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Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34^{cdc2} function

Kathleen L.Gould[1], Sergio Moreno, David J.Owen, Shelley Sazer and Paul Nurse

Cell Cycle Group, Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK

[1]Present address: Department of Cell Biology, School of Medicine, Vanderbilt University, Nashville, TN 37232, USA

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Eukaryotic cell cycle progression requires the periodic activation and inactivation of a protein-serine/threonine kinase which in fission yeast is encoded by the *cdc2*$^+$ gene. The activity of this gene product, p34^{cdc2}, is controlled by numerous interactions with other proteins and by its phosphorylation state. In fission yeast, p34^{cdc2} is phosphorylated on two sites, one of which has been identified as Tyr15. Dephosphorylation of Tyr15 regulates the initiation of mitosis. To understand more completely the regulation of p34^{cdc2} kinase activity, we have identified the second site of phosphorylation as Thr167, a residue conserved amongst all p34^{cdc2} homologues. By analysing the phenotypes of cells expressing various position 167 mutations and performing *in vitro* experiments, we establish that Thr167 phosphorylation is required for p34^{cdc2} kinase activity at mitosis and is involved in the association of p34^{cdc2} with cyclin B. Dephosphorylation of Thr167 might also play a role in the exit from mitosis.

Key words: cell cycle/phosphorylation/protein kinase/ *Schizosaccharomyces pombe* p34^{cdc2}

Introduction

The p34^{cdc2} protein kinase is a key regulator of eukaryotic cell cycle progression (reviewed by Lohka, 1989; Murray and Kirschner, 1989; Draetta, 1990; Nurse, 1990). In the fission and budding yeasts, p34^{cdc2} protein kinase activity controls both the initiation of DNA replication and the entry into mitosis (Nurse and Bissett, 1981; Piggott *et al.*, 1982; Reed and Wittenberg, 1990; Surana *et al.*, 1991). Homologues of p34^{cdc2} have been identified in all multicellular eukaryotes examined (Draetta *et al.*, 1987; Lee and Nurse, 1987; Cisek and Corden, 1989; Krek and Nigg, 1989; Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990; Spurr *et al.*, 1990; Hirt *et al.*, 1991) where p34^{cdc2} regulates entry into mitosis (Th'ng *et al.*, 1990) as a component of M-phase promoting factor (MPF) (Dunphy *et al.*, 1988; Gautier *et al.*, 1988) and probably controls the initiation of DNA replication as well (Blow and Nurse, 1990; D'Urso *et al.*, 1990; Furukawa *et al.*, 1990).

Studies in a variety of cells and cell-free systems have shown that p34^{cdc2} protein kinase activity is periodic in the cell cycle peaking at the G_2/M-phase transition (Mendenhall *et al.*, 1987; Draetta and Beach, 1988; Labbe *et al.*, 1988; Booher *et al.*, 1989; Dunphy and Newport, 1989; Felix *et al.*, 1989; Gautier *et al.*, 1989; Moreno *et al.*, 1989; Pines and Hunter, 1989; Pondaven *et al.*, 1990; Krek and Nigg, 1991; Surana *et al.*, 1991). Further, the regulation of its activation involves a complicated series of post-translational modifications and subunit associations (reviewed by Lohka, 1989; Draetta, 1990; Lewin, 1990; Nurse, 1990; Pines and Hunter, 1990; Freeman and Donoghue, 1991). In particular, activation of p34^{cdc2} is associated with its binding to a cyclin molecule (Draetta and Beach, 1988; Booher *et al.*, 1989; Felix *et al.*, 1989; Labbe *et al.*, 1989a; Meijer *et al.*, 1989; Pines and Hunter, 1989; Gautier *et al.*, 1990; Solomon *et al.*, 1990) while inactivation of p34^{cdc2} involves cyclin destruction (Draetta *et al.*, 1989; Murray *et al.*, 1989; Felix *et al.*, 1990; Ghiara *et al.*, 1991). In yeasts, two classes of cyclin-like proteins have been identified which appear to regulate p34^{cdc2} function either during the G_1 phase (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990) or during the G_2 phase (Booher and Beach, 1987, 1988; Hagan *et al.*, 1988; Moreno *et al.*, 1989; Ghiara *et al.*, 1991; Surana *et al.*, 1991) of the cell cycle. However, a complex of p34^{cdc2} bound to cyclin is not necessarily active (Gautier and Maller, 1991). The timing of p34^{cdc2} activation is regulated further by the phosphorylation state of p34^{cdc2} (Draetta *et al.*, 1988; Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Gould *et al.*, 1990; Jessus *et al.*, 1990; Pondaven *et al.*, 1990; Solomon *et al.*, 1990; Krek and Nigg, 1991; Lundgren *et al.*, 1991; Norbury *et al.*, 1991).

p34^{cdc2} is phosphorylated on up to four different sites depending on the cell type (Draetta *et al.*, 1988; Gould and Nurse, 1989; Morla *et al.*, 1989; Norbury *et al.*, 1991; Solomon *et al.*, 1990; Krek and Nigg, 1991). Phosphorylation of each site varies during the cell cycle indicating that each has a potential regulatory function (Draetta *et al.*, 1988; Dunphy and Newport, 1989; Gould and Nurse, 1989; Labbe *et al.*, 1989b; Morla *et al.*, 1989; Norbury *et al.*, 1991; Pondaven *et al.*, 1990; Solomon *et al.*, 1990; Krek and Nigg, 1991). We have previously shown that in fission yeast, p34^{cdc2} is phosphorylated on just two sites during a normal cell cycle, one of which is Tyr15 (Gould and Nurse, 1989). Tyr15 is conserved amongst all p34^{cdc2} proteins and has been identified as the site of tyrosine phosphorylation in the chicken (Krek and Nigg, 1991) and mammalian (Norbury *et al.*, 1991) p34^{cdc2} homologues. The role of Tyr15 phosphorylation is to inhibit activation of p34^{cdc2} (Gould and Nurse, 1989) that is bound to cyclin (Solomon *et al.*, 1990). Phosphorylation of Tyr15 is dependent upon the presence of both the mitotic inhibitor, p107^{wee1} (Russell and Nurse, 1987; Featherstone and Russell, 1991), and another gene, *mik1*, which encodes a p107^{wee1}-related molecule (Lundgren *et al.*, 1991). The mitotic activator p80^{cdc25} (Russell and Nurse, 1986; Ducommun *et al.*, 1990; Moreno *et al.*, 1991b), directly or indirectly regulates Tyr15 de-

phosphorylation (Gould *et al.*, 1990; Kumagai and Dunphy, 1991). In higher eukaryotes, Thr14 has also been identified as a major site of phosphorylation (Norbury *et al.*, 1991; Krek and Nigg, 1991). Phosphorylation of Thr14 acts in conjunction with Tyr15 phosphorylation to inhibit $p34^{cdc2}$ activation (Norbury *et al.*, 1991). A serine phosphorylation site has also been mapped in chicken $p34^{cdc2}$ although its function is not known (Krek and Nigg, 1991).

We had observed previously that $p34^{cdc2}$ was phosphorylated on a threonine residue in addition to Tyr15 (Gould and Nurse, 1989). Here, we report the identity of the phosphorylated residue as Thr167. The comparable site is present and is most likely phosphorylated in all $p34^{cdc2}$ homologues (Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). To examine the function of Thr167 phosphorylation in cells, we have replaced Thr167 with several different amino acids by site-directed mutagenesis and expressed the mutant $p34^{cdc2}$ proteins in fission yeast. We have found that in contrast to the inhibitory effect of Tyr15 phosphorylation, Thr167 phosphorylation is essential for $p34^{cdc2}$ kinase activity at mitosis. It appears to be involved in the stable association of $p34^{cdc2}$ with $p56^{cdc13}$ and dephosphorylation of this residue might play a role in exit from mitosis.

Results

Localization of the threonine phosphorylation site

Tryptic cleavage of ^{32}P-labelled *Schizosaccharomyces pombe* $p34^{cdc2}$ generated only two phosphopeptides, termed 1 and 2 (Gould and Nurse, 1989). Phosphopeptide 1 contained phosphorylated Tyr15 and phosphopeptide 2 contained phosphothreonine (Gould and Nurse, 1989). The electrophoretic mobility of phosphopeptide 2 on thin-layer cellulose plates at pH 4.7 relative to a neutral dye marker and to phosphopeptide 1 (data not shown) indicated that the peptide contained a single phosphate moiety and suggested that *S.pombe* $p34^{cdc2}$ was phosphorylated at a single threonine.

To identify this target threonine, we assumed that the site of threonine phosphorylation would be conserved throughout evolution. A sequence comparison of *S.pombe* (Hindley and Phear, 1984), *Saccharomyces cerevisiae* (Lorincz and Reed, 1984), human (Lee and Nurse, 1987), chicken (Krek and Nigg, 1989), *Drosophila melanogaster* (Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990) and murine (Cisek and Corden, 1989; Spurr *et al.*, 1990) $p34^{cdc2}$ homologues revealed that six threonines were present in all of them, threonines at amino acid positions 14, 47, 167, 172, 189 and 228 of the *S.pombe* protein.

