The EMBO Journal vol.14 no.12 pp.2760–2771, 1995

A pre-start checkpoint preventing mitosis in fission yeast acts independently of p34^{cdc2} tyrosine phosphorylation

Jacqueline Hayles[1] and Paul Nurse

ICRF Cell Cycle Laboratory, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

[1]Corresponding author

We have monitored the tyrosine (Y15) phosphorylated and dephosphorylated forms of p34^{cdc2} from *Schizosaccharomyces pombe* as cells proceed through the cell cycle. Y15 is dephosphorylated in G_1 before start and becomes phosphorylated only after cells pass start and enter late G_1. This transition is associated with a switch from one checkpoint which restrains mitosis in pre-start G_1, by a mechanism independent from Y15 phosphorylation, to a second checkpoint acting post-start during late G_1 and S phase operating through Y15 phosphorylation. The pre-start checkpoint may act by preventing formation of the p34^{cdc2}/p56^{cdc13} complex. The complex between Y15-phosphorylated p34^{cdc2} and p56^{cdc13} accumulates during S phase and G_2, but the level generated is not solely dependent on the amount of p34^{cdc2} and p56^{cdc13} present in the cell. The extent of p56^{cdc13} breakdown at the end of mitosis may be determined by the amount complexed with p34^{cdc2}. We have also shown that an insoluble form of p34^{cdc2} is associated with the progression of the cell through late G_1 into S phase.

Key words: checkpoints/mitosis/p34^{cdc2}/*Schizosaccharomyces pombe*/tyrosine phosphorylation

Introduction

The 34 kDa protein kinase p34^{cdc2} and mitotic B cyclin play key roles in regulating the eukaryotic cell cycle (Nurse, 1990). In the fission yeast *Schizosaccharomyces pombe*, p34^{cdc2} is required in late G_1 at start when the cell becomes committed to the cell cycle, and then again in late G_2 at the onset of mitosis (Nurse and Bissett, 1981). The level of p34^{cdc2} is constant through the cell cycle, but p34^{cdc2} protein kinase activity is sharply periodic, rising to a peak at the G_2/M transition (Moreno *et al.*, 1989). This increase in kinase activity brings about mitosis, and if p34^{cdc2} is prematurely activated then mitosis is advanced (Gould and Nurse, 1989). Kinase activation requires association with the B-cyclin p56^{cdc13} encoded by *cdc13*$^+$, but this does not normally determine the timing of activation. This is determined by dephosphorylation of a tyrosine (Y15) located in the catalytic site of the enzyme (Gould and Nurse, 1989; Endicott *et al.*, 1994), brought about by the phosphatase p80^{cdc25} encoded by *cdc25*$^+$ (Millar *et al.*, 1991; Strausfeld *et al.*, 1991). Three other phosphatases, *pyp1*, *pyp2* and *pyp3* (Millar *et al.*, 1992a,b; Ottilie *et al.*, 1992), have also been shown to influence the regulation of p34^{cdc2} activation. The phosphatase activity of p80^{cdc25} is counteracted by the tyrosine/threonine protein kinases encoded by the *wee1* (Featherstone and Russell, 1991; Parker *et al.*, 1992; McGowan and Russell, 1993) and *mik1* genes (Lundgren *et al.*, 1991) which phosphorylate Y15.

In fission yeast, p34^{cdc2} activity is also required for checkpoints preventing the onset of mitosis before the completion of S phase (Enoch and Nurse, 1990) and the onset of S phase in the absence of mitosis (Broek *et al.*, 1991). Checkpoint controls preventing premature entry into mitosis when DNA replication is incomplete act through the tyrosine phosphorylation state of the p34^{cdc2}/cyclin B complex (Dasso and Newport, 1990; Enoch and Nurse, 1990; Enoch *et al.*, 1991; Kumagai and Dunphy, 1991).

In order to describe more fully the changes in the Y15 phosphorylation state of p34^{cdc2} protein during the cell cycle, we have investigated extraction procedures and gel systems in an attempt to resolve the different forms of p34^{cdc2}. We show here that p34^{cdc2} from fission yeast can be resolved into two forms, one of which is phosphorylated on Y15. Monitoring phosphorylation changes during the cell cycle has shown that there is no detectable tyrosine-phosphorylated p34^{cdc2} in G_1 before start and that tyrosine-phosphorylated p34^{cdc2} only begins to appear after completion of the *cdc10* gene function at start.

The lack of tyrosine phosphorylation on p34^{cdc2} during the pre-start period suggests that the checkpoint preventing entry into mitosis by modulating the level of tyrosine phosphorylation of p34^{cdc2} is not operative during this period of the cell cycle. To examine this further, we investigated the three checkpoint-defective mutants *mik1Δ wee1-50*, *chk1Δ (rad27)* and *hus1-14* (Lundgren *et al.*, 1991; Enoch *et al.*, 1992; Sheldrick and Carr, 1993; Walworth *et al.*, 1993) and found that none of these enter mitosis from the pre-start G_1 period. They do, however, enter prematurely into mitosis from a post-start period when cells leak past the *cdc10* block point. These results are contrary to previously published data for the *wee1-50 mik1Δ* and *chk1Δ* (*rad27*) mutants (Lundgren *et al.*, 1991; Sheldrick and Carr, 1993).

P34^{cdc2} and p56^{cdc13} have previously been found to be present in two fractions: a high-speed supernatant (soluble fraction) and pellet (insoluble fraction) (Hayles *et al.*, 1994). We have monitored p34^{cdc2} forms in both these fractions during the cell cycle and find that during normal vegetative growth there is no difference between the two fractions which are both present throughout the cell cycle. However, during spore germination and after heat treatment of a *cdc2-L7* mutant, insoluble p34^{cdc2} is the predominant form found when cells undergo the G_1 to S phase transition, suggesting that this form of p34^{cdc2} may have activity in G_1 at start.

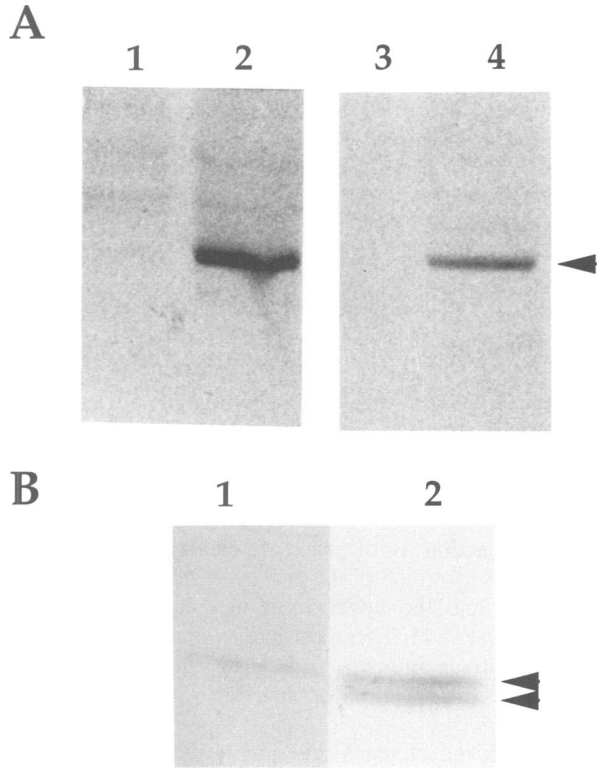

Fig. 1. (**A**) Immunoblots of soluble (lanes 1 and 2) and insoluble (lanes 3 and 4) fractions of *S.pombe* protein prepared from the human replacement strain HY1 (lanes 1 and 3) and wild type (lanes 2 and 4), and probed with a C-terminal $p34^{cdc2}$ antibody, PN24. The arrow marks the position of $p34^{cdc2}$. No doublet is observed as the extracts were electrophoresed using a mini gel system which does not resolve the two bands. (**B**) An immunoblot of a PN24 immunoprecipitate of total protein from *S.pombe* probed with the anti-phosphotyrosine antibody PY72 (lane 1) and PN24 (lane 2). The arrows mark the two bands of $p34^{cdc2}$.

Results

The PN24 antibody specifically recognizes $p34^{cdc2}$

In a number of organisms there are several proteins of 34 kDa which are similar in sequence to $p34^{cdc2}$ (Meyerson *et al.*, 1992). Although such gene products have not been reported in fission yeast, we first established that the antibody used in this study was specific for $p34^{cdc2}$. The rabbit polyclonal antibody PN24 (Simanis and Nurse, 1986), raised against the C-terminal six amino acids of $p34^{cdc2}$, was used for Western blotting of soluble and insoluble extracts from a wild-type strain (972) and a yeast strain (HY1) in which the endogenous $cdc2^+$ gene had been replaced by the human CDC2 gene (MacNeill and Nurse, 1993). Human $p34^{CDC2}$ differs completely in the C-terminal region of amino acids from *S.pombe* $p34^{cdc2}$, and as a consequence does not react with the PN24 antibody.

The $p34^{cdc2}$ protein was readily detected in both soluble and insoluble extracts from wild-type cells (Figure 1A, lanes 2 and 4), with about twice as much in the soluble as in the insoluble extract. No 34 kDa proteins were detected in either extract from the HY1 strain (Figure 1A, lanes 1 and 3). This experiment establishes that PN24 is specific for $p34^{cdc2}$ and does not recognize other 34 kDa proteins from *S.pombe*.

