitin to proteins can cause metaphase arrest. These systems may well be required for Cdc13 destruction, but since they cause metaphase arrest, the phenotypes of these mutants support the idea that specific proteolysis of other unknown proteins is required for sister chromatid separation. The latter two genes, $sds22^+$ and $dis2^+$–$sds21^+$ encode regulatory and catalytic subunits of type-1 protein phosphatase respectively (Ohkura *et al.*, 1989; Stone *et al.*, 1993), suggesting that the ubiquitin-mediated proteolysis machinery may be negatively regulated by phosphorylation, and/or that the phosphorylation state of target proteins might affect their susceptibility to proteolysis.

The effect of strong expression of N-terminus of Cdc13 in G_1-arrested cells

Overexpression of the N-70 in *cdc10* G_1-arrested cells appeared to promote entry into S phase and subsequent progression into M phase, whereas the same fragment with a mutated destruction box maintained a G_1 arrest. This result suggests that unknown proteins which promote S phase are also susceptible to destruction box-dependent, ubiquitin-mediated proteolysis. A key question is what are the proteins which promote and initiate DNA replication from the *cdc10* G_1 arrest point. We would argue that it cannot be Cdc13, because we found that overexpression of Cdc13 with a mutated destruction box (Cdc13/Des2) did not stimulate DNA replication although premature mitosis was observed. A similar result was reported by Hayles *et al.* (1994), who tested the effects of strong expression of Cdc13 together with Cdc2 in these circumstances. We surmise that unknown proteins other than cyclins are stabilized in this situation, which promote the initiation of S phase. One of the likely candidates is Cdc18, an unstable protein whose production normally requires Cdc10 and which is required for initiating DNA replication. Cdc18 is normally synthesized *de novo* prior to S phase and disappears in each cell cycle after S phase has been completed (Nishitani and Nurse, 1995; Muzi-Falconi *et al.*, 1996). In fact, we did find that Cdc18 was somewhat stabilized by the presence of N-70, but not by N-70/Des2 (H.Yamano, unpublished data). However, inspection of the sequence of Cdc18 does not reveal anything like a destruction box, and the stabilization of Cdc18 was much weaker than that of Cdc13 when N-70 was expressed in G_1-arrested cells (H.Yamano, unpublished data). Probably the *cdc18* mRNA levels are very low in this situation (Kelly *et al.*, 1993).

We doubt whether Cdc18 is the only protein that is stabilized by the presence of N-70 (it should be recalled that forced expression of Cdc18 cannot rescue a strain that completely lacks Cdc10). It is possible that other proteins that are necessary for initiating DNA replication are regulated by ubiquitin-mediated, destruction box-dependent proteolysis. One inference, by analogy to regulation of DNA replication in budding yeast, is that pre-initiation complexes are probably not formed in cells arrested in G_1 by a *cdc10* mutation. As a result, even when Cdc13 is overexpressed and forms active kinase with Cdc2 under this circumstances, it cannot trigger the initiation of DNA synthesis. However, because N-70 can stabilize other proteins that promote the formation of competent pre-initiation complexes, in addition to Cdc13, DNA replication occurs when N-70 is expressed in *cdc10* G_1-arrested cells. Heichman and Roberts (1996) recently found that mutations in *CDC16* and *CDC27*, which encode two components of the APC complex involved in ubiquitin-mediated proteolysis, caused re-replication of DNA within a single cell cycle, consistent with the idea that regulated proteolysis is important for the control of initiation of replication (see Wuarin and Nurse, 1996 for discussion).

The role of N-70

As described above, high levels of N-70 can inhibit mitotic ubiquitin-mediated proteolysis *in vivo,* and the domain comprising the N-terminal 70 residues of Cdc13 could act as a mitotic destruction module for other proteins when assayed in the *Xenopus* cell-free system. Proteolysis *in vitro* worked equally well whether the destruction box was located at the N- or C-terminus of the Gal4 fusion protein, suggesting that N-70 contains all the necessary elements for recognition by ubiquitin-mediated proteolytic machinery and subsequent proteolysis. Since N-70 with a mutated destruction box (N-70/Des2) does not function as a destruction module either *in vivo* or *in vitro*, the destruction box clearly plays an essential role for programmed proteolysis (Funabiki *et al.*, 1996), but we do not know how much more besides the destruction box itself is required for this function. Nor is it clear whether the inhibition stems from a relatively non-specific sequestration of ubiquitin by N-70, or whether proteins that interact specifically with the destruction box are diverted from their normal functions. These are important topics for future investigation.

Materials and methods

Fission yeast strains and methods

An *S.pombe* haploid wild-type h^- strain and its derivatives were used. All media and growth condition are as described by Moreno *et al.* (1991). Temperature-sensitive mutants used were *cdc25-22* (Fantes *et al.*, 1979) and *cdc10-V50* (Marks *et al.*, 1992), which were normally grown at the permissive temperature of 25°C, whereas 36°C was used as the restrictive temperature. Wild-type cells were cultured at 32°C. Transformation of *S.pombe* was performed by the high-efficiency lithium method (Okazaki *et al.*, 1990).