Two different partial proteolytic mapping procedures were used to localize the phosphorylated threonine: chemical cleavage with *N*-chlorosuccinimide (NCS) and enzymatic cleavage with *Staphylococcus aureus* V8 protease. NCS cleavage of $p34^{cdc2}$ gave rise to four major fragments of ~32–19 kDa. By immunoblotting with antibodies specific for either the N-terminal or the C-terminal seven amino acids of $p34^{cdc2}$ (Simanis and Nurse, 1986), it was found that these four fragments contained the N terminus of the protein but lacked the C terminus (data not shown, schematized in Figure 1A). Phosphoamino acid analyses of the four ^{32}P-labelled NCS fragments showed that all four contained both phosphotyrosine and phosphothreonine (data not shown). Thus, threonine 228 was eliminated as a possible phosphorylation site since it is not within all four NCS fragments which contained the phosphorylated threonine. Partial V8 protease digestion yielded four different fragments which ranged between ~10 and ~29 kDa (Gould and Nurse, 1989; Fleig and Nurse, 1991). Again by immunoblotting with antibodies specific for the termini of $p34^{cdc2}$, it was determined that the largest V8 fragment contained the C-terminus while the smallest fragment of ~10 kDa contained the N-terminus (data not shown, schematized in Figure 1A). The 10 kDa fragment also reacted with an

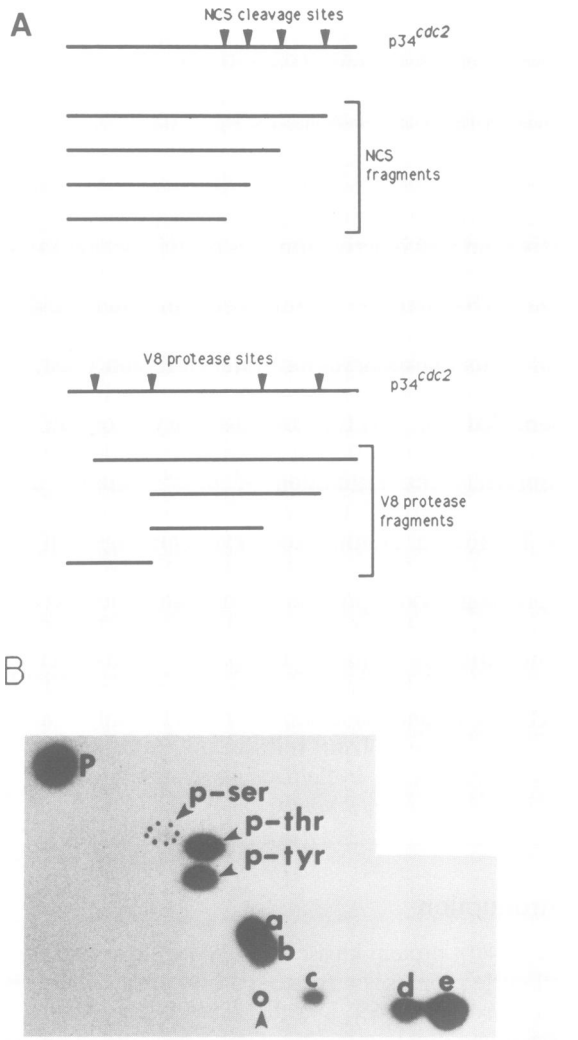

Fig. 1. Locating the site of threonine phosphorylation. (**A**) Schematic representations of the sites of NCS and V8 protease clipping and the resultant fragments which helped to localize the site of threonine phosphorylation. These representations are based on the ability of the fragments to be recognized by antibodies directed against various peptides and bacterially produced fragments. We do not know exactly where the two intermediate-sized V8 fragments are derived from and their placement is only approximate. (**B**) Phosphoamino acid analysis of $p34^{cdc2}$. Wild-type *S.pombe* cells were labelled with [^{32}P]orthophosphate, $p34^{cdc2}$ was immunoprecipitated with PN24 from denatured lysates, resolved by electrophoresis on SDS–polyacrylamide gel, transferred to immobilon-P and partially hydrolysed in acid. The phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis. Autoradiography was for 5 days at −70°C with pre-sensitized Kodak XAR5 film and intensifying screens. Letters a–e indicate phosphopeptides and o indicates the position of the origin. P, free phosphate; p-ser, phosphoserine; p-thr, phosphothreonine; p-tyr, phosphotyrosine.

antibody raised against a peptide containing amino acids 42–57 of p34^{cdc2} (the PSTAIRE peptide, Lee and Nurse, 1987) (data not shown). Phosphoamino acid analysis revealed that whereas the three larger V8 fragments contained only phosphothreonine, the smallest fragment contained phosphotyrosine only (Fleig and Nurse, 1991). Therefore, threonines 14 and 47 were eliminated as candidates since both are located within the N-terminal V8 fragment which did not contain phosphothreonine leaving threonine 167, 172 or 189 as the potential target.

Another piece of evidence regarding the identity of the phosphorylated threonine came from an examination of the ^{32}P-labelled partial acid hydrolysis products of p34^{cdc2}. In addition to the phosphoamino acids, a series of incompletely hydrolysed small phosphopeptides and free ^{32}P could be visualized by autoradiography of a two-dimensional separation of p34^{cdc2} phosphoamino acids (Figure 1B). There are two partial products above the origin (a and b) and three to the right of the origin (c, d and e) (Figure 1B). Rehydrolysis in acid of products a and b together yielded both phosphotyrosine and phosphothreonine whereas that of c, d and e together produced only phosphothreonine (data not shown). The electrophoretic migration of products c–e toward the negative electrode at pH 1.9 could only be explained by the presence of a positively charged amino acid(s) very close to the phosphorylated threonine residue. Of the three remaining candidates (threonines 167, 172 and 189), only threonine 167 (Thr167) is near a basic amino acid (histidine 168) and, therefore, it seemed likely to be the site of phosphorylation.

To confirm that Thr167 was indeed the single site of threonine phosphorylation in *S.pombe* p34^{cdc2} and to discover what effect, if any, phosphorylation at this site had on p34^{cdc2} function, the codon specifying Thr at position 167 was replaced with those encoding either alanine (A167), serine (S167), glutamic acid (E167), aspartic acid (D167) or tyrosine (Y167) by site-directed mutagenesis of the *cdc2*$^{+}$ cDNA. A double mutant was also constructed, *cdc2*-F15/A167, in which Tyr15 was changed to phenylalanine and Thr167 was changed to alanine. The mutant and wild-type cDNAs were each subcloned into the *S.pombe* expression vector, pMNS21L (Maundrell, 1990), such that transcription of the mutants was driven by the thiamine repressible *nmt1* promoter. Each mutant was then expressed in HY1 (a gift from Stuart MacNeill), an *S.pombe* strain in which the wild-type *cdc2* protein coding region has been replaced with the human *CDC2* cDNA. Mutant *S.pombe cdc2* proteins expressed in HY1 can be examined biochemically using antibodies which recognize *S.pombe* p34^{cdc2} but do not recognize human p34^{cdc2} (4711 or PN24, described in Materials and methods). To permit analyses of the non-functional mutant proteins, it was found necessary (see below) to select HY1 strains in which the pMNS21L plasmids containing the mutant *cdc2* cDNAs had integrated into the genome and were maintained stably. This was not necessary and was not done in the cases of the *cdc2*$^{+}$ and *cdc2*-S167 cDNAs which had no deleterious effects on the cells. HY1 cells expressing various *cdc2*

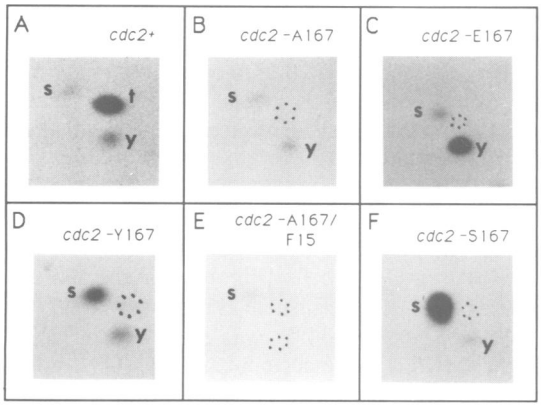

Fig. 2. Phosphoamino acid analyses of p34^{cdc2} proteins. HY1 strains expressing wild-type or one of five p34^{cdc2} mutants were labelled with [^{32}P]orthophosphate. The p34^{cdc2} proteins were immunoprecipitated with PN24, resolved on a SDS–polyacrylamide gel, transferred to immobilon-P, and partially hydrolysed with acid. The resultant phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis. The exposure times of the autoradiographs were 4–6 days at −70°C with pre-sensitized Kodak XAR5 film and intensifying screens. s, phosphoserine; t, phosphothreonine; y, phosphotyrosine.

A

	Rescue of *cdc2* ^{ts}
vector control	–
cdc2+	+
cdc2 -A167	–
cdc2 -S167	+
cdc2 -E167	±
cdc2 -D167	–
cdc2 -Y167	–
cdc2 -F15/A167	–

B

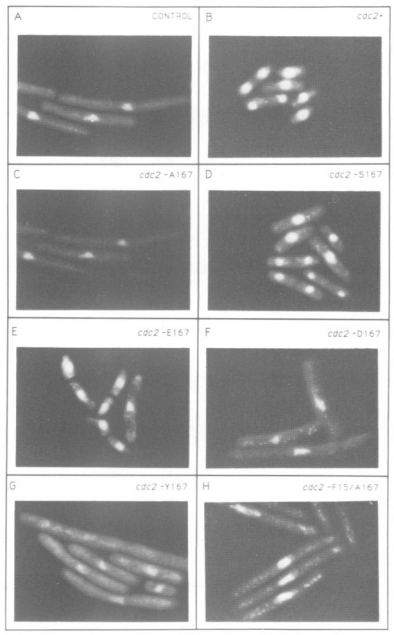

Fig. 3. Ability of mutants to rescue *cdc2*^{ts}. (**A**) The ability of various *cdc2* mutants to rescue growth of the *cdc2*-33 strain at the non-permissive temperature of 36°C. (**B**) Cells expressing the various *cdc2* mutants were grown to a density of ~5 × 10^{6} cells/ml at 25°C, transferred to 36°C for 4 h, fixed, and stained with DAPI.

proteins in the presence of thiamine were labelled with [^{32}P]orthophosphate, and the *S.pombe cdc2* proteins were immunoprecipitated with PN24, transferred to immobilon-P and subjected to phosphoamino acid analyses. Wild-type *S.pombe* p34^{cdc2} contained all three phosphoamino acids (Figure 2A) in the same relative amounts as we have reported previously (Gould and Nurse, 1989; Fleig and Nurse, 1991). The p34$^{cdc2\text{-}A167}$, p34$^{cdc2\text{-}E167}$ and p34$^{cdc2\text{-}Y167}$ proteins lacked phosphothreonine but still contained phosphotyrosine (Figure 2B, C and D). Both phosphothreonine and phosphotyrosine were absent from p34$^{cdc2\text{-}F15/A167}$ indicating that positions 167 and 15 were the only major phosphoacceptor sites in the protein (Figure 2E). The *cdc2*-S167 protein was not phosphorylated on threonine but was heavily phosphorylated on serine residues (Figure 2F). Further analysis of partial acid hydrolysis products and phosphopeptides from p34$^{cdc2\text{-}S167}$ showed that Ser167 was indeed phosphorylated and accounted for all of the observed phosphoserine (data not shown). These data firmly establish that Thr167 is the site of phosphorylation.