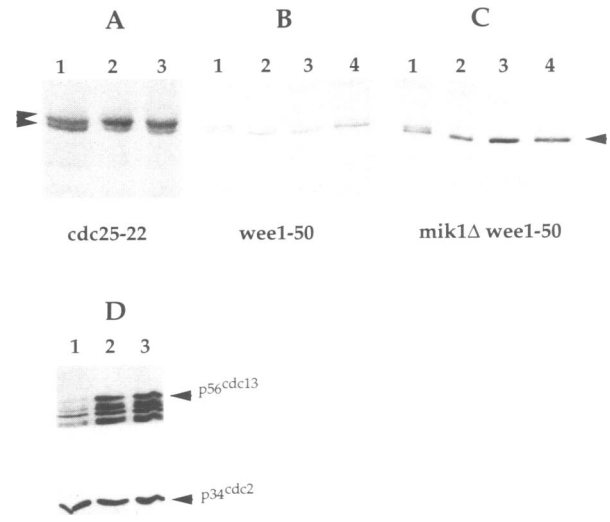

Fig. 2. Immunoblots with PN24 of soluble extracts from (**A**) *cdc25-22* which becomes blocked in G_2 at the restrictive temperature, (**B**) *wee1-50* which is advanced into mitosis with reduced Y15 phosphorylation at the restrictive temperature, (**C**) *mik1Δ wee1-50* strain which undergoes mitotic catastrophe with no Y15 phosphorylation at the restrictive temperature. Mutants were grown to early exponential phase at 25°C and then shifted to 36°C for 0 h (lane 1), 2 h (lane 2), 4 h (lane 3) and 6 h (lane 4) for *wee1-50* and *mik1Δ wee1-50*. The arrows mark $p34^{cdc2}$. (**D**) $p13^{suc1}$ precipitates from a time course of a *cdc25-22* mutant shifted to 36°C for 0 h (lane 1), 2 h (lane 2), 4 h (lane 3) were Western blotted with PN24 (lower panel) and SP4 (upper panel). Two hours after shift up to 36°C, the cells were blocked in G_2 and had accumulated high levels of the $p34^{cdc2}/p56^{cdc13}$ complex. The $p56^{cdc13}$ becomes readily degraded and the arrow marks the intact protein.

Resolution by SDS–PAGE of tyrosine-phosphorylated and dephosphorylated $p34^{cdc2}$

$p34^{cdc2}$ extracted from cells of multicellular eukaryotes has been observed to run as multiple bands (Morla *et al.*, 1989; Norbury *et al.*, 1991; Osmani *et al.*, 1994), but such isoforms have not been previously detected in extracts prepared from fission or budding yeast. We have found that running an 18 cm 12.5% SDS–polyacrylamide gel (see Materials and methods) resolves $p34^{cdc2}$ into a doublet in both the soluble and insoluble fractions. If these two fractions are mixed together or electrophoresed side by side, only two bands with the same mobility are detected (data not shown), showing that different isoforms of $p34^{cdc2}$ from fission yeast are resolved into two bands and these two bands are detected in both the soluble and the insoluble fractions. *Schizosaccharomyces pombe* $p34^{cdc2}$ is phosphorylated on Y15 and T167 (Gould and Nurse, 1989; Gould *et al.*, 1991). To identify which band contained Y15-phosphorylated $p34^{cdc2}$, we immunoprecipitated with PN24 and the subsequent immunoprecipitate was electrophoresed and Western blotted with PN24 (Figure 1B, lane 2) or with the anti-phosphotyrosine antibody PY72 (Figure 1B, lane 1). Only the upper band was detected by PY72, establishing that this form contains phosphorylated Y15.

This was confirmed using mutant strains altered in the level of Y15-phosphorylated $p34^{cdc2}$. Extracts were prepared from a temperature-sensitive *cdc25-22* mutant which arrests in G_2 with inactive $p34^{cdc2}$ phosphorylated

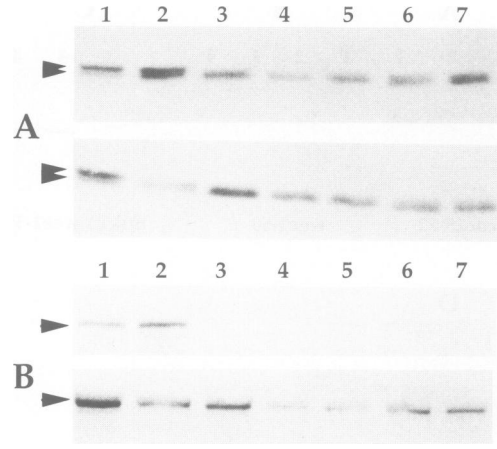

Fig. 3. Protein from the soluble (A and B, upper panels) and insoluble (A and B, lower panels) fractions was prepared from a *cdc25-22* mutant blocked at the restrictive temperature of 36°C (lane 1), then shifted down to the permissive temperature for 20 min (lane 2), 40 min (lane 3), 60 min (lane 4), 80 min (lane 5), 100 min (lane 6) and 120 min (lane 7). Protein samples were immunoblotted with PN24 (**A**) and SP4 (**B**), and the arrows show the proteins. Coomassie Blue staining of the electrophoresed protein samples showed that all lanes were equally loaded.

on Y15 (Gould and Nurse, 1989; Moreno *et al.*, 1989). Western blotting with PN24 showed that the upper band was substantially increased compared with the same strain grown at the permissive temperature of 25°C when cells proceed through the cell cycle normally (Figure 2A, compare track 1 with tracks 2 and 3). In the next experiments, extracts were prepared from a temperature-sensitive *wee1-50* strain incubated at the restrictive temperature of 36°C when cells are viable but enter mitosis at a small cell size, and a *mik1Δ wee1-50* strain which is lethal because of premature entry into mitosis (Lundgren *et al.*, 1991). $p107^{wee1}$ is a threonine/tyrosine kinase which phosphorylates $p34^{cdc2}$ on Y15 (Featherstone and Russell, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). *Wee1-50* cells at 36°C have reduced levels of Y15-phosphorylated $p34^{cdc2}$ (Lundgren *et al.*, 1991). Western blotting with PN24, of protein from a *wee1-50* strain grown at 36°C, shows that the upper band, although still present, is reduced (Figure 2B, lanes 2, 3 and 4). The *mik1*-encoded protein kinase has homology with the $p107^{wee1}$ kinase and a *mik1Δ wee1-50* strain at 36°C has no Y15-phosphorylated $p34^{cdc2}$ (Lundgren *et al.*, 1991). Western blotting of this strain after shift to 36°C shows that the upper band is undetectable (Figure 2C, lanes 2, 3 and 4) compared with cells grown at the permissive temperature (Figure 2C, lane 1). These results are all consistent with only the upper band of $p34^{cdc2}$ being phosphorylated on Y15.

Only a subpopulation of $p34^{cdc2}$ is phosphorylated on tyrosine

Having established that only the slower migrating band of $p34^{cdc2}$ contains Y15-phosphorylated $p34^{cdc2}$, we examined the level of Y15 phosphorylation as cells pass synchronously through mitosis into G_1. To do this, we used cells released from a late G_2 block imposed by holding the *cdc25-22* mutant strain at 36°C (Figure 3). As already shown (Figure 2A), at the *cdc25-22* block point there is a considerable increase in the upper form to a level where it represents ~80% of the total in both the soluble fraction (Figure 3A, lane 1, upper panel) and the insoluble fraction (Figure 3A, lanes 1, lower panel). Within 20 min of shift to 25°C, the level of the lower form is increased, especially in the soluble fraction (Figure 3A, upper panel, lane 2). By 40 min when the cells are at the end of mitosis, as determined by the loss of $p56^{cdc13}$ (Figure 3B, lane 3, upper panel), Y15-phosphorylated $p34^{cdc2}$ is barely detectable (Figure 3A, lane 3, upper and lower panels).

Cell cycle changes were examined further using a synchronous wild-type culture prepared by elutriation (Aves *et al.*, 1985). The peak of septation occurred (Figure 4E) just before the increase in cell number (data not shown). The doublet is resolved in both fractions [Figure 4A (soluble) and B (insoluble)], but is clearer in the insoluble fraction. Both forms are clearly present at about equal levels for much of the cell cycle, but the upper form is reduced and the lower form increased in amount in lanes 2–4 and 9–11. These lanes represent cells proceeding through mitosis into G_1, indicating that a reduction in tyrosine phosphorylation occurs during this stage of the cell cycle.

Although the changes are qualitatively similar in the two synchronous cultures, they differ quantitatively. In the selection synchronous culture, the upper Y15-phosphorylated form never exceeds the lower form and is usually somewhat lower in amount. In contrast, at the *cdc25-22* block point the upper form is substantially increased. To examine this point further, we monitored the levels of cyclin B, $p56^{cdc13}$, using the SP4 antibody (Moreno *et al.*, 1989) during the same synchronous cultures (Figures 3B and 4C and D). In most eukaryotic cells cyclin B levels are periodic, becoming reduced as cells exit mitosis and $p34^{cdc2}$ protein kinase activity falls. Interestingly, only a small reduction in the level of $p56^{cdc13}$ was observed in the selection synchronous culture as cells proceeded through mitosis. This drop is more clearly evident in the insoluble fraction (Figure 4D, lanes 3–4 and 10–11). The level in the soluble fraction (Figure 4C) shows some fluctuation, but the $p56^{cdc13}$ in this fraction is more unstable after cell breakage and degradation obscures the changes in level. In contrast, a dramatic drop was observed in the *cdc25-22* block and release synchonized culture, particularly with the soluble fraction (see tracks 2–3, Figure 3B, upper panel).