To induce expression from the *nmt1* promoter, cells were grown in minimal medium containing 2 μM thiamine to mid-exponential phase, spun down and washed three times with minimal medium and resuspended in fresh medium lacking thiamine at a density calculated to produce 3×10^6 cells/ml after 14 h of induction. Flow cytometric analysis of DNA content was performed on a Becton-Dickinson FACScan using propidium iodide staining of cells as described by Sazer and Sherwood (1990). To determine the septation index, cells were stained with Calcofluor as described by Mitchison and Nurse (1985).

The indestructible Cdc13 integrant strain was constructed as follows. Plasmid pREP41(Cdc13/Des2) (see below) was cut with *Pst*I and *Sac*I. The fragment containing the intermediate *nmt1* promoter (REP41), *cdc13* destruction box mutant gene and *nmt1* terminator was cloned into the integration plasmid pJK148 (a gift from Dr P.Nurse) at the *Pst*I–*Sac*I site. The resulting plasmid pJK–REP41–cdc13/Des2 was linearized using the Tth 111I restriction enzyme and transformed into a *leu1-32* strain. Stable Leu^+ transformants were isolated and confirmed by Southern hybridization. The integrant used in this paper contained three tandem copies of *cdc13*/Des2 integrated into the *leu1* locus.

Plasmid construction

The 5′ and 3′ UTR sequences of *Xenopus* B2 mRNA are helpful for the translation of heterologous mRNAs in *Xenopus* egg extracts. To construct a version of Cdc13 with these translation-enhancing UTRs, we first introduced an *Nde*I site at the initiating methionine and a *Bam*HI site just after the termination codon of a full-length clone of *Xenopus* cyclin B2 in pGEM1 (plasmid pHY22). The *cdc13* coding sequence was isolated by PCR and the full-length *Nde*I–*Bam*HI fragment used to replace the *Xenopus* cyclin B2 ORF of pHY22. Destruction box mutants were constructed out using mutagenic PCR essentially as described by Horton and Pease (1991). The first destruction box mutant (Des1) changed the sequence RQHLLANTL to AQHALANTL and the second destruction box mutant (Des2) changed RHALDDVSN to AHAADDVSN. The mutations were checked by sequencing with the United States Biochemicals Sequenase kit (Cleveland, OH). Cdc13(Δ67) lacking the N-terminal 67 residues was also prepared by PCR and subcloned into the pHY22 backbone.

To construct the plasmids that express Gal4(1–147) DNA binding domain (Gal4bd) fused with the N-terminal 70 residues of Cdc13 [Gal4bd–(N-70) or Gal4bd–(N-70/Des2)], the N-terminal 70 residues of Cdc13 (N-70) or the same fragment with a mutated destruction box (N-70/Des2) were isolated by PCR and subcloned into pGBN9 (from

Dr M.Yanagida, Kyoto University, Japan). This vector contains an *Nde*I site introduced into the cloning cassette of pGBT9 (Clontech). The resulting plasmids, pGBN9(N-70) or pGBN(N-70/Des2) were cut to completion with *Bam*HI and partially with *Hin*dIII to isolate full-length fragments containing Gal4bd fused to N-70 or N-70/Des2, which were subcloned into pHY22 after converting the *Nde*I site in the vector and the *Hin*dIII site in the insert to blunt ends. For the plasmids expressing (N-70)–Gal4bd or (N-70/Des2)–Gal4bd, PCR fragments containing N-70 or N-70/Des2 with a *Hin*dIII site introduced at the 5′ end and an *Nde*I site at the 3′ end were subcloned cloned into pGBN9 cut to completion with *Nde*I and partially cut with *Hin*dIII, thereby losing the Gal4bd. After checking for the correctness of the intermediate construct, the resulting plasmids pGBN(N-70) or pGBN(N-70/Des2) were cut with *Nde*I and *Bam*HI, and the *Nde*I–*Bam*HI PCR fragment containing Gal4bd with a termination codon was ligated into them. Finally, the resulting plasmids pGBN(N-70)–Gal4db or pGBN(N-70/Des2)–Gal4bd were cut with *Bam*HI and *Hin*dIII. The fragments containing (N-70)–Gal4bd or (N-70/Des2)–Gal4bd were subcloned into pHY22 after converting the *Nde*I site in the vector and the *Hin*dIII site in the insert to blunt ends.

For expression in *S.pombe*, the thiamine-repressible *nmt1* promoter-containing plasmids pREP1, pREP41 and pREP81 were used. pREP41 and pREP81 have modified promoters with expression levels that are 10 and 100 times less than that of pREP1, respectively (Basi *et al.*, 1993; Maundrell, 1993). *Nde*I–*Bam*HI fragments containing full-length *cdc13* and destruction box mutants were excised from the corresponding pHY22-based plasmids described above and subcloned into pREP41 and pREP81. N-70 or N-70/Des2 were excised with *Nde*I and *Bam*HI from pGBN9(N-70) or pGBN9(N-70/Des2), respectively, and subcloned into pREP1.