The small amount of serine phosphorylation observed in many phosphoamino acid analyses of *S.pombe* p34^{cdc2} (see Figure 2A–E) appears to be artefactual (Fleig and Nurse, 1991). If immunoprecipitates of ^{32}P-labelled *S.pombe* p34^{cdc2} are subjected to tryptic digestion or partial digestion with either V8 protease or *N*-chlorosuccinimide, phosphoserine is not recovered in any fragment or phosphopeptide (Gould and Nurse, 1989; Fleig and Nurse, 1991). This indicates that the phosphoserine contamination is derived from a multitude of minor contaminants and is not due to a major contaminating protein. Further evidence supporting the notion that *S.pombe* p34^{cdc2} is not phosphorylated on serine is that every serine residue in the C-terminal third of the protein including the serine residue corresponding to serine 283, the site phosphorylated in chicken p34^{cdc2} (Krek and Nigg, 1991), has been changed to an alanine without affecting the amount of serine phosphorylation detected in *S.pombe* p34^{cdc2} immunoprecipitates (U.N.Fleig and P.Nurse, unpublished data).

Phenotypes of p34^{cdc2} mutants

To examine the functional consequence of Thr167 phosphorylation, each mutant was tested for its ability to rescue the temperature-sensitive (ts) *cdc2* strain, *cdc2*-33. This mutant arrests at both the G_1/S and G_2/M transition points at the non-permissive temperature and produces highly elongated cells. Each mutant in pMNS21L was transformed into *cdc2*-33 and colonies containing the plasmids were allowed to form at the permissive temperature of 25°C. Although thiamine was included in the growth medium to prevent overexpression from the *nmt1* promoter, moderate expression of p34^{cdc2} was still detectable under these conditions (see below and Fleig and Nurse, 1991). The ability of the mutants to complement ts p34^{cdc2} was then assessed by replica-plating to the restrictive temperature of 36°C still in the presence of thiamine. Only wild-type *cdc2* and the *cdc2*-S167 mutants were able to fully complement *cdc2^{ts}* (Figure 3A). The *cdc2*-E167 mutant gave rise to slow-growing colonies at 36°C either when replica-plated from the permissive temperature or when placed directly at 36°C after transformation into *cdc2^{ts}* strains. The other mutants did not support any colony growth. The inability of p34$^{cdc2\text{-}A167}$ to complement ts *cdc2* agrees with an earlier report (Booher and Beach, 1986). The phenotypes of cells expressing these mutants in liquid culture after 4 h at 36°C are shown in Figure 3B. Cells expressing p34^{cdc2+} or p34$^{cdc2\text{-}S167}$ were phenotypically wild-type, p34$^{cdc2\text{-}E167}$-expressing cells generally contained one or more septa and two or more nuclei, and cells expressing the other mutants were highly elongated and contained a single nucleus (Figure 3B).

Overproduction of the mutant cdc2 protein

As show above, the non-phosphorylatable mutants at position 167 were unable to complement *cdc2^{ts}* when they were expressed at low to moderate levels. To examine whether higher than normal levels could compensate for their defects as is the case for certain ts *cdc2* mutants (Durkacz *et al.*, 1985), thiamine was removed from the media to allow high levels of expression from the *nmt1* promoter. Overexpression did not change the ability of the mutants to rescue *cdc2^{ts}*. Moreover, in the HY1 strain or at the permissive temperature in the *cdc2*-33 strain, overproduction of these mutants led to cell elongation and cessation of division. The phenotypes of HY1 cells during induction of p34$^{cdc2\text{-}A167}$ or p34$^{cdc2\text{-}E167}$ are shown in Figure 4A and B, respectively. Twelve to fourteen hours after thiamine was removed from the media, the cells began to elongate. This timing coincides with upregulation of *nmt1*$^{+}$ promoter activity (Maundrell, 1990) and overexpression of the mutant proteins (see below; Fleig and Nurse, 1991). In the case of p34$^{cdc2\text{-}E167}$, cells became multiply septated, branched and accumulated multiple nuclei (Figure 4B). The toxicity of these non-complementing mutants explained our difficulty in maintaining them episomally on high copy-number vectors and why it was necessary to obtain integrants of these mutants under thiamine-repressible control in order to study their protein products.

Kinase activity of the mutant cdc2 proteins

The first property of these non-functional position 167 mutants we examined was their ability to function as protein kinases. Each position 167 mutant was tested for histone H1 kinase activity in both the HY1 and *cdc2*-33 strains. In HY1, each mutant was expressed from the *nmt1* promoter derepressed for 0, 6, 18 or 24 h and isolated from equal amounts of cell lysates by immunoprecipitation with 4711. These immunoprecipitates were divided in half. The abundance of p34^{cdc2} was determined using one half and the other half was assayed for kinase activity towards the exogenous substrate, histone H1. The specificity of 4711 for *S.pombe* p34^{cdc2} is demonstrated in Figure 5, panels A, B, H and I. There was no 4711-reactive p34^{cdc2} in immune complexes from HY1 cells (Figure 5A) and the immune complexes did not have histone H1 kinase activity (Figure 5H). When *S.pombe* p34^{cdc2} was expressed in HY1 from episomal pMNS21L, the accumulation of p34^{cdc2} during the time course of derepression was detected easily (Figure 5B) and the immune complexes displayed histone H1 kinase activity (Figure 5I). These results demonstrated that we were able to assay the protein kinase activity of the *S.pombe* mutants introduced into HY1 and that p34^{cdc2Hs} did not contribute to the activity measured in this assay. Furthermore, protein kinase activity did not increase in parallel with increasing levels of p34^{cdc2} indicating that some other component which was necessary for kinase activation was

limiting in the cells. p34$^{cdc2\text{-}S167}$, like p34^{cdc2+}, accumulated to high levels during derepression of the *nmt1* promoter (Figure 5C) and had histone H1 kinase activity (Figure 5J). p34$^{cdc2\text{-}A167}$ accumulated also but to lower levels because its gene was integrated as a single copy (Figure 5D). p34$^{cdc2\text{-}A167}$ lacked histone H1 kinase activity (Figure 5K).

In a second experiment, the kinase activities of two other mutants were compared with p34^{cdc2+}. In this experiment, a background histone kinase activity was observed but neither p34$^{cdc2\text{-}E167}$ nor p34$^{cdc2\text{-}F15/A167}$ demonstrated any protein kinase activity above this background (Figure 5L and M) although the proteins were present in the immune complexes (Figure 5E and F). Also in this second experiment, the linearity of the immunoblotting and protein kinase assays was examined by diluting 2-, 4- and 8-fold an immunoprecipitate of p34^{cdc2+} from a 24 h-induced culture. Both the level of protein and protein kinase activity decreased in a linear manner indicating that the activity detected in this assay is an accurate reflection of the amount of active histone H1 kinase in an immune complex (Figure 5G and N).

When the mutants were assayed for histone H1 kinase activity in a *cdc2*^{ts} background, the same results were obtained. Lysates were made from cells growing at the permissive temperature (25°C) and from cells shifted to the non-permissive temperature (36°C) for 2 and 4 h. Only p34^{cdc2+} and p34$^{cdc2\text{-}S167}$ were active at 40°C both in immune complexes and in lysates assayed directly (data not shown). The *cdc2*-A167, *cdc2*-E167 and *cdc2*-F15/A167 proteins showed no evidence of histone H1 kinase activity under any of these conditions (data not shown).