Evidence from other organisms shows that Y15 phosphorylation of $p34^{cdc2}$ occurs when $p34^{cdc2}$ is in a complex with cyclin B (Solomon *et al.*, 1990; Meijer *et al.*, 1991). This suggests that in a *cdc25-22* mutant at 36°C, when the majority of $p34^{cdc2}$ is in the Y15-phosphorylated form (Figure 2A, lane 1 compared with lanes 2 and 3), a higher level of the $p34^{cdc2}/p56^{cdc13}$ complex has accumulated. In *Xenopus*, binding to $p34^{cdc2}$ has been shown to be necessary for degradation of cyclin B2 (Stewart *et al.*, 1994; van der Velden and Lohka, 1994). The differences we observe in the periodicity of cyclin levels between the two synchronization methods may be due to the amount of cyclin complexed with $p34^{cdc2}$ in these different situations. At the *cdc25-22* block in *S.pombe*, more $p34^{cdc2}/p56^{cdc13}$ complex may

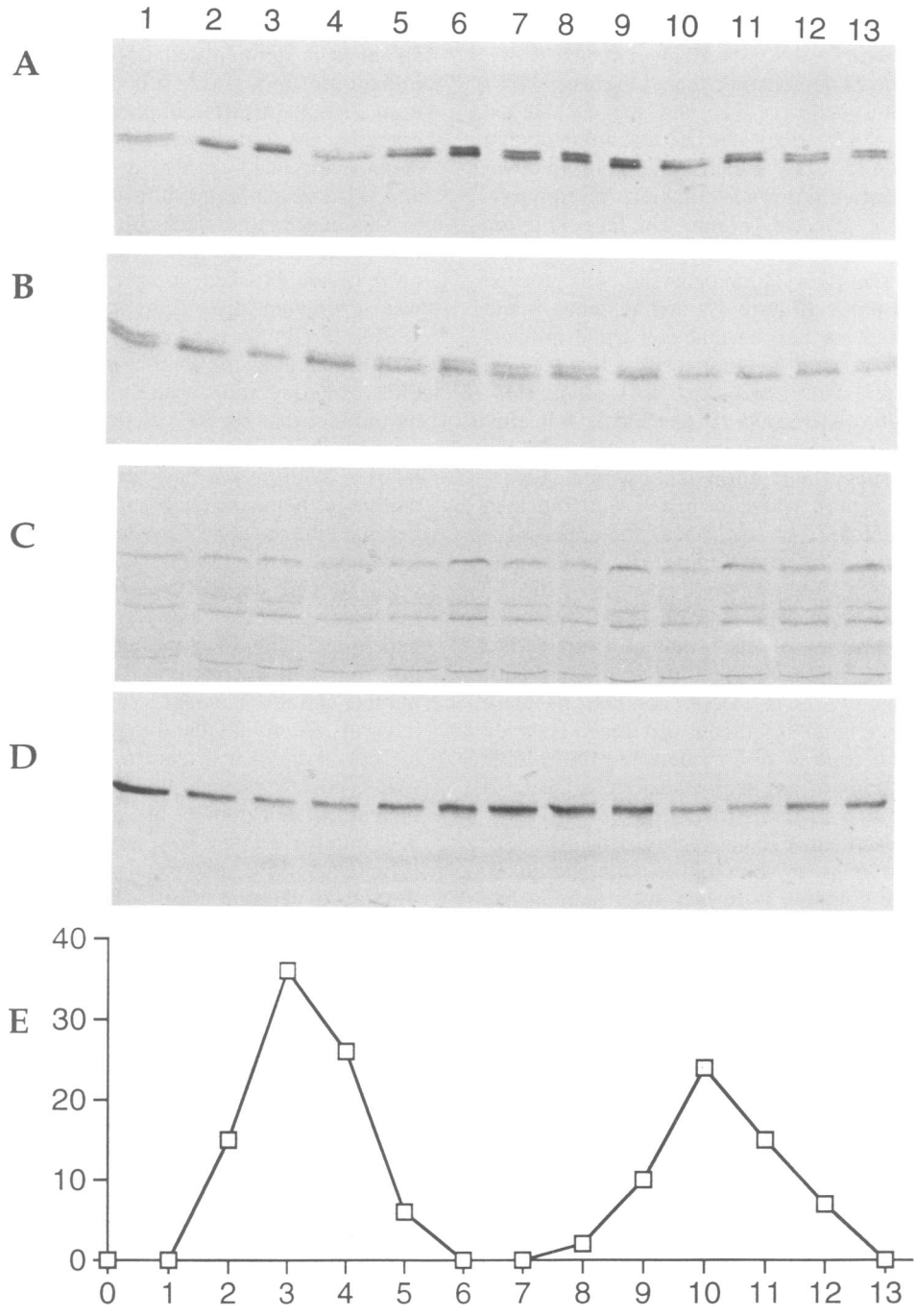

Fig. 4. A synchronous culture was prepared using an elutriator rotor and protein samples were prepared every 20 min from time 0 min (lane 1) to 280 min (lane 13) from the soluble (**A** and **C**) and the insoluble (**B** and **D**) fractions. The peaks of septation are shown in (**E**). The protein was immunoblotted with PN24 (A and B) and SP4
(C and D) as shown by the arrows.

accumulate and it is this cyclin B that is degraded at the end of mitosis. It is possible that the increased amount of p34^{cdc2} in the soluble fraction after release from the block (Figure 3A, lane 2, upper panel) and the greater level of cyclin B degradation in this fraction (Figure 3B, upper panel) may reflect a translocation of the complex before cyclin degradation into the soluble fraction. To test whether there is an increase in the level of the p34^{cdc2}/p56^{cdc13} complex, we used p13^{suc1} beads (Brizuela *et al.*, 1987) to precipitate the complex from *cdc25-22* cells as they became blocked at the G_2/M transition after shift to 36°C. The precipitates were Western blotted with PN24 and SP4 to monitor the level of the complex. We found that by 2 h after the shift to the restrictive temperature cells were blocked at the G_2/M border and there was an increase in the level of p56^{cdc13} complexed with p34^{cdc2} compared with exponential growing cells (Figure 2D, lane 1 compared with lanes 2 and 3). The higher level of complex detected in *cdc25-22* arrested cells may reflect an accumulation of the p34^{cdc2}/p56^{cdc13} complex, supporting the proposal that only the p56^{cdc13} complexed with p34^{cdc2} is degraded at mitosis.

p34^{cdc2} is not rephosphorylated on Y15 until cells have completed start

The above experiments demonstrate that at mitosis p34^{cdc2} becomes dephosphorylated on Y15 and suggest that on exit from mitosis p34^{cdc2} does not become immediately phosphorylated on Y15. Sixty minutes after shifting down *cdc25-22* to 25°C, when mitosis is complete, phosphorylation on Y15 is not detected (Figure 3A, lane 4, upper and lower panels). The selection synchronous culture indicates that Y15 phosphorylation occurs as cells proceed through G_1 into S phase (Figure 4A and B, lanes 4 and 11). To examine this further, several cell cycle mutants were used to block cells at G_1 prior to start (*cdc10-129*) and in late G_1 after start (*cdc20-M10* and *cdc22-M45*) (Nurse *et al.*, 1976). At the *cdc10-129* block 4 h after shifting to the restrictive temperature of 36°C, only the lower Y15 dephosphorylated form is observed (Figure 5B, lane 9, lower panel), whilst in a post-start block in G_1 imposed by *cdc20-M10* or *cdc22-M45* the upper Y15-phosphorylated form is present (Figure 5B, lanes 3 and 6, lower panel). The Y15-phosphorylated form of p34^{cdc2} appears as cells pass start. The fluorescent-activated cell scanning (FACS) profile for each mutant (Figure 5A) shows that all three mutants arrest by 4 h after shift up with predominantly a 1C DNA content. In these experiments, there was no significant difference between the phosphorylation state of p34^{cdc2} between the soluble and insoluble fractions or any major change in the level of p34^{cdc2} in these two fractions (data not shown).

As previously described, Y15 phosphorylation of p34^{cdc2} only occurs when it is in a complex with cyclin B, suggesting that the complex is formed after start in late G_1. We investigated whether the complex was present in the *cdc10-129*, *cdc20-M10* and *cdc22-M45* mutants by precipitating p34^{cdc2} with p13^{suc1} beads, and Western blotting with PN24 and SP4. In exponential growth and at time points as the cells from the asynchronous cultures gradually undergo cell cycle arrest, the complex is detectable (Figure 5C, lanes 1–3, 5–7 and 9–11). In a *cdc10-129* mutant, 4 h after shift up, only a very low level of the complex can be detected (Hayles *et al.*, 1994, and Figure 5C, lane 12), whereas in extracts from *cdc20-M10* and *cdc22-M45* the complex is easily detected after 4 h at 36°C when the majority of the cells are still in G_1 (Figure 5C, lanes 4 and 8). This result indicates that the p34^{cdc2}/p56^{cdc13} complex is not present in pre-start cells.

The lack of p34^{cdc2}/p56^{cdc13} complex is due in part at least to the low level of p56^{cdc13} found in G_1. No p56^{cdc13} was detected in total protein extracts from *cdc10-129* cells arrested in G_1 after 4 h at 36°C (Figure 5B, lane 9, upper panel), whereas it is easily detected in the *cdc20-M10* and *cdc22-M45* cells after the same time at 36°C (Figure 5B, lanes 3 and 6, upper panel).

A pre-start G_1 checkpoint does not affect the level of Y15 p34^{cdc2} phosphorylation

A checkpoint control has been identified that prevents entry into mitosis when DNA replication is blocked (Dasso and Newport, 1990; Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Weinert and Hartwell, 1993). This checkpoint modulates the level of tyrosine phosphorylation of p34^{cdc2} (Dasso and Newport, 1990; Enoch *et al.*, 1991) and in *S.pombe* several genes have been identified that are involved in the transduction of the signal from unreplicated DNA to p34^{cdc2} preventing the initiation of mitosis (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992). A different checkpoint mechanism must be operative pre-start since, as shown above, p34^{cdc2} is already dephosphorylated on Y15 at this stage of the cell cycle and yet does not enter mitosis.