Preparation of rabbit polyclonal antibodies against Cdc13 and mouse monoclonal antibody against Cdc2

N-terminally His_{10}-tagged full-length Cdc13 was expressed in *Escherichia coli* and bound to Ni–NTA beads under denaturing conditions as described by the supplier (Qiagen). The beads were injected into a rabbit with Freund's adjuvant followed by standard immunization procedure for raising polyclonal antibodies (Harlow and Lane, 1988).

A peptide spanning the C-terminal 14 residues of Cdc2 (AKRALQQN-YLRDFH) was coupled to KLH and injected to mice in Freund's complete adjuvant. The injections were repeated after 28 days and every 14 days for 10 weeks (in Freund's incomplete adjuvant). A hyperimmune mouse was injected intravenously with BSA coupled to the same peptide. On day 3 the spleen was removed and its cells fused with SP2/0 myeloma cells as described by Harlow and Lane (1988). Wells giving a good signal in the ELISA assay using BSA-coupled peptide were selected and subsequently confirmed as anti-Cdc2 antibodies by immunoblotting an extract of *S.pombe*. mAb Y100 was found to give a strong clean signal, and was used for the immunoblots shown in this paper.

Preparation of yeast extract, immunoblotting and histone H1 kinase assay

Yeast extracts were prepared essentially as described by Stone *et al.* (1993). Cultures were grown to $3–5\times10^6$ cells/ml, harvested by centrifugation, washed in 1 ml of lysis buffer (50 mM Tris–HCl, pH 8.0, 0.4 M NaCl, 10 mM EDTA, 5 mM EGTA, 80 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM 2-mercaptoethanol, 1 mM PMSF). Cells were disrupted in the lysis buffer by vortexing vigorously with glass beads. Cell debris was removed by a 1 min spin in a microcentrifuge and the protein concentration determined by a Bradford assay. Equal amounts of protein (20–50 μg) were analysed by SDS–PAGE, electrophoretically transferred to nitrocellulose and immunoblotted with anti-Cdc13 antisera or the mouse monoclonal anti-Cdc2 antibody Y100 described above (Harlow and Lane, 1988). For synchronous cultures, *cdc25* mutant cells were shifted from 36°C to 25°C and harvested quickly at the indicated time points using a stop buffer (Simanis and Nurse, 1986) and freezing the cell pellet in liquid nitrogen. Proteins were subsequently extracted as described above.

For histone H1 kinase assay, Cdc2 was purified by affinity chromatography on $p13^{suc1}$ beads and washed five times with H1 kinase buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT). The beads were incubated in 20 μl of this buffer containing 100 μM ATP, 5 μCi of [γ-^{32}P]ATP and 5 μg of histone H1 (Sigma) for 10 min at 30°C. The reaction was terminated by addition of SDS sample buffer and boiling. The samples were analysed by SDS–PAGE and quantified by a Molecular Dynamics PhosphorImager using ImageQuant software.

mRNA analysis

Total RNA was prepared from fission yeast cells as described by Russell and Hall (1983). RNAs were analysed by electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde (Fourney *et al.*, 1988). Probes for blotting were prepared from DNA fragments containing full-length *cdc13* or *ura4* by random oligonucleotide priming with [^{32}P]dCTP using the Megaprime DNA Labelling system (Amersham, UK).

Cyclin destruction assay in Xenopus egg extract

Cyclin destruction assays were carried out essentially as previously described by Stewart *et al.* (1994). Substrates for destruction assays were prepared by translation of mRNA encoding test constructs for 2 h in the presence of [^{35}S]methionine in a mixture containing 90% reticulocyte lysate and 10% frog egg extract, or in 100% reticulocyte lysate for the experiment shown in Figure 4. Small aliquots of the translation reactions were added to fresh CSF extract (giving a final concentration of 80% CSF extract), and cycloheximide (100 μg/ml) was added to block further protein synthesis. Destruction of cyclins was triggered by the addition of $CaCl_2$ to 0.4 mM final concentration. Samples were taken at intervals after adding $CaCl_2$, analysed by SDS–PAGE, and the intensities of the labelled cyclin bands were quantified by a Molecular Dynamics PhosphorImager using ImageQuant software.

Indirect immunofluorescence microscopy

Established immunofluorescence procedures using aldehyde fixation were followed (Hagan and Hyams, 1988). Microtubules were stained by anti-α-tubulin mAb TAT1 (Woods *et al.*, 1989), the gift of Dr K.Gull, University of Manchester, UK, followed by CY3-conjugated sheep anti-mouse IgG (Sigma). Chromosomal DNA was stained with 0.2 μg/ml DAPI.

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