Thr167 dephosphorylation correlates with inhibition of activity

The data presented above suggested that in contrast to the inhibitory effect of Tyr15 phosphorylation (Gould and Nurse, 1989; Gould *et al.*, 1990; Jessus *et al.*, 1990; Pondaven *et al.*, 1990; Kumagai and Dunphy, 1991), Thr167 phosphorylation was a prerequisite for p34^{cdc2} function. To determine if Thr167 phosphorylation was required for phosphotransferase activity *per se*, we sought to remove phosphate from this residue enzymatically and assay the dephosphorylated protein. First, we explored whether or not protein phosphatase 2A (PP2A) could dephosphorylate Thr167. An immunoprecipitate of ^{32}P-labelled p34^{cdc2}

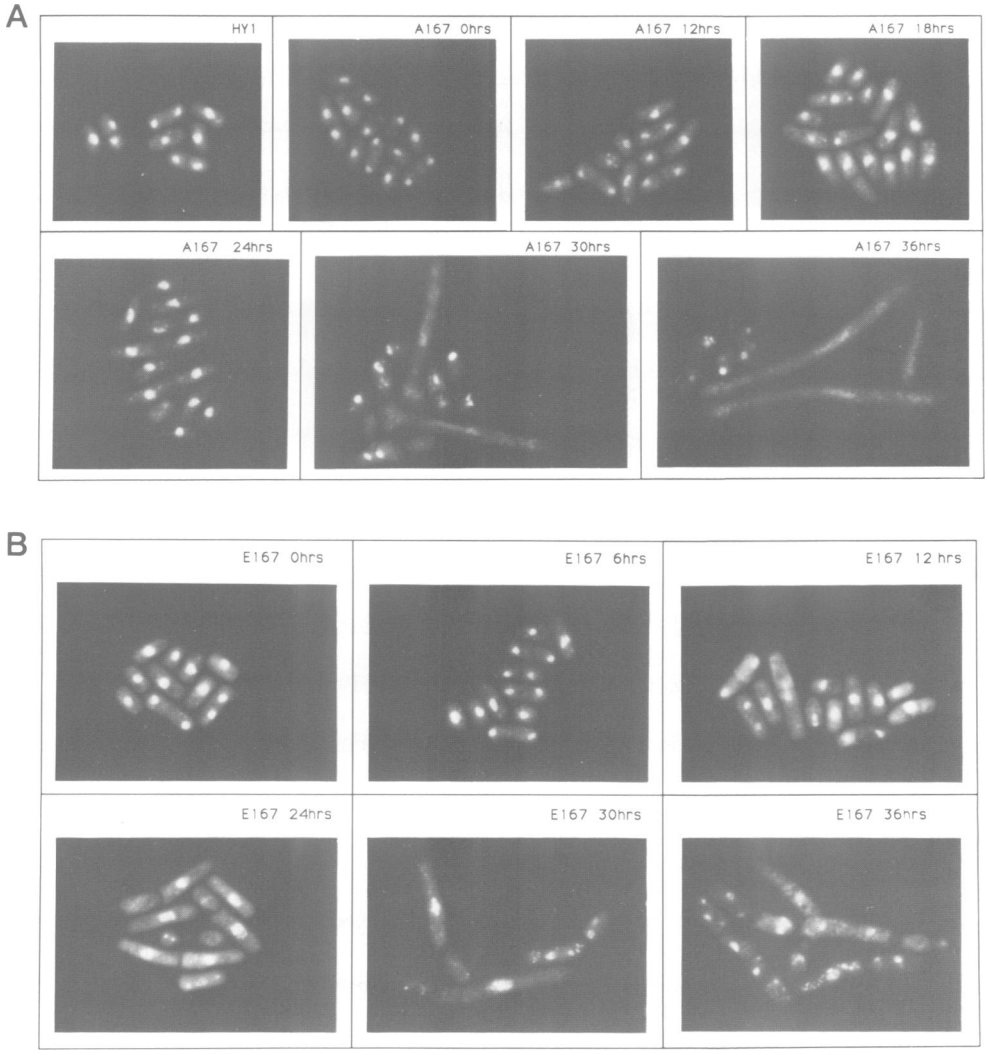

Fig. 4. Photomicrographs of the induction of p34$^{cdc2\text{-}A167}$ and p34$^{cdc2\text{-}E167}$. HY1 (A) and HY1 with integrated pMNS21L plasmids expressing p34$^{cdc2\text{-}A167}$ (**A**) or p34$^{cdc2\text{-}E167}$ (**B**) in the presence of 5 μg/ml thiamine were washed in a large volume of thiamine-free media three times and resuspended once again in thiamine-free media. Samples of cells were taken at the indicated times, fixed, and stained with DAPI.

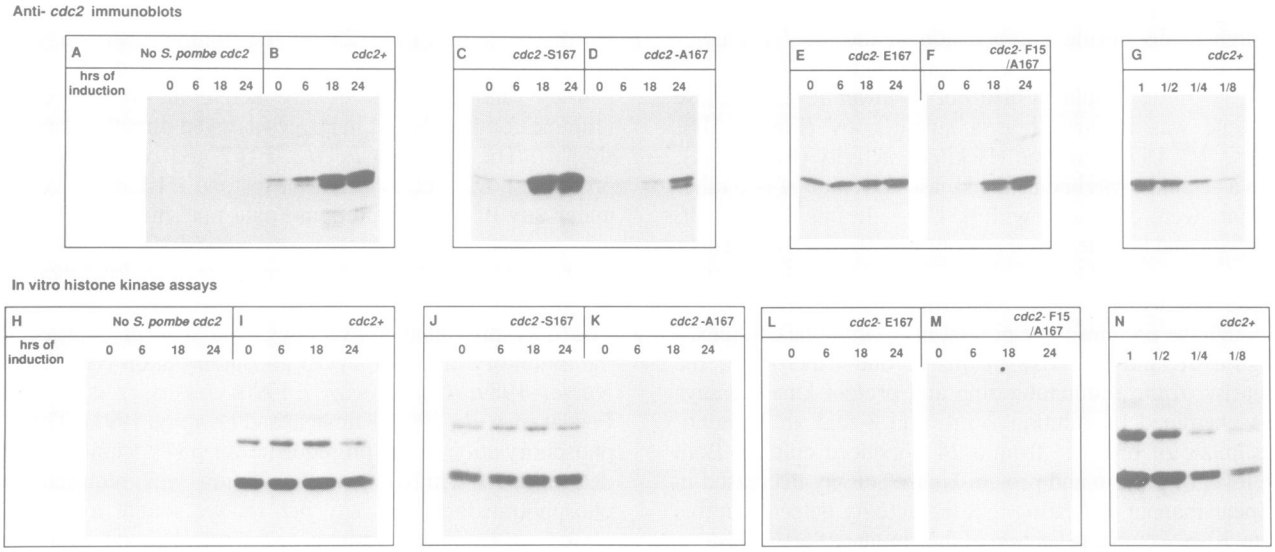

Fig. 5. Histone H1 kinase activities of p34^{cdc2} mutants. HY1 cells expressing either no *S.pombe* p34^{cdc2} (panels **A** and **H**), wild-type p34^{cdc2} (panels **B** and **I**, also panels **G** and **N**), or the indicated *S.pombe* p34^{cdc2} mutants (panels **C**, **D**, **E**, **F**, **J**, **K**, **L** and **M**) were grown in media containing 5 μg/ml thiamine. They were then washed thoroughly in thiamine-free media to allow derepression of the *nmt1* promoter. Equivalent amounts of cells were taken at the indicated times after derepression and lysed. The p34^{cdc2} proteins were isolated by immunoprecipitation with 4711 and the immunoprecipitates were divided into two equal portions. In the case of the samples shown in panels G and N, the immunoprecipitate of wild-type p34^{cdc2} from a culture derepressed for 24 h was diluted 1-, 2-, 4- and 8-fold. Panels A–G. One half of each immunoprecipitate was resolved on an SDS–polyacrylamide gel and transferred to immobilon-P. The blot was incubated with 4711 followed by [^{125}I]protein A. The blots were exposed to presensitized Kodak XAR5 film with intensifying screens for 16 h. The major band on the autoradiographs corresponds to p34^{cdc2} and the minor band of ~50 kDa corresponds to the immunoglobulin heavy chain. Panels H–N. The other half of each immunoprecipitate shown in panels A–G was assayed for histone H1 kinase activity. After being heated to 100°C for 2 min in 2 × gel sample buffer, the reactions were resolved on SDS–polyacrylamide gels and subjected to autoradiography with Kodak XAR5 film. The kinase reaction of each sample is shown underneath the immunoblot from the same sample. Exposure times were 16 h at −70°C with intensifying screens. The major band visualized corresponds to histone H1, and the minor band might be related to p56^{cdc13}.

was generated from cells arrested in late G_2 due to a ts mutation in the *cdc25* gene. The ^{32}P-labelled p34^{cdc2} immunoprecipitate was divided in half; one half was treated with PP2A and the other with buffer alone. PP2A removed much of the phosphothreonine from the protein and also some phosphotyrosine (Figure 6A). At a higher concentration, PP2A removed most of the phosphate from p34^{cdc2} (Figure 6B). In parallel experiments, unlabelled immunoprecipitates were divided in half, treated with protein phosphatases or buffers alone, and divided once again to assay histone H1 kinase activity and p34^{cdc2} abundance. At the block point of *cdc25*-22 in late G_2, the protein kinase activity of p34^{cdc2} was low and was slightly decreased by PP2A treatment (Figure 6C). Fifteen minutes after release into mitosis, the protein kinase activity of the untreated half had risen significantly as expected from previous reports (Booher *et al.*, 1989; Moreno *et al.*, 1990) while that of the PP2A-treated half remained low (Figure 6C). As we showed before, treatment with the T-cell protein-tyrosine phosphatase also leads to activation of p34^{cdc2} isolated from cells blocked in late G_2 (Gould *et al.*, 1990; Figure 6D, lane 2). PP2A treatment prevented this activation whether added after (Figure 6D, lane 4) or before (Figure 6D, lane 8) the protein-tyrosine phosphatase. PP2A treatment alone resulted in a small inhibition of the basal activity (Figure 6D, lane 6; also Figure 6C) and these treatments did not alter the level of p34^{cdc2} (Figure 6E).