This conclusion is difficult to reconcile with the report that when cells with p34^{cdc2} completely dephosphorylated on Y15 are blocked at start using a *cdc10-129 mik1Δ wee1-50* mutant, they can enter mitosis (Lundgren *et al.*, 1991). If p34^{cdc2} is already dephosphorylated on Y15, then eliminating the kinases responsible should have no effect. Interpretation of these experiments is complicated by the fact that *cdc10^{ts}* cells can eventually leak past the start block point (our unpublished observations and see later). Therefore we have examined the *mik1Δ wee1-50* mutant further using the *cdc10-V50 mik1Δ wee1-50* mutant because the *cdc10-V50* allele blocks more effectively (Reymond *et al.*, 1992). Wild-type, *cdc10-V50*, *mik1Δ wee1-50* and *cdc10-V50 mik1Δ wee1-50* strains were nitrogen starved to arrest them in G_1 (see Figure 7A, panels 1, 2, 7 and 8, time 0 h), and were then inoculated into minimal medium at 36°C. Septation index, cell number and FACS analysis samples were taken at 1 hourly intervals. We found that the *mik1Δ wee1-50* mutant started to septate at ~4 h (Figure 6A, left-hand panel) and that wild-type cells septate at ~5 h. Both strains showed an increase in cell number at ~5 h, although the increase in cell number in the *mik1Δ wee1-50* strain was slower (Figure 6A, right-hand panel) because the cells were going through an aberrant mitosis (Figure 7B, panel 1) and were slow to undergo cell separation. These cells show a variety of mitotic abnormalities, including a cut phenotype (Hirano *et al.*, 1986) when the nucleus is severed by the septum or a single nucleus on one side of the septum. No septation was seen in the *cdc10-V50 mik1Δ wee1-50* strain until 7–8 h and no cell number increase occurred during the course of the experiment (Figure 6A), showing that the *mik1Δ wee1-50* strain arrested pre-start does not enter mitosis. The appearance of septa in the *cdc10-V50 mik1Δ wee1-50* strain at the end of the experiment corresponded to the time at which the *cdc10-V50* strain started to leak through the cell cycle block (see Figure 7A, panel 8, time 7 h). The *cdc10-V50* and the *cdc10-V50 mik1Δ wee1-50* strains became elongated (Figure 7B, panels 2 and 8) compared with the *mik1Δ wee1-50* and wild-type strains (Figure 7B, panels 1 and 7), indicating that these cells are blocked in cell cycle progression and were not going into mitosis. From these data, we conclude that, contrary to earlier reports, the *mik1Δ wee1-50* strain cannot enter a premature mitosis from the pre-start interval in G_1.

We have examined two other checkpoint mutants, *chk1Δ (rad27)* (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994) and *hus1-14* (Enoch *et al.*, 1992), to see if these are defective in the pre- or post-start checkpoint. The *hus1-14* mutant enters mitosis when DNA replication is blocked with hydroxyurea and is also radiation sensitive; *chk1Δ(rad27)* arrests normally in hydroxyurea, but enters mitosis when DNA is damaged. The mutants were crossed into a *cdc10-V50* strain and then treated as for the *mik1Δ wee1-50* strains. The single mutants undergo S phase and cell division slightly later than the wild-type strain (Figure

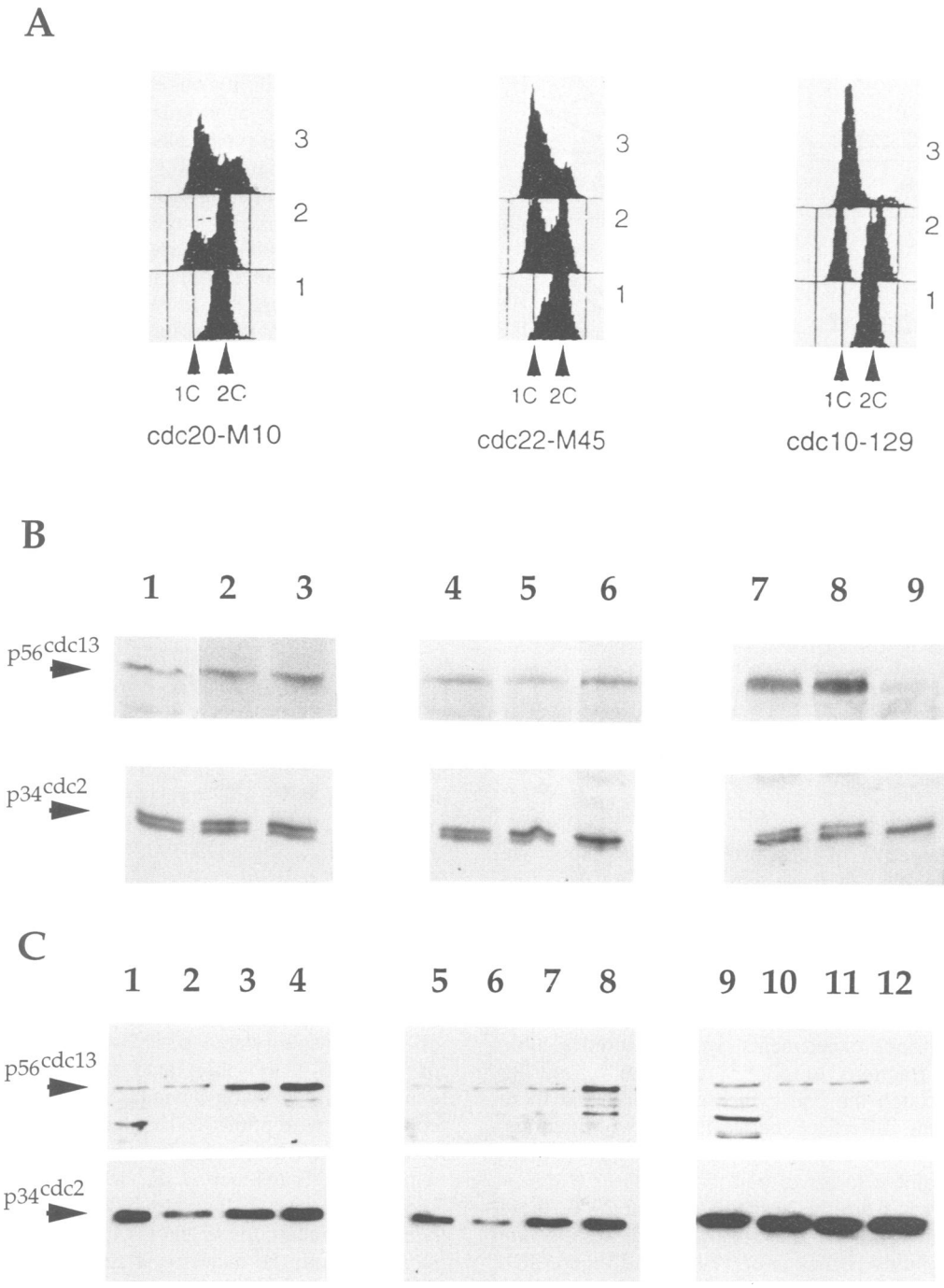

Fig. 5. (**A**) FACS samples of *cdc20-M10*, *cdc22-M45* and *cdc10-129* mutants were taken after shifting to 36°C for 0 h (1), 2 h (2) and 4 h (3). By 4 h, the majority of the cells had arrested in G_1, although there was some leak through in the *cdc20-M10* and *cdc22-M45* mutants. (**B**) Protein from the soluble fraction of *cdc20-M10* (lanes 1–3), *cdc22-M45* (lanes 4–6) and total protein from *cdc10-129* (lanes 7–9) was prepared from exponentially growing cells (lanes 1, 4 and 7) and after shift up at 2 h (lanes 2, 5 and 8) and 4 h (lanes 3, 6 and 9). The protein was immunoblotted with SP4 (upper panel) and PN24 (lower panel). The arrows show the protein. The Western blot using SP4 (lanes 7–9) was developed for longer to show that only very low levels of $p56^{cdc13}$ are detected after 4 h at 36°C. (**C**) Precipitates of the $p34^{cdc2}/p56^{cdc13}$ complex were carried out using $p13^{suc1}$ beads from *cdc20-M10* (lanes 1–4), *cdc22-M45* (lanes 5–8) and *cdc10-129* (lanes 9–12). In precipitates from exponentially growing cells from each strain (lanes 1, 5 and 9) the complex can be seen. After 2 h at 36°C (lanes 2, 6 and 10) and 3 h at 36°C (lanes 3, 7 and 11), the complex is still present but is reduced in level in the *cdc10-129* strain, and by 4 h (lanes 4, 8 and 12) the complex is barely detected in the *cdc10-129* strain but is still clearly detected in *cdc20-M10* and *cdc22-M45*. The arrows show the proteins.