Thr167 phosphorylation and subunit associations

Two other proteins are known to bind directly to p34^{cdc2} in *S.pombe*, p56^{cdc13} (a cyclin B homologue) (Booher *et al.*, 1989) and p13^{suc1} (Brizuela *et al.*, 1987; Booher *et al.*, 1989), and the presence of p56^{cdc13} is required for p34^{cdc2} protein kinase activation (Moreno *et al.*, 1989). Since the mutants which could not be phosphorylated at position 167 lacked protein kinase activity, we explored the possibility that Thr167 phosphorylation was a prerequisite for binding to p56^{cdc13} or to p13^{suc1}. If they were unable to bind p56^{cdc13}, this would explain their lack of protein kinase activity. Lysates were made from HY1 expressing either no *S.pombe* p34^{cdc2}, p34^{cdc2+} or p34$^{cdc2\text{-}A167}$, and subjected to immunoprecipitation with either antibodies to p34^{cdc2} (4711), p56^{cdc13} or p13^{suc1} coupled to agarose beads. The immunoprecipitates were resolved on a SDS–polyacrylamide gel, transferred to immobilon-P and then blotted with PN24. Both p34^{cdc2+} and p34$^{cdc2\text{-}A167}$ were detected in 4711 immunoprecipitates (Figure 7A) and both were also capable of binding to p13^{suc1} beads (Figure 7B). Both were also immunoprecipitated by anti-p56^{cdc13} serum (Figure 7C), but the amount of p34$^{cdc2\text{-}A167}$ was considerably reduced compared with p34^{cdc2+}.

Thr167 phosphorylation at G_1/S?

As shown above, Thr167 phosphorylation is critical for p34^{cdc2} function and is probably required for p34^{cdc2} phosphotransferase activity at the G_2/M transition. To determine if Thr167 phosphorylation has a role at the G_1/S phase transition as well, we examined the DNA content of *cdc2*^{ts} strains containing an integrated copy of either *cdc2*-A167 or *cdc2*-E167 by flow cytometry. As expected, the *cdc2*-L7 and *cdc2*-33 strains arrested with a 1C as well as a 2C DNA content within 1 h of temperature shift

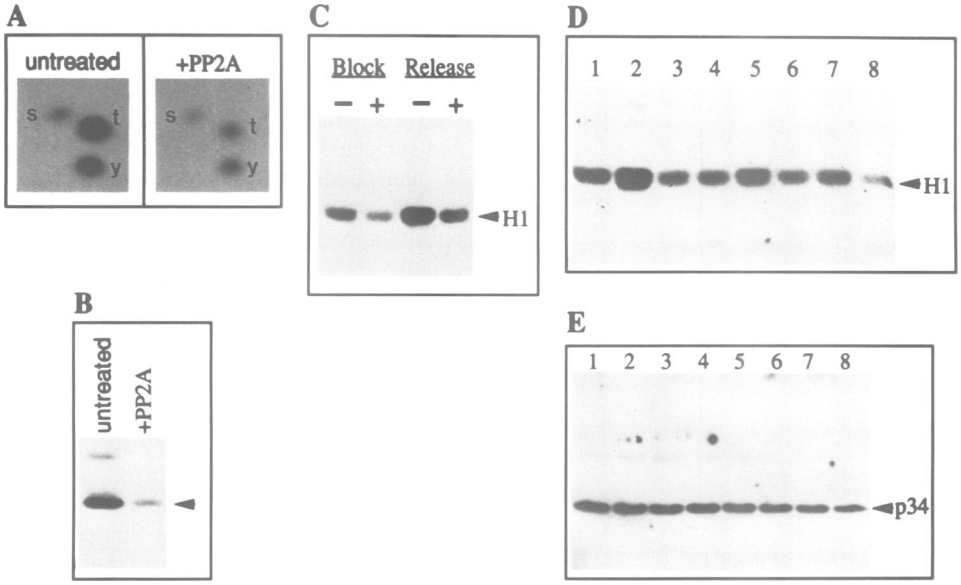

Fig. 6. PP2A inactivates histone H1 kinase activity. (**A**) p34^{cdc2} was immunoprecipitated with 4711 from *cdc25*-22 cells labelled with [^{32}P]orthophosphate and growth-arrested in late G_2 at 36°C. One half of the immunoprecipitate was treated with PP2A buffer alone and the other half with buffer and 10 mU/ml PP2A for 30 min at 30°C. The immunoprecipitates were resolved on a SDS−polyacrylamide gel, transferred to immobilon-P, and partially hydrolysed in acid. The phosphoamino acids were separated by two-dimensional thin-layer electrophoresis and visualized by autoradiography for 5 days at −70°C with pre-sensitized Kodak XAR5 film and an intensifying screen. s, phosphoserine; t, phosphothreonine; y, phosphotyrosine. (**B**) p34^{cdc2} was immunoprecipitated with 4711 from *cdc25*-22 cells labelled with [^{32}P]orthophosphate and growth-arrested at 36°C. One half of the immunoprecipitate was treated with PP2A buffer alone and the other half with buffer and 10 times as much PP2A, 100 mU/ml, as in (A) for 30 min at 30°C. The immunoprecipitates were resolved on a SDS−15% polyacrylamide gel, transferred to immobilon-P, and visualized by autoradiography for 16 h at −70°C with pre-sensitized Kodak XAR5 film and an intensifying screen. The arrow indicates the position of p34^{cdc2}. (**C**) p34^{cdc2} was immunoprecipitated with 4711 from *cdc25*-22 cells which had been arrested in late G_2 by being held at 36°C (Block) or had been released from this block for 15 min by rapid cooling to 25°C (Release). The immunoprecipitates were then split in half. One half of each was treated with PP2A buffer alone and the other half of each was treated with buffer and 10 mU/ml PP2A for 30 in at 30°C. All four samples were then assayed for histone H1 kinase activity. The histone H1 kinase reactions were resolved on an SDS−polyacrylamide gel and the phosphorylated histone H1 was visualized by autoradiography with pre-sensitized Kodak XAR5 at −70°C and an intensifying screen for 16 h. (**D**) p34^{cdc2} was immunoprecipitated with 4711 from *cdc25*-22 cells which had been arrested in late G_2 by being held at 36°C. The immunoprecipitate was then split into four and each again in half. One half of each was incubated in buffer alone while their counterparts were treated with phosphatases. After washing into H15 buffer, the histone H1 kinase activity of one half of the eight samples was assayed. The reactions were separated on an SDS−polyacrylamide gel, transferred to immobilon-P, and incubated with 4711 followed by [^{125}I]protein A. Histone H1 kinase activity was visualized by autoradiography. Lane 2 is from a sample treated with the T-cell protein-tyrosine phosphatase for 30 min at 30°C. Lane 4 is from a sample treated as in lane 2 but for an additional 30 min at 30°C with 10 mU/ml PP2A. Lane 6 is from a sample treated for 30 min at 30°C with 10 mU/ml PP2A. Lane 8 is from a sample treated as in lane 6 but for an additional 30 min at 30°C with the T-cell protein-tyrosine phosphatase. Lanes 1, 3, 5 and 7 are from controls which were treated identically to samples in lanes 2, 4, 6 and 8, respectively, except that no phosphatases were added. (**E**) The other half of each immunoprecipitate shown in (D) was resolved on an SDS−polyacrylamide gel, transferred to immobilon-P, incubated with 4711 and then with [^{125}I]protein A. p34^{cdc2} was visualized by autoradiography for 16 h. H1, histone H1.

indicative of both a G_1 and a G_2 block (Figure 8A and C). Cells of both strains leaked through the G_1 block by 4 h at 36°C, arrested with a 2C DNA content and persisted in this state for at least 6 h (Figure 8A and C; data not shown).

A peak corresponding to a 1C DNA content was seen reproducibly in the *cdc2*-L7 strain expressing p34$^{cdc2\text{-}A167}$ at 36°C although it was more transient than in the parental strain (Figure 8B). By DAPI staining, these cells appeared elongated with a single interphase nucleus (data not shown). In contrast, cells with a 1C DNA content were never observed in the *cdc2*^{ts} strain expressing p34$^{cdc2\text{-}E167}$ at 36°C (Figure 8D). Instead, cells with a greater than 2C DNA content appeared after 4 h at 36°C. The phenotypes of these cells are shown in Figure 9A. During the temperature shift, cells with several septa and nuclei accumulated (Figure 9A). The flow cytometric analysis coupled with the DAPI staining showed that these cells continued to replicate and separate their DNA to daughter cells but did not always complete cell division before proceeding into the next round of DNA synthesis. These data are consistent with the ability of this mutant to support enough growth for the formation of slow growing colonies on plates at 36°C in *cdc2*^{ts} strains (see Figure 3).

Anti-tubulin immunofluorescence revealed an unusually high number of mitotic spindles in the *cdc2*-33 strain expressing p34$^{cdc2\text{-}E167}$ after 4 h at 36°C (Figure 9B, panels A and B); some cells contained two spindles (Figure 9B, panel C). The DNA was often distributed abnormally in relation to the spindle, either along its length or at its centre (Figure 9B, panels D and E). To provide quantitative evidence that p34$^{cdc2\text{-}E167}$-expressing cells spent a longer than normal time in mitosis, the septation index was determined as cells were shifted to 36°C. At time 0, the septation index was 15% (higher than the normal 7%). By 6 h after temperature shift, 93% of the cells contained at least one septum (Table I).

Tyr15 but not Thr167 phosphorylation is limiting

In previous ^{32}P-labelling experiments, we noticed that the amount of ^{32}P-labelled p34^{cdc2} was greater in cells which

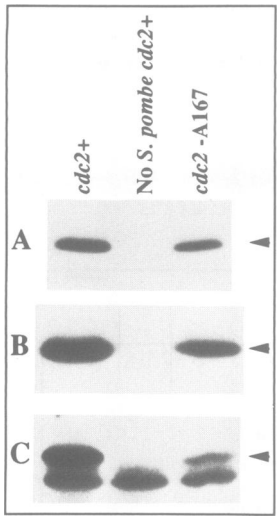

Fig. 7. Lysates were made from HY1 expressing $p34^{cdc2+}$, HY1, and HY1 expressing $p34^{cdc2\text{-}A167}$ and subjected to immunoprecipitation with 4711 (**A**), $p13^{suc1}$ coupled to agarose beads (**B**), or antibodies to $p56^{cdc13}$ (**C**). The immunoprecipitates were run on an SDS–polyacrylamide gel, transferred to immobilon-P, and probed with PN24 followed by [^{125}I]protein A. The presence of $p34^{cdc2}$, indicated by the arrows, was visualized by autoradiography of the relevant portions of the immunoblot. The nature of the lower band in (C) is not known. Exposure times at −70°C with Kodak XAR5 film and intensifying screens were 4 h (A), 16 h (B) and 5 days (C).

overexpressed $p34^{cdc2}$. To investigate whether this was a uniform increase or specific to one phosphorylation site, we labelled with [^{32}P]orthophosphate a strain expressing 5–20 times more $p34^{cdc2}$ than wild-type in parallel with a wild-type strain. $p34^{cdc2}$ was immunoprecipitated from each and subjected to phosphoamino acid analysis. The amount of phosphothreonine on $p34^{cdc2}$ increased significantly in the strain overexpressing $p34^{cdc2}$ while the level of phosphotyrosine did not increase to nearly the same extent (Figure 10A and B).

Thr167 and Tyr15 phosphorylation can occur in S phase

We previously examined the phosphoamino acid content of $p34^{cdc2}$ in asynchronous populations, and in *cdc* mutants blocked in late G_2 or in mitosis. To see if Thr167 and Tyr15 phosphorylations occurred earlier in the cell cycle, we examined the phosphoamino acid content of $p34^{cdc2}$ in cells which had not yet completed DNA synthesis. Wild-type cells which had been pre-treated with hydroxyurea were labelled with [^{32}P]orthophosphate in the continuing presence of hydroxyurea. $p34^{cdc2}$ was isolated by immunoprecipitation and its phosphoamino acid content was determined. Both Thr167 and Tyr15 were phosphorylated under these conditions to equivalent levels indicating that these phosphorylation events can occur before the completion of DNA synthesis (Figure 10C).

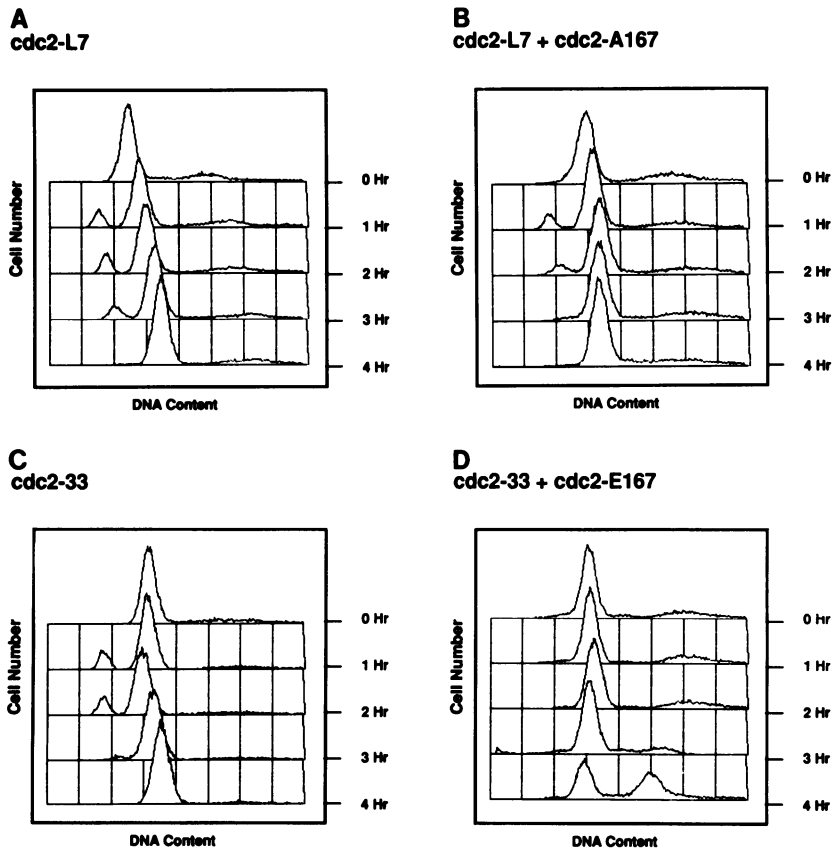

Fig. 8. Flow cytometric analysis of DNA content of $cdc2^{ts}$ cells expressing $p34^{cdc2}$ mutants. Cells were grown to mid-exponential phase at the permissive temperature of 25°C in the presence of 5 μg/ml thiamine and then shifted at t = 0 to the restrictive temperature of 36°C for 4 h. Samples were withdrawn hourly, fixed in ethanol and stained with propidium iodide. Linear fluorescence histograms show relative DNA content in arbitrary units on the horizontal axis and the cell number on the vertical axis. (**A**) *cdc2*-L7 cells; (**B**) *cdc2*-L7 cells expressing $p34^{cdc2\text{-}A167}$; (**C**) *cdc2*-33 cells; (**D**) *cdc2*-33 cells expressing *cdc2*-E167.

Discussion

We showed previously that *S.pombe* p34^{cdc2} was phosphorylated on two major sites, one of which we identified as Tyr15 (Gould and Nurse, 1989). In this study, we have identified the second site of phosphorylation in *S.pombe* p34^{cdc2} as Thr167. Serine but not alanine, glutamic acid, aspartic acid or tyrosine can substitute for threonine at residue 167. The Ser167 mutant protein is indistinguishable from the wild-type protein functionally and Ser167 can be phosphorylated. Thr167 is present in all sequenced p34^{cdc2} proteins (Figure 11) and is likely to be a major site of phosphorylation in vertebrate p34^{cdc2} as well as in the *S.pombe* protein although it has not been identified definitively in other cell types (Krek and Nigg, 1990; Solomon *et al.*, 1990; Lee *et al.*, 1991; Norbury *et al.*, 1991). In vertebrate cells, Thr14 is a third major site of p34^{cdc2} phosphorylation (Krek and Nigg, 1991; Norbury *et al.*, 1991). We have not detected Thr14 phosphorylation of *S.pombe* p34^{cdc2} under ordinary growth conditions.

In contrast to phosphorylation at Tyr15 which inhibits p34^{cdc2} kinase activity, our evidence indicates that phosphorylation at Thr167 is required for p34^{cdc2} function and that the two phosphorylation events are completely independent. With one exception which will be discussed below, mutant p34^{cdc2} proteins which have non-phosphorylatable amino acids in place of Thr167 cannot complement ts *cdc2* mutants and lack histone H1 protein kinase activity *in vitro*. Although non-functional, these mutant proteins are stable, phosphorylated on tyrosine, and their overexpression in the presence of wild-type p34^{cdc2} leads to cell elongation and a block in cell division. Thus, these mutant p34^{cdc2} proteins appear to retain the capacity to interact with and even titrate away other proteins with which the wild-type protein must interact in order to function normally. These data argue that the structure of the mutant proteins is not dissimilar to wild-type but that the mutants are inactive as protein kinases. Dominant negative mutants of p34^{cdc2} with similar properties have been described before (Mendenhall *et al.*, 1988; Fleig and Nurse, 1991).

In our previous study of *S.pombe* p34^{cdc2} phosphorylation, we found that both Tyr15 and Thr167 were phosphorylated during G_2 and that phosphorylation of both residues decreased as cells passed into and through mitosis (Gould and Nurse, 1989). Although we did not observe complete dephosphorylation of either site at any time and were unable to discern if one dephosphorylation event preceded the other as cells entered mitosis (Gould and Nurse, 1989), activation of p34^{cdc2} *in vitro* was associated with tyrosine but not threonine dephosphorylation (Gould *et al.*, 1990). In *Xenopus* oocyte extracts and in cells where good cell cycle