6B and C, and Figure 7, panels 3, 5 and 7, time 4 h). However, *cdc10-V50 chk1Δ*, *cdc10-V50 hus1-14* and *cdc10-V50* became arrested at the *cdc10* block point (Figure 7A, panels 4, 6 and 8) and started to elongate (Figure 7B, panels 4, 6 and 8). There was no evidence of entry into mitosis until 7 h when the *cdc10-V50* mutant had just started to leak through the block, as indicated by a small fraction of cells with a greater than 1C DNA content (Figure 7A, panel 8, 7 h). These double mutants then start to septate (Figure 6B and C, left-hand panels)

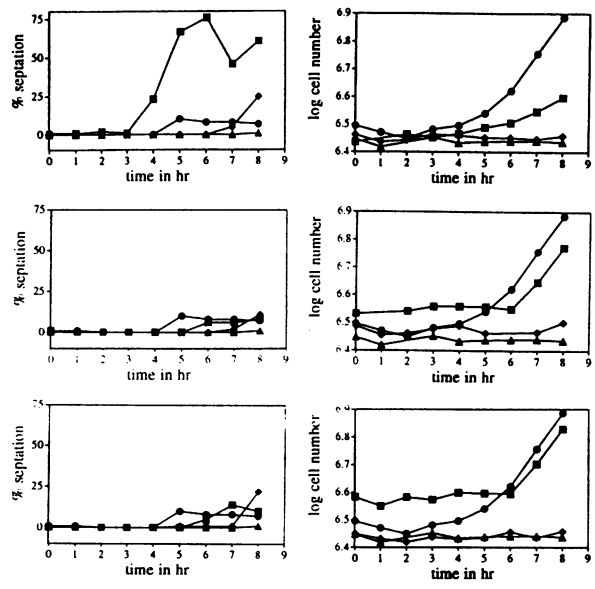

Fig. 6. Per cent septation (left-hand panel) and $\log_{10}$ cell number increase (right-hand panel) for (**A**) *mik1Δ wee1-50* (closed squares), *cdc10-V50 mik1Δ wee1-50* (closed diamonds), wild type (972) (closed circles) and *cdc10-V50* (closed triangles), (**B**) *chk1Δ(rad27)* (closed squares), *cdc10-V50 chk1Δ (rad27)* (closed diamonds), wild type (972) (closed circles) and *cdc10-V50* (closed triangles) and (**C**) *hus1-14* (closed squares), *cdc10-V50 hus1-14* (closed diamonds) wild type (972) (closed circles) and *cdc10-V50* (closed triangles).

as they are defective in the post-start checkpoints. We conclude that neither of these two mutants are defective in a pre-start G_1 checkpoint since they do not enter mitosis when arrested at the *cdc10-V50* block point.

During re-replication the predominant form of p34^{cdc2} is found in an insoluble cellular fraction

During the previous experiments, we monitored soluble and insoluble fractions of p34^{cdc2} and p56^{cdc13} during progression through the cell cycle, and found that there is no significant difference between the two fractions. During heat treatment of the temperature-sensitive mutant *cdc2-L7*, the soluble forms of p34^{cdc2} and p56^{cdc13} disappear. On recovery from the heat treatment at 29°C, these cells reset from G_2 to G_1 and undergo S phase, causing an increase in ploidy [Broek *et al.* (1991) and Figure 8C]. This resetting from G_2 to G_1 is associated with loss of the p34^{cdc2}/p56^{cdc13} mitotic complex (Hayles *et al.*, 1994). We repeated this experiment and looked to see what happened to the insoluble fractions of p34^{cdc2} and p56^{cdc13} upon recovery at 29°C. We found that both proteins remained detectable throughout the recovery time course (Figure 8A and B, lower panels). Around the time of S phase, there is a reduction in the level of p56^{cdc13} from the insoluble fraction (Figure 8A, lower panel, lane 3), consistent with the low level of p56^{cdc13} observed in late G_1 (Hayles *et al.*, 1994). The soluble form of p34^{cdc2} only began to appear around the time of S phase (Figure 8B, upper panel, lane 4) and the soluble form of p56^{cdc13} was still not detectable at this time (Figure 8A, upper panel). This result supports the earlier data showing that the resetting from G_2 to G_1 is correlated with disappearance of the soluble fraction of p34^{cdc2} (Broek *et al.*, 1991) and suggests that it is the insoluble fraction of p34^{cdc2} which persists that may be responsible for the G_1 function of p34^{cdc2}. This possibility was examined further in a situation where cells have a protracted G_1 phase. Germinating spores from wild-type cells undergo S phase ~5–8 h (Nurse and Thuriaux, 1977) after addition of medium at 32°C. In the germinating spores, we found that the soluble fraction of p34^{cdc2} only begins to appear around the time of S phase (Figure 8D, upper panel, lane 4), whereas the insoluble form is present from the begining of the experiment (Figure 8D, lower panel). These data support the suggestion that the insoluble fraction may be functional for the start activity of p34^{cdc2}.

Discussion

Tyrosine phosphorylation of p34^{cdc2} in *S.pombe* inhibits activation of the p34^{cdc2}/p56^{cdc13} complex (Gould and Nurse, 1989). In higher eukaryotes, threonine 14 is also phosphorylated and this, in conjunction with Y15 phosphorylation, prevents activation of the mitotic complex (Krek and Nigg, 1991; Norbury *et al.*, 1991). The Y15 inhibitory phosphorylation of p34^{cdc2} does not take place until the p34^{cdc2}/cyclin B complex has formed (Solomon *et al.*, 1990; Meijer *et al.*, 1991). Tyrosine phosphorylation of p34^{cdc2} in *S.pombe* has previously been demonstrated using anti-phosphotyrosine antibodies or ^{32}P labelling (Gould and Nurse, 1989). These methods have given no indication of the total amount of p34^{cdc2} that is phosphorylated on Y15 compared with Y15 unphosphorylated levels. Using SDS–PAGE to resolve p34^{cdc2} as a doublet, we have been able to estimate the level of Y15-phosphorylated and dephosphorylated p34^{cdc2} at different stages of the cell cycle. As cells pass synchronously through the cell cycle, the level of Y15-phosphorylated p34^{cdc2} oscillates, becoming reduced during mitosis and G_1, and increased as cells proceed through G_2. The level of Y15-phosphorylated p34^{cdc2} never exceeds the level of the Y15-dephosphorylated form, suggesting that in exponentially growing *S.pombe* cells not all the p34^{cdc2} is complexed with p56^{cdc13}. This also appears to be the case in sea urchin, where only a fraction of p34^{cdc2} is complexed with cyclin B (Meijer *et al.*, 1991). However, we have shown that in a cell cycle block imposed by using a *cdc25-22* mutant, the level of Y15-phosphorylated p34^{cdc2} increases until the majority of p34^{cdc2} is in this form and this increase is correlated with an increase in the level of p34^{cdc2}/p56^{cdc13} complex.

p56^{cdc13} levels oscillate during the cell cycle in a selection synchronous culture, particularly in the insoluble fraction, but there was no evidence that either the soluble or insoluble fractions were completely degraded at mitosis. In contrast, in extracts prepared from blocking and releasing a *cdc25-22* strain all the soluble p56^{cdc13} is degraded at the end of mitosis and the insoluble fraction is reduced in level (Figure 3B, bottom panel). Given that the level of the p34^{cdc2}/p56^{cdc13} complex is increased, it is likely that it is the p56^{cdc13} in the complex which is degraded at mitosis. Cells containing a greater proportion of p56^{cdc13} in the mitotic complex will show a greater p56^{cdc13} degradation after mitosis. If this view is correct, then we would expect that only some of p56^{cdc13} is complexed with p34^{cdc2} in a pre-activatable MPF complex in normal

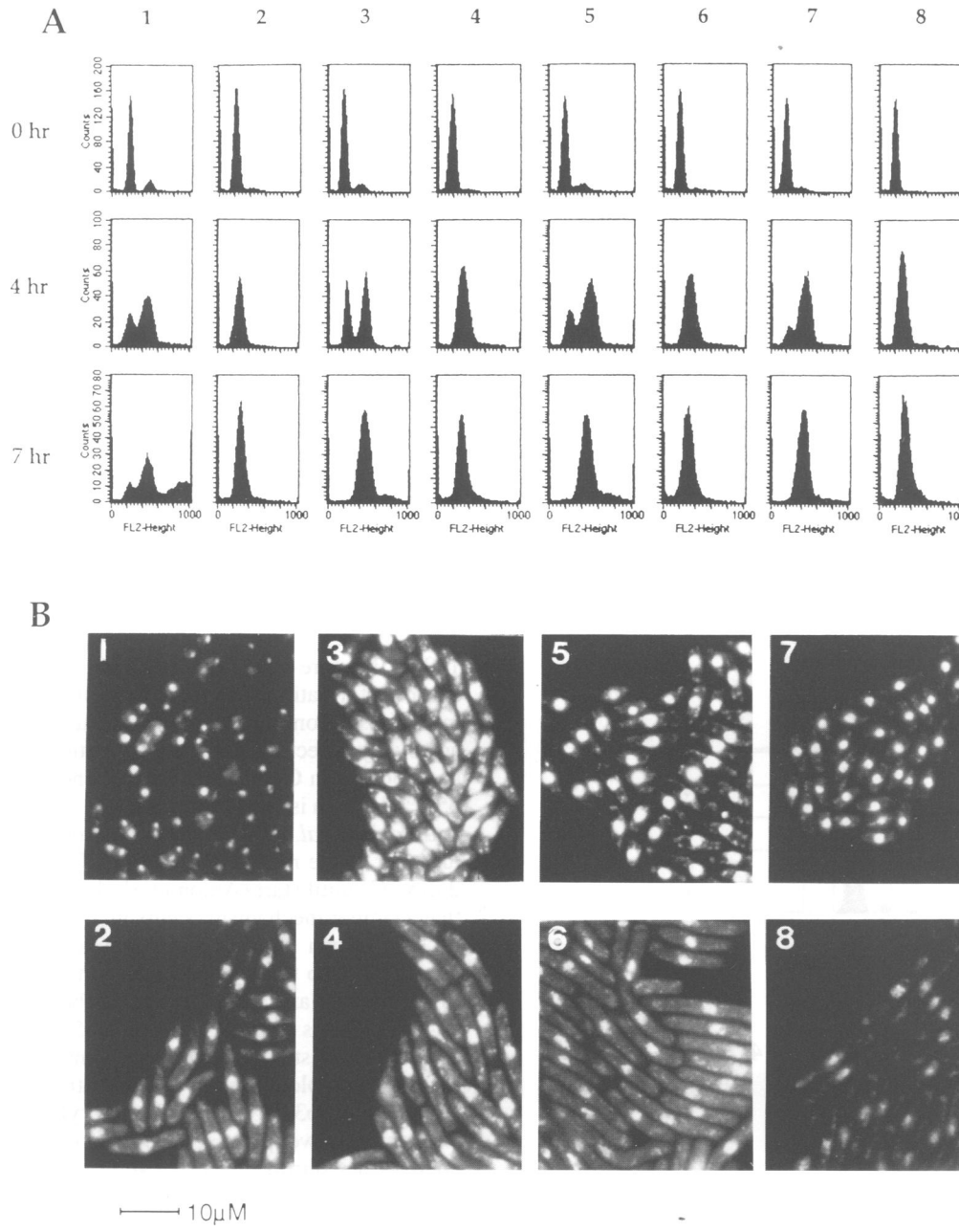

Fig. 7. (**A**) FACS analysis of *mik1Δ wee1-50* (panel 1), *cdc10-V50 mik1Δ wee1-50* (panel 2), *chk1Δ (rad27)* (panel 3), *cdc10-V50 chk1Δ (rad27)* (panel 4), *hus1-14* (panel 5), *cdc10-V50 hus1-14* (panel 6), wild type (panel 7) and *cdc10-V50* (panel 8). Data are shown after addition of nitrogen after 0 h at 36°C (top row), after 4 h at 36°C when the mutants in a *cdc10*$^{+}$ background and wild type are proceeding through S phase (middle row), and after 7 h at 36°C (bottom row) when the *chk1Δ (rad27)*, *hus1-14* mutants and wild type have a 2C DNA content, *mik1Δ wee1-50* has cut and cells have from a <1C to 4C DNA content. The mutants in a *cdc10-V50* background and *cdc10-V50* have a predominantly 1C DNA content, but a small shoulder of S phase cells appears in the *cdc10-V50* mutant at 7 h, indicating a leak through the block point. The 1C peak is at 250 on the *x*-axis and the 2C peak at 500. There is some drift of the peaks when the cells are elongating (panels 2, 4, 6 and 8) (Sazer and Sherwood, 1990). (**B**) Cells after 7 h at 36°C: (1) *mik1Δ wee1-50*, (2) *cdc10-V50 mik1Δ wee1-50*, (3) *chk1Δ (rad27)*, (4) *cdc10-V50 chk1Δ (rad27)*, (5) *hus1-14*, (6) *cdc10-V50 hus1-14*, (7) wild type, (8) *cdc10-V50*. By 7 h all the mutants in a *cdc10*$^{+}$ background and wild type had started to undergo cell division, whereas the mutants in a *cdc10-V50* background and *cdc10-V50* showed no increase in cell number and were elongating.

exponentially growing cells. This suggests that the rate-limiting step for the formation of the complex in these cells is not simply the levels of p34^{cdc2} or cyclin B, and that the formation of the complex is regulated by some as yet unidentified process.

What happens to Y15 phosphorylation of p34^{cdc2} after cells exit from mitosis and when does the p34^{cdc2}/p56^{cdc13} complex reform? We cannot detect any p34^{cdc2} Y15 phosphorylation or p34^{cdc2}/p56^{cdc13} complex in cells arrested at the *cdc10-129* block point in G_1 at start, whilst Y15-phosphorylated p34^{cdc2} and the p34^{cdc2}/p56^{cdc13} complex are present in cells blocked after start using the *cdc20-M10* or *cdc22-M45* mutants. It has previously been shown that if a complex between p34^{cdc2}/p56^{cdc13} is made in cells blocked in G_1 at start, then the cells can enter mitosis without undergoing DNA replication (Hayles *et al.*,

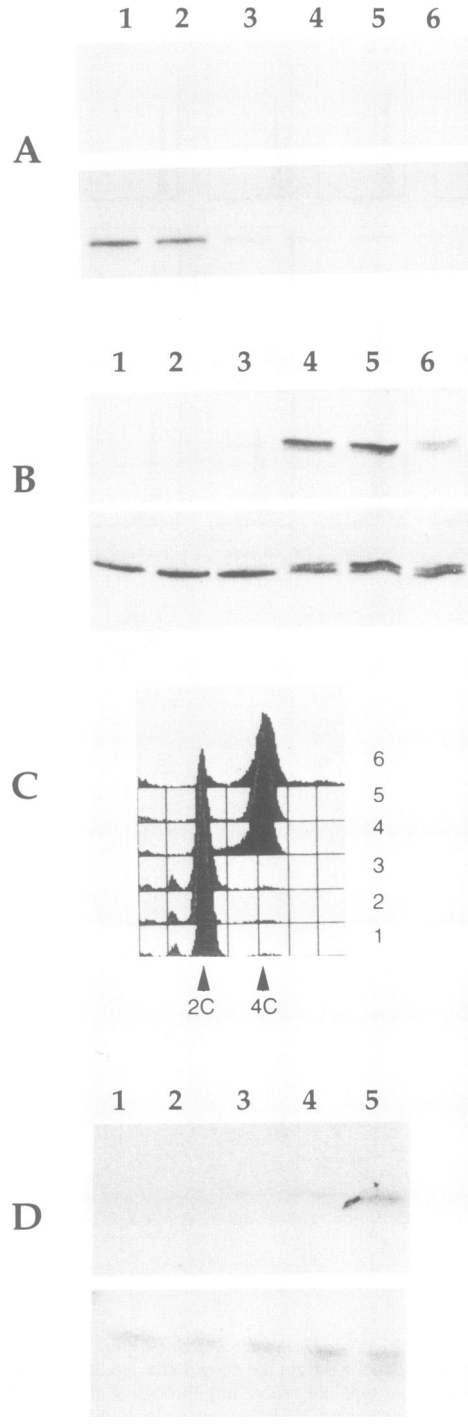

Fig. 8. A *cdc2-L7* strain was heat treated and then allowed to re-replicate its DNA at 29°C. Protein samples from the soluble (A and B, upper panels) and the insoluble (A and B, lower panels) fractions were prepared at 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 3 h (lane 4), 4 h (lane 5) and 5 h (lane 6) after shifting to the permissive temperature of 29°C. The protein was immunoblotted with SP4 (**A**) and PN24 (**B**). FACS samples were taken at the same time points (**C**). Re-replication occurs between 2 and 3 h after shift down. (**D**) Soluble (upper panel) and insoluble protein (lower panel) extracts from germinating wild-type spores, lane 1 at 2 h, lane 2 at 4 h, lane 3 at 6 h, lane 4 at 8 h and lane 5 at 10 h, after inoculating into minimal medium at 32°C. S phase occurred between 6 and 8 h.

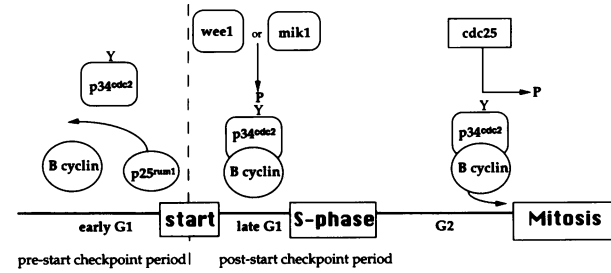

Fig. 9. Model of pre- and post-start checkpoint controls in fission yeast cell cycle. In G_1, no $p34^{cdc2}/p56^{cdc13}$ complex is present and only a low level of $p56^{cdc13}$ is present. The inhibition of complex formation may involve the activity of the *rum1* gene product, $p25^{rum1}$. As cells pass start, the level of $p56^{cdc13}$ increases and $p34^{cdc2}/p56^{cdc13}$ complex forms and is phosphorylated on Y15 by $p107^{wee1}$ and the *mik1* gene product, thus preventing premature entry into mitosis. When cells have completed S phase and reach the cell size requirement for mitosis, $p80^{cdc25}$ dephosphorylates Y15, the complex is activated and cells enter mitosis. Checkpoints preventing entry into mitosis act pre-start by inactivation of the $p34^{cdc2}$/cyclin B complex, perhaps by disruption of the complex or turnover of cyclin B, and post-start through the Y15 state of $p34^{cdc2}$.

1994). The pre-start G_1 checkpoint may act to prevent complex formation. The *rum1* gene product prevents entry into mitosis from G_1 (Moreno and Nurse, 1994) and could act by by affecting complex formation between $p34^{cdc2}$ and $p56^{cdc13}$ in G_1, perhaps by influencing the turnover of $p56^{cdc13}$ which is found only at a very low level [this paper and Hayles *et al.* (1994)]. In *Saccharomyces cerevisiae*, the turnover of the mitotic B cyclin, CLB2, protein continues during G_1 until start (Amon *et al.*, 1994) and it is possible that a similar mechanism occurs in *S.pombe*. After passage of start, when cells become committed not only to S phase, but also to the subsequent mitosis, $p56^{cdc13}$ begins to accumulate and a complex forms between $p56^{cdc13}$ and $p34^{cdc2}$. This is kept inactive by Y15 phosphorylation of $p34^{cdc2}$. After start, a new checkpoint is activated which monitors completion of DNA replication and acts through the state of $p34^{cdc2}$ Y15 phosphorylation. From these experiments, we propose that two different checkpoint controls act during the cell cycle, one pre-start and the other post-start, which act sequentially to prevent mitosis until S phase is completed (see Figure 9). Cells that are defective in the pre-start checkpoint, such as *rum1Δ cdc10-129*, enter mitosis when blocked pre-start, but if these cells leak past start they block in S phase and do not enter mitosis because the post-start checkpoint is still functional (Moreno and Nurse, 1994).