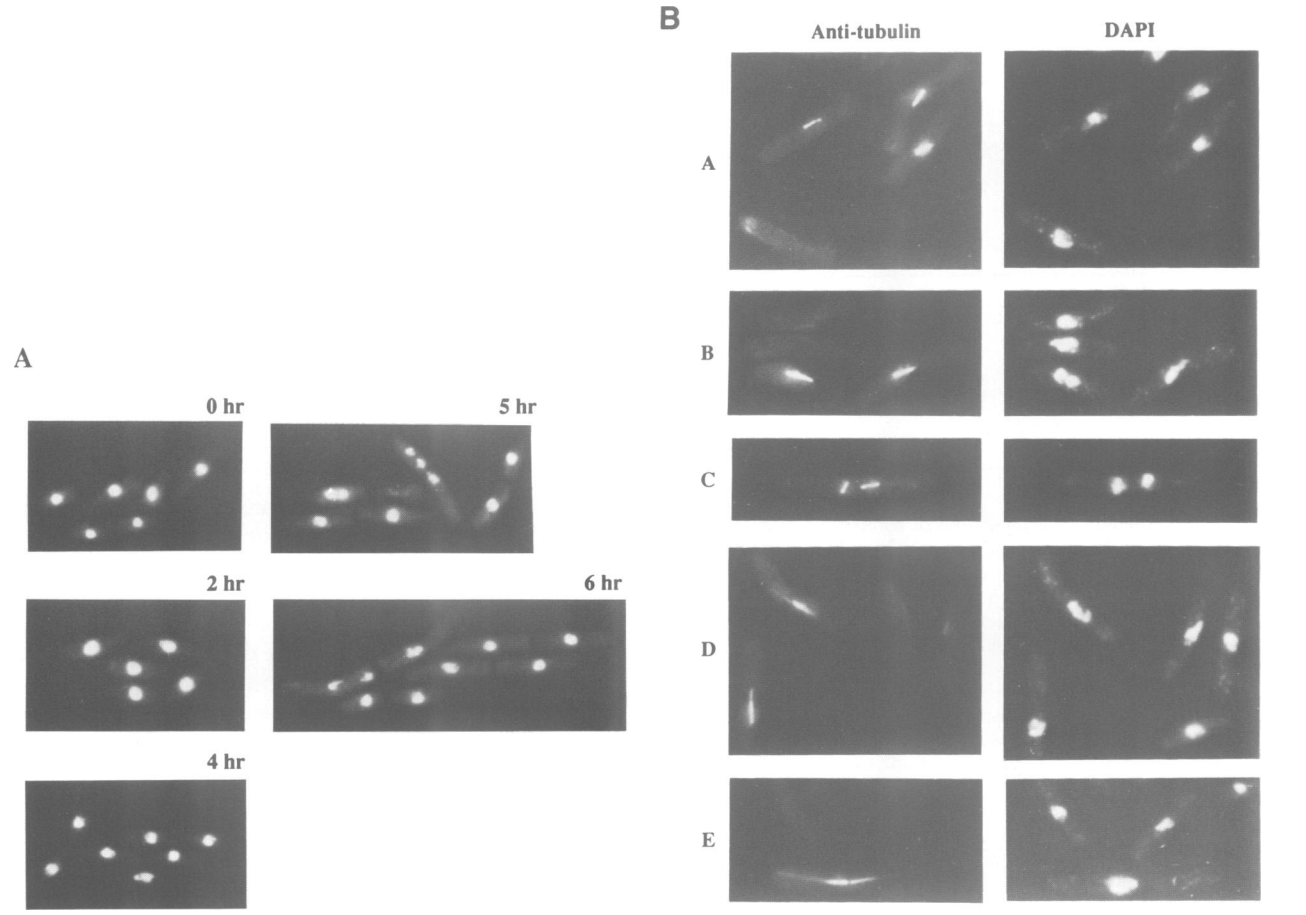

Fig. 9. Photomicrographs of cells expressing p34$^{cdc2\text{-}E167}$. (**A**) The *cdc2*-33 strain expressing p34$^{cdc2\text{-}E167}$ was grown at 25°C to a density of 5 × 10^6 cells/ml in the presence of 5 μg/ml thiamine and then placed at 36°C for the indicated number of hours. Aliquots of cells were fixed as described in the legend to Figure 8 and stained with DAPI. (**B**) The same cells as in (A) were taken at 4 h after shift to 36°C, fixed for immunofluorescence, and stained both with a rabbit anti-tubulin antibody followed by Texas Red-conjugated goat anti-rabbit Ig and with DAPI.

synchrony was achieved, $p34^{cdc2}$ in mitosis was found to be phosphorylated on a threonine but not on Tyr15 (Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). The phosphorylated threonine seen in these studies is almost certainly the equivalent of Thr167 and our molecular genetic studies are consistent with the result that $p34^{cdc2}$ is phosphorylated at Thr167 at a time when it is active.

We have found that Thr167 and Tyr15 become phosphorylated in the presence of hydroxyurea, indicating that these phosphorylation events are not dependent upon the completion of DNA synthesis and can occur during S phase, consistent with what has been observed in other cell types (Norbury *et al.*,1991; Krek and Nigg, 1991). However, in cells with a longer G_1 phase than is characteristic of fission yeast, ^{32}P-labelling studies have provided evidence that neither Thr167 nor Tyr15 is phosphorylated during G_1 (Lee *et al.*, 1988; Morla *et al.*, 1989; Krek and Nigg, 1990; Norbury *et al.*, 1991). These data suggest that Thr167 phosphorylation might not be involved in $p34^{cdc2}$ function at the G_1/S transition and appears only after S phase is initiated. Our flow cytometric analysis of a $cdc2^{ts}$ strain expressing $p34^{cdc2\text{-}A167}$ is of interest in this context. In a $cdc2^{ts}$ strain, the presence of $p34^{cdc2\text{-}A167}$ at the non-permissive temperature reduced the length of time those cells arrested in G_1. Although this is not what is expected if Thr167 phosphorylation was important for $p34^{cdc2}$ function in G_1, the effects of $p34^{cdc2\text{-}A167}$ on the G_1 block were small. Therefore, although this result makes the role of Thr167 phosphorylation in G_1 difficult to interpret, the combined observations on the timing of Thr167 and Tyr15 phosphorylation in several cell systems have raised the possibility that the strategy for regulating $p34^{cdc2}$ activity at the G_1/S transition, both positively and negatively, might be entirely different from what is utilized at the G_2/M transition.

In combination with *in vivo* ^{32}P-labelling studies which showed that Thr167 dephosphorylation occurred as cells passed through mitosis (Gould and Nurse, 1989), the phenotype of cells expressing $p34^{cdc2\text{-}E167}$ indicate that Thr167 dephosphorylation as well as phosphorylation might be important for normal cell cycle progression. We replaced Thr167 with the negatively charged residues, aspartate and glutamate, in an attempt to mimic constitutive phosphorylation at position 167. This approach has been used successfully for the investigation of regulatory phosphorylation events in the cAMP-dependent protein kinase (Levin and Zoller, 1990) and in isocitrate dehydrogenase (Thorness and Koshland, 1987; Dean and Koshland, 1990; Hurley *et al.*, 1990). In the former studies, aspartate seems to have more closely approximated the phosphorylated residue functionally than glutamate but in $p34^{cdc2}$, only the glutamate substitution gave a different phenotype than the alanine substitution. $p34^{cdc2\text{-}E167}$ was the only non-phosphorylated position 167 mutant to rescue, albeit poorly, $cdc2^{ts}$ mutants. Curiously, this mutant displayed no *in vitro* protein kinase activity indicating that although it can function in cells, its ability to form appropriate complexes might be compromised and the immune complex protein kinase assay might be too stringent a measure of its functional capacity. Flow cytometric analysis demonstrated that a $cdc2^{ts}$ strain expressing this mutant could pass through the G_1/S and G_2/M transitions repeatedly. However, cells accumulated

Table I.

Time	% Septated cells
0	15
1	18
2	22
3	33
4	55
5	78
6	93

The *cdc2*-33 strain expressing $p34^{cdc2\text{-}E167}$ was grown at 25°C to a density of 5×10^6 cells/ml and then placed at 36°C for the indicated number of hours, fixed and stained with DAPI. Cells were observed microscopically and the percentage of cells containing a septum was determined by counting 500 cells at each time point.

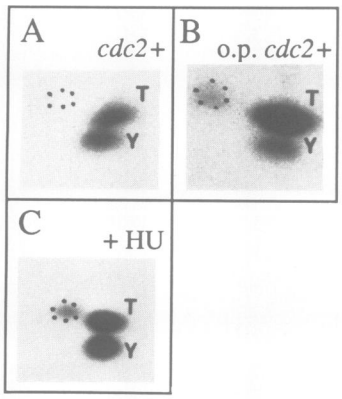

Fig. 10. Phosphoamino acid analyses. Equal numbers of wild-type *S.pombe* cells (**A**), cells overexpressing by ~5- to 20-fold $p34^{cdc2}$ from the multicopy plasmid pIRT2 (**B**), or wild-type *S.pombe* treated with hydroxyurea (**C**) were labelled with [^{32}P]orthophosphate, lysed and subjected to immunoprecipitation with PN24. Immunoprecipitates were resolved on SDS–polyacrylamide gels, transferred to immobilon-P, and partially hydrolysed in acid. The phosphoamino acids were separated in two dimensions by thin-layer electrophoresis. T, phosphothreonine; Y, phosphotyrosine; o.p., overproduced; HU, hydroxyurea. The dotted circles indicate the position of phosphoserine.

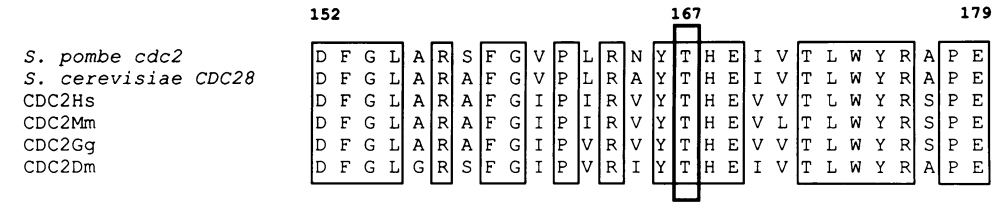

Fig. 11. Sequence alignment of $p34^{cdc2}$ proteins in the region of Thr167. Hs, human, Mm, mouse; Gg, chicken; Dm, *D.melanogaster*. Thr167 is boxed in bold lines.

septa and mitotic spindles which indicates that they were delayed in transit through mitosis. These results are consistent with the possibility that E167 does indeed mimic constitutive Thr167 phosphorylation and that p34^{cdc2} must ordinarily be entirely dephosphorylated in order to complete the cell cycle. Studies in cell free *Xenopus* extracts (Murray *et al.*, 1989) and in budding yeast (Ghiara *et al.*, 1991) have shown that cyclin B must be degraded in order to inactivate histone H1 kinase activity and exit from the mitotic state. Our results raise the possibility that p34^{cdc2} dephosphorylation might also have a role in the exit from mitosis.