We have shown that *mik1Δ wee1-50* is not defective in the pre-start G_1 checkpoint. When arrested at the *cdc10* block point in G_1, the cells elongate and do not enter mitosis. As the cells leak through the block point, then the mutant can enter premature mitosis as the cells are defective in the post-start checkpoint. The signal affecting Y15 phosphorylation and preventing entry into mitosis in these post-start cells may be generated by the replication complex and involve the *cdc18*, *cut5/rad4* gene and *cdt1* products (Kelly *et al.*, 1993; Saka and Yanagida, 1993; Hofmann and Beach, 1994).

The checkpoint monitoring DNA damage is thought to act through a different pathway, but to have overlapping functions with the checkpoint monitoring completion of

DNA replication (Sheldrick and Carr, 1993). The radiation checkpoint is thought not to act through Y15 phosphorylation of p34cdc2/p56cdc13, but to inactivate the complex some other way. It may be that the mechanism used for this checkpoint is the same as that used for the pre-start checkpoint, and in this case we might expect mutants to be identified that are defective both pre- and post-start. However, we do not believe that mutants of *chk1Δ (rad27)* and *hus1-14* are defective pre-start. The *chk1Δ (rad27)* mutant has been postulated to be defective in both pre- and post-start checkpoints, but not in the monitoring of completion of DNA synthesis (Sheldrick and Carr, 1993). We have re-examined this mutant and find that under the conditions that we use, it becomes blocked at the *cdc10-V50* arrest point pre-start, continues to elongate and does not enter mitosis until cells leak through the *cdc10* block point.

The checkpoint control restraining mitosis from early G_1 may also function in higher eukaryotes. In mammalian cells, p34CDC2 is thought to be dephosphorylated on tyrosine during G_1 (Morla *et al.*, 1989), and yet they do not enter mitosis from G_1. It is possible that in mammalian cells the p34CDC2 and cyclin B complex formation is inhibited in G_1 to prevent inappropriate entry into mitosis by an analogous mechanism to that operative in *S.pombe*.

p34cdc2 and p56cdc13 are found in an insoluble as well as a soluble cell fraction (Hayles *et al.*, 1994). The nature of the insoluble fraction is not known, but may be derived from the cytoskeleton (Wittenberg *et al.*, 1987) or the nuclear matrix (Gallagher *et al.*, 1993). We have shown that the insoluble fraction may be associated with G_1 progression. The soluble form of p34cdc2 disappears after nitrogen starvation and heat treatment (see Materials and methods) of the *cdc2-L7* and *cdc13-117* mutants, whereas the insoluble form is still present (Hayles *et al.*, 1994). Under these conditions, cells re-replicate their DNA. We have extended the study of the insoluble fraction of p34cdc2 and p56cdc13 during re-replication in a *cdc2-L7* mutant. The insoluble fraction remains present throughout the course of re-replication at the permissive temperature, whereas the soluble fraction only begins to appear around the time of S phase. In germinating spores, we found that again the soluble form is barely detectable until around the time of S phase, whereas the insoluble form is present throughout G_1 in the germinating wild-type spores. Although we cannot rule out that a low level of p34cdc2 soluble fraction is sufficient to bring about DNA replication, we find that the predominant form found when cells undergo S phase in germinating wild-type spores, and after heat treatment of the *cdc2-L7* mutant, is in the insoluble fraction.

The insoluble fraction may be derived from a cell compartment in which p34cdc2 and p56cdc13 are associated with other proteins which may stabilize them, thus making them less temperature sensitive and less prone to denaturation and degradation. This may be one of the reasons that *cdc2ts* mutants are less easy to block in G_1 at start than in G_2 (MacNeill *et al.*, 1991). The insolubility of this form of p34cdc2 means that it is not readily available for biochemical analysis and has probably been excluded from previous studies. p34CDC28 from *S.cerevisiae* (Wittenberg *et al.*, 1987) and a PSTAIRE reactive 34 kDa protein from mammalian cells have also been found in an insoluble form (Bailly *et al.*, 1989), suggesting that this insoluble form of p34cdc2 and possibly other cyclin-dependent kinases (CDKs) may be found in other organisms. Given that a primary role for these CDKs in late G_1 is preparation of the cells for S phase (Cardoso *et al.*, 1993), the insoluble forms may be derived from the nuclear matrix where they would be closely associated with DNA replication.

Materials and methods

Yeast strains and media

All strains used were all derived from the wild-type strains 972h$^-$ and 975h$^+$ (Hayles and Nurse, 1992). All media were prepared as previously described (Moreno *et al.*, 1991).

Yeast methods

FACS and cell number counting are described in Moreno *et al.* (1991). Septa were counted using Calcafluor (Mitchison and Nurse, 1985), DNA was stained using DAPI (4′,6, diamidino-2-phenylindole). Temperature shift experiments were all carried out as previously described in Moreno and Klar (1991). The synchronous culture was prepared using an elutriator rotor (Aves *et al.*, 1985) and spore germination as described in Moreno *et al.* (1989). The heat treatment experiments were performed according to Broek *et al.* (1989). An exponentially growing culture of *cdc2-L7* was shifted to 36°C in medium lacking a nitrogen source for 4.5 h, heat treated for 45 min at 49°C, then shifted down to 29°C in yeast extract medium. Samples were taken at 1 h intervals during recovery.

Protein methods

The soluble and insoluble fractions were prepared as previously described (Hayles *et al.*, 1994). SDS–PAGE using a 12.5% 18 cm gel, prepared from a 30:0.8 acrylamide:bis acrylamide solution using >99% pure SDS from Sigma (L-6026) or Gibco BRL (5525UB), was carried out as described in Laemmli (1970). The gel was electrophoresed at 30 mA/gel with cooling until the 14 kDa marker had run off the bottom of the gel. Western blotting was carried out using Immobilon P (Millipore) according to the manufacturer's instructions. The blots were probed with either anti-cdc2 antibody PN24 (Simanis and Nurse, 1986) or anti-cdc13 antibody SP4 (Moreno *et al.*, 1989) and the protein visualized using alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma) or ECL (Amersham) for p13suc1 precipitates. We have found that the doublet is not always easily resolved and may sometimes run as a broad band. A procedure using Sigma SDS has been used to resolve the difference between α and β tubulins using one-dimensional PAGE (Best *et al.*, 1981). We have tried >95% pure Sigma SDS (L5750) and find that the doublet is more easily resolved, but that the bands are rather smeared.

Immunoprecipitations were prepared as described in Gould and Nurse (1989) and p13suc1 precipitates were carried out as described in Hayles *et al.* (1994). These were electrophoresed and Western blotted, then probed with either PN24, SP4 or anti-phosphotyrosine antibody PY72 (a gift from T.Hunter and R.Schulte).

Acknowledgements

We would like to thank all our colleagues in the Cell Cycle Laboratory at ICRF for very useful discussions and help in the preparation of this paper, especially Bodo Stern and Matthew O'Connell. We also thank Nancy Walworth for the *chk1Δ* strain.

References

Al-Khodairy,F. and Carr,A.M. (1992) DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. *EMBO J.*, **11**, 1343–1350.

Al-Khodairy,F., Fotou,E., Sheldrick,K.S., Griffiths,D.J.F., Lehman,A.R. and Carr,A.M. (1994) Identification and characterisation of new elements involved in checkpoint and feedback controls in fission yeast. *Mol. Biol. Cell*, **5**, 147–160.

Amon,A., Irniger,S. and Nasmyth,K. (1994) Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell*, **77**, 1037–1050.

Aves,S., Durkacz,B., Carr,T. and Nurse,P. (1985) Cloning, sequencing

and transcriptional control of the *Schizosaccharomyces pombe* cdc10 'start' gene. *EMBO J.*, **4**, 457–463.

Bailly,E., Doree,M., Nurse,P. and Bornens,M. (1989) p34^{cdc2} is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. *EMBO J.*, 3985–3995.

Best,D., Warr,P.J. and Gull,K. (1981) Influence of the composition of commercial sodium dodecyl sulphate preparations on the separation of α and β tubulin during polyacrylamide gel electrophoresis. *Anal. Biochem.*, **114**, 281–284.

Brizuela,L., Draetta,G. and Beach,D. (1987) p13^{suc1} acts in the fission yeast cell cycle as a component of the p34^{cdc2} protein kinase. *EMBO J.*, **6**, 3507–3514.

Broek,D., Bartlett,R., Crawford,K. and Nurse,P. (1991) Involvement of p34^{cdc2} in establishing the dependency of S phase on mitosis. *Nature*, **349**, 388–393.

Cardoso,M.C., Leonhardt,H. and Nadal-Ginard,B. (1993) Reversal of terminal differentiation and control of DNA replication: cyclinA and cdk2 specifically localise at subnuclear sites of DNA replication. *Cell*, **74**, 979–992.

Dasso,M. and Newport,J. (1990) Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis *in vitro*: studies in *Xenopus*. *Cell*, **61**, 811–823.

Endicott,J.A., Nurse,P. and Johnson,L.N. (1994) Mutational analysis supports a structural model for the cell cycle protein kinase p34^{cdc2}. *Protein Eng.*, **7**, 243–253.

Enoch,T. and Nurse,P. (1990) Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell*, **60**, 665–673.

Enoch,T., Gould,K. and Nurse,P. (1991) Mitotic Checkpoint Control in Fission Yeast. *Cold Spring Harbor Symp Quant Biol.*, **56**, 409–416.

Enoch,T., Carr,A. and Nurse,P. (1992) Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes Dev.*, **6**, 2035–2046.

Featherstone,C. and Russell,P. (1991) Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature*, **349**, 808–811.

Gallagher,I.M., Alfa,C.E. and Hyams,J.S. (1993) P63$^{(cdc13)}$, a B-type cyclin, is associated with both the nucleolar and chromatin domains of the fission yeast nucleus. *Mol. Biol. Cell*, **4**, 1087–1096.

Gould,K.L. and Nurse,P. (1989) Tyrosine phosphorylation of the fission yeast cdc2^{+} protein kinase regulates entry into mitosis. *Nature*, **342**, 39–45.

Gould,K.L., Moreno,S., Owen,D.J., Sazer,S. and Nurse,P. (1991) Phosphorylation at Th 167 is required for *Schizosaccharomyces pombe* p34^{cdc2} function. *EMBO J.*, **10**, 3297–3309.

Hayles,J. and Nurse,P. (1992) Genetics of the fission yeast *Schizosaccharomyces pombe*. *Annu. Rev. Genet.*, **26**, 373–402.

Hayles,J., Fisher,D., Woollard,A. and Nurse,P. (1994) Temporal order of S-phase and mitosis in fission yeast is determined by the state of the p34^{cdc2}/mitotic B cyclin complex. *Cell*, **78**, 813–822.

Hirano,T., Funahashi,S., Uemura,T. and Yanagida,M. (1986) Isolation and characterization of *Schizosaccharomyces pombe* cut mutants that block nuclear division but not cytokinesis. *EMBO J.*, **5**, 2973–2979.

Hofmann,J.F.X. and Beach,D. (1994) *cdt1* is an essential target of the cdc10/sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. *EMBO J.*, **13**, 425–434.

Kelly,T.J., Martin,G.S., Forsburg,S.L., Stephen,R.J., Russo,A. and Nurse,P. (1993) The fission yeast *cdc18*$^{+}$ gene product couples S-phase to start and mitosis. *Cell*, **74**, 371–382.

Krek,W. and Nigg,E.A. (1991) Mutations of p34^{cdc2} phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34^{cdc2} kinase activation in vertebrates. *EMBO J.*, **10**, 3331–3341.

Kumagai,A. and Dunphy,W.G. (1991) The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell*, **64**, 903–914.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lundgren,K., Walworth,N., Booher,R., Dembski,M., Kirschner,M. and Beach,D. (1991) mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell*, **64**, 1111–1122.

MacNeill,S.A. and Nurse,P. (1993) Genetic analysis of human p34^{CDC2} function in fission yeast. *Mol. Gen. Genet.*, **240**, 315–322.

MacNeill,S., Creanor,J. and Nurse,P. (1991) Isolation, characterisation and molecular cloning of new mutant alleles in the fission yeast p34^{cdc2} in establishing the dependency of S-phase on mitosis. *Mol. Gen. Genet.*, **229**, 109–118.

McGowan,C.H. and Russell,P. (1993) Human wee1 kinase inhibits cell-division by phosphorylating p34^{cdc2} exclusively on tyr15. *EMBO J.*, **12**, 75–85.

Meijer,L., Azzi,L. and Wang,J.Y.J. (1991) Cyclin B targets p34^{cdc2} for tyrosine phosphorylation. *EMBO J.*, **10**, 1545–1554.

Meyerson,M., Enders,G.H., Wu,C., Su,L.K., Gorka,C., Nelson,C., Harlow,E. and Tsai,L.H. (1992) A family of *cdc2*-related protein kinases. *EMBO J.*, **11**, 2909–2917.

Millar,J., McGowan,C., Lenaers,G., Jones,R. and Russell,P. (1991) p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast. *EMBO J.*, **10**, 4301–4309.

Millar,J.B.A., Lenaers,G. and Russell,P. (1992a) Pyp3 PTPase acts as a mitotic inducer in fission yeast. *EMBO J.*, **11**, 4933–4941.

Millar,J.B.A., Russell,P., Dixon,J.E. and Guan,K.L. (1992b) Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. *EMBO J.*, **11**, 4943–4952.

Mitchison,J. and Nurse,P. (1985) Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.*, **75**, 357–376.

Moreno,S. and Nurse,P. (1994) Regulation of progression through the G1 phase of the cell cycle by the *rum1*$^{+}$ gene. *Nature*, **367**, 236–242.

Moreno,S., Hayles,J. and Nurse,P. (1989) Regulation of p34^{cdc2} protein kinase during mitosis. *Cell*, **58**, 361–372.

Moreno,S., Klar,A. and Nurse,P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.*, **194**, 795–823.

Morla,A.O., Draetta, G.,Beach,D. and Wang,J.Y.J. (1989) Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. *Cell*, **58**, 193–203.

Norbury,C., Blow,J. and Nurse,P. (1991) Regulatory phosphorylation of the p34^{cdc2} protein kinase in vertebrates. *EMBO J.*, **10**, 3321–3329.

Nurse,P. (1990) Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503–508.

Nurse,P. and Bissett,Y. (1981) Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature*, **292**, 558–560.

Nurse,P. and Thuriaux,P. (1977) Controls over the timing of DNA replication during the cell cycle of fission yeast. *Exp. Cell Res.*, **107**, 365–375.

Nurse,P., Thuriaux,P. and Nasmyth,K. (1976) Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.*, **146**, 167–178.

Osmani,A.H., van Peij,N., Mischke,M., O'Connell,M.J. and Osmani,S.A. (1994) A single p34^{cdc2} protein kinase (encoded by nimX^{cdc2}) is required at G1 and G2 in *Aspergillus nidulans*. *J. Cell Sci.*, **107**, 1519–1528.

Ottilie,S., Chernoff,J., Hannig,G., Hoffman,C.S. and Erikson,R.L. (1992) The fission yeast genes *pyp1*$^{+}$ and *pyp2*$^{+}$ encode protein tyrosine phosphatases that negatively regulate mitosis. *Mol. Cell. Biol.*, **12**, 5571–5580.

Parker,L.L., Atherton-Fessler,S. and Piwnica-Worms,H. (1992) p107^{wee1} is a dual-specificity kinase that phosphorylates p34^{cdc2} on tyrosine 15. *Proc. Natl Acad. Sci. USA*, **89**, 2917–2921.

Parker,L.L. and Piwnica-Worms,H. (1992) Inactivation of the p34^{cdc2}–cyclin B complex by the human WEE1 tyrosine kinase. *Science*, **257**, 1955–1957.

Reymond,A., Schmidt,S. and Simanis,V. (1992) Mutations in the *cdc10* start gene of *Schizosaccharomyces pombe* implicate the region of homology between *cdc10* and *SWI6* as important for p85^{cdc10} function. *Mol. Gen. Genet.*, **234**, 449–456.

Saka,Y. and Yanagida,M. (1993) Fission yeast *cut5*$^{+}$, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene *rad4*$^{+}$. *Cell*, **74**, 383–393.

Sazer,S. and Sherwood,S.W. (1990) Mitochondrial growth and DNA synthesis occur in the absence of nuclear DNA replication in fission yeast. *J. Cell Sci.*, **97**, 509–516.

Sheldrick,K.S. and Carr,A.M. (1993) Feedback controls and G2 checkpoints: fission yeast as a model system. *BioEssays*, **15**, 775–781.

Simanis,V. and Nurse,P. (1986) The cell cycle control gene *cdc2*$^{+}$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell*, **45**, 261–268.

Solomon,M.J., Glotzer,M., Lee,T.H., Phillippe,M. and Kirschner,M. (1990) Cyclin activation of p34^{cdc2}. *Cell*, **63**, 1013–1024.

Stewart,E., Koyayashi,H., Harrison,D. and Hunt,T. (1994) Destruction of cyclins A and B2, but not B1, requires binding to p34^{cdc2}. *EMBO J.*, **13**, 584–594.

Strausfeld,U., Labbé,J.-C., Fesquet,D., Cavadore,J.C., Picard,A., Sadhu,K., Russell,P. and Dorée,M. (1991) Dephosphorylation and

activation of a p34^{cdc2}/cyclin B complex *in vitro* by human CDC25 protein. *Nature*, **351**, 242–245.

van der Velden,H.M.W. and Lohka,M.J. (1994) Cell cycle-regulated degradation of *Xenopus* cyclin B2 requires binding to p34^{cdc2}. *Mol. Cell. Biol.*, **5**, 713–724.

Walworth,N., Davey,S. and Beach,D. (1993) Fission yeast *chk1* protein kinase links the rad checkpoint pathway to *cdc2*. *Nature*, **363**, 368–371.

Weinert,T.A. and Hartwell,L.H. (1993) Cell cycle arrest of *cdc* mutants and specificity of the *RAD9* checkpoint. *Genetics*, **134**, 63–80.

Wittenberg,C., Richardson,S.L. and Reed,S.I. (1987) Subcellular localisation of a protein kinase required for cell cycle initiation in *Saccharomyces cerevisiae*: evidence for an association between the CDC28 gene product and the insoluble cytoplasmic matrix. *J. Cell Biol.*, **105**, 1527–1538.

Received on January 30, 1995; revised on March 28, 1995