Additional evidence that Thr167 phosphorylation is required for p34^{cdc2} function is provided by experiments examining the consequence of Thr167 dephosphorylation to p34^{cdc2} activity *in vitro*. Experiments with okadaic acid under conditions where it specifically inhibits PP2A have shown that PP2A activity negatively regulates p34^{cdc2} kinase activation in *Xenopus* egg extracts (Felix *et al.*, 1990) and that inhibiting PP2A can lead to early activation of p34^{cdc2} protein kinase activity in several cell systems (Goris *et al.*, 1989; Picard *et al.*, 1989; Yamashita *et al.*, 1990). Indeed, an endogenous activity, termed INH, which inhibited MPF activation in *Xenopus* oocyte extracts was recently shown to be a form of PP2A (Lee *et al.*, 1991). Lee *et al.* (1991) showed that PP2A treatment of p34^{cdc2} resulted in dephosphorylation of at least one phosphopeptide from p34^{cdc2} and that the overall dephosphorylation of p34^{cdc2} paralleled inactivation of histone H1 protein kinase activity. We have found that PP2A and protein phosphatase 1 (PP1) (data not shown) dephosphorylated p34^{cdc2} *in vitro* primarily at Thr167 and that these treatments resulted in a loss of p34^{cdc2} protein kinase activity. As noted by Lee *et al.* (1991), dephosphorylation of cyclin B complexed to p34^{cdc2} also occurs upon PP2A treatment (Lee *et al.*, 1991; our unpublished observations) and it is possible that cyclin dephosphorylation or dephosphorylation of other components bound to p34^{cdc2} is the event responsible for the observed inactivation of histone H1 kinase activity. Since it is not known whether or not cyclin phosphorylation is important for the activity of the p34^{cdc2}–cyclin complex or if other components might be present in these complexes, it is difficult to interpret fully the results of these experiments. However, these results raise the interesting question of what protein phosphatase removes phosphate from Thr167 *in vivo* and at what stage of the cell cycle.

Two isoforms of each PP2A (Kinoshita *et al.*, 1990) and PP1 (Booher and Beach 1989; Ohkura *et al.*, 1989) have been identified in *S.pombe* and both types of phosphatase have been linked to the regulation of the cell cycle (Booher and Beach, 1989; Okhura *et al.*, 1989; Kinoshita *et al.*, 1990). PP1 has also been identified as a regulator of the *Aspergillus nidulans* (Doonan and Morris, 1989) and *D.melanogaster* (Axton *et al.*, 1990) cell cycles. In *S.pombe*, deletion of *ppa2* which encodes the major PP2A activity leads to slow cell growth and reduced cell size at mitosis (Kinoshita *et al.*, 1990). Although there are probably many substrates of PP2A in cells, if Thr167 were one of its targets and the phosphatase activity was reduced, early entry into mitosis and perhaps a delay in exit from mitosis is exactly what one would expect. PP1 activity is required for exit from mitosis (Ohkura *et al.*, 1989; Kinoshita *et al.*, 1990) and also interacts genetically with regulators of p34^{cdc2} activation, *cdc25* and *wee1* (Booher and Beach, 1989). Given these genetic interactions and *in vitro* results, it would be interesting to learn whether the phosphorylation state or activity of p34^{cdc2} was affected by deleting or overexpressing these phosphatase genes.

p34^{cdc2} protein kinase activity in *S.pombe* requires the presence of a cyclin subunit and the ability of p34^{cdc2} to bind to it (Booher and Beach, 1987, 1988; Booher *et al.*, 1989; Moreno *et al.*, 1989). Our results indicate that Thr167 phosphorylation might be required for the stable association between p34^{cdc2} and cyclin B. We found that the non-phosphorylatable mutant, p34$^{cdc2\text{-}A167}$, was co-immunoprecipitated with antibodies to p56^{cdc13} but that the amount of p34$^{cdc2\text{-}A167}$ brought down in these co-immunoprecipitation experiments compared with the wild-type protein was significantly less (see Figure 7). Furthermore, mutants which cannot be phosphorylated on Thr167 contain phosphorylated Tyr15 but at an apparently reduced level (see Figure 2). Since Tyr15 phosphorylation is thought to occur only after cyclin B binding (Solomon *et al.*, 1990), it could be that although it is not a prerequisite for an initial interaction with cyclin B, Thr167 phosphorylation is required to form a stable cyclin B–p34^{cdc2} complex. Overproduction of p34^{cdc2+} in cells leads to a large increase in phosphorylation of Thr167 but not of Tyr15 (Figure 10). Since the amount of cyclin B in cells is probably limiting in this experiment, this result suggests that Thr167 phosphorylation might take place without prior association with cyclin B whereas phosphorylation of Tyr15 does not. In contrast, both Thr167 and Tyr15 phosphorylation increase when DNA replication is inhibited with hydroxyurea. This result is consistent with the observation that both cyclin B (Dasso and Newport, 1990) and p34^{cdc2} tyrosine phosphorylation increase (Kumagai and Dunphy, 1991) when DNA replication is blocked in *Xenopus* extracts. Although experiments in *Xenopus* cell-free extracts indicated that modification of p34^{cdc2} by phosphorylation occurs only after binding to cyclin B, these experiments would not have detected an early phosphorylation event which does not turn over during the course of the ^{32}P-labelling (Solomon *et al.*, 1990).

Thr167 lies in a highly conserved region of p34^{cdc2} (Figure 11) and in a sequence alignment is in the same position as the regulatory threonine phosphorylation site in MAP kinase (Boulton *et al.*, 1990; Hanks, 1991; Payne *et al.*, 1981). Like Thr167 phosphorylation of p34^{cdc2}, threonine phosphorylation of MAP kinase is required for its protein kinase activity (Anderson *et al.*, 1990). Despite their proximity to autophosphorylation sites in the cAMP-dependent protein kinase (Levin and Zoller, 1990) and in numerous protein-tyrosine kinases (Hanks, 1991), there is no evidence for the autophosphorylation of these sites. In fact, dephosphorylation of these threonines *in vitro* followed by the addition of Mg/ATP does not result in their re-phosphorylation (Payne *et al.*, 1991; our unpublished data). Thus, either Thr167 is the target of another protein kinase or Thr167 phosphorylation is an autophosphorylation event regulated by an unknown cellular factor.

Materials and methods

Strains and cell culture

The strains used in this study were the wild-type *S.pombe* strain 972 h$^-$ (Gutz *et al.*, 1974), *cdc25*-22 h$^-$, *cdc2*-33 *leu1*-32 h$^-$, *cdc2*-L7 *leu1*-32 h$^+$ and HY1 (*cdc2*::CDC2Hs, *leu1*-32, *his3*-237 h$^-$). The HY1 strain in

which the human *CDC2* cDNA has replaced the *S.pombe cdc2*$^+$ gene was provided by Stuart MacNeill. Cells were grown in minimal medium (Nurse, 1975) containing the appropriate supplements in the presence or absence of 5 μg/ml thiamine. For [^{32}P]orthophosphate labelling, cells were grown overnight to a density of $5 \times 10^6 - 1 \times 10^7$ cells/ml in phosphate-free minimal medium (Moreno *et al.*, 1991a) with 100 μg/ml NaH_2PO_4 and appropriate supplements. Cells were filtered, resuspended at 5×10^6 cells/ml in 5 ml phosphate-free minimal medium with 50 μg/ml NaH_2PO_4, and labelled with 1–2 mCi [^{32}P]orthophosphate (NEN) for 3–4 h. Hydroxyurea when used was added to cultures at a final concentration of 12 mM. For experiments which involved a temperature shift, cells were grown to a density of 5×10^6 cells/ml at 25°C and then placed at the restrictive temperature of 36°C. Transformations were carried out as described (Moreno *et al.*, 1991).

Oligonucleotide mutagenesis and plasmids

The *cdc2*$^+$ cDNA with a *Nde*I site at the initiation codon and a *Bam*HI site shortly after the stop codon (Gould and Nurse, 1989) in the phagemid pTZ19R (Pharmacia) was mutagenized using the Amersham *in vitro* mutagenesis kit according to the manufacturer's instructions. The following 20-base long oligonucleotides were used.

(1) CGGAACTAT**TCG**CATGAGAT for Ser167
(2) CGGAACTAT**GCG**CATGAGAT for Ala167
(3) CGGAACTAT**TAT**CATGAGAT for Tyr167
(4) CGGAACTAT**GAT**CATGAGAT for Asp167
(5) CGGAACTAT**GAT**CATGAGAT for Glu167
(6) GAAGGAACC**TTT**GGCGTTGT for Phe15.

The coding regions of the mutant cDNAs were removed from pTZ19R by digesting with *Nde*I and *Bam*HI and cloned into pMNS21L under control of the thiamine-repressible *nmt1* promoter (Maundrell, 1990) using standard recombinant DNA techniques (Maniatis *et al.*, 1982). The resultant plasmids were then sequenced throughout the coding region of *cdc2* using the dideoxynucleotide techniques suggested in a Sequenase kit manual (US Biochemicals).

Immunoprecipitation, immunoblotting and protein kinase assays

Approximately 2×10^8 cells were washed once in 4 ml 0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, 50 mM NaF and lysed by vortexing with glass beads in 10 μl HB15 buffer (25 mM MOPS, pH 7.2, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM EDTA, 15 mM $MgCl_2$, 1 mM DTT, 100 μM sodium orthovanadate, 1 mM PMSF, 5 μg/ml leupeptin, 1% Nonidet P-40, 130 μM TLCK and 130 μM TPCK). For immunoprecipitation with the anti-C-terminal peptide antibody, PN24 (Simanis and Nurse, 1986), 250 μl of B buffer was added (10 M sodium phosphate, pH 7.0, 0.5% SDS, 1 mM DTT, 1 mM EDTA, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 1 mM PMSF, 4 μg/ml leupeptin and 4 μg/ml aprotinin), the lysate was brought to 100°C for 2 min and then diluted with 750 μl of buffer N (10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 5 μg/ml leupeptin, 1 mM PMSF). For immunoprecipitation with an antiserum raised against bacterially produced fission yeast p34^{cdc2}, 4711 (Gould and Nurse, 1989), 1 ml of buffer N was added. After two 30 min clearing spins in a microfuge, incubations with PN24 and 4711 were performed in antibody excess for 1 h on ice. Protein A–Sepharose was then added for 30 min at 4°C and the immune complexes were washed thoroughly with buffer N. For immunoblotting, the immune complexes were heated to 100°C for 2 min in 1 × SDS-gel sample buffer, resolved on SDS–15% polyacrylamide gels, transferred to immobilon-P (Millipore), and incubated with 400-fold dilutions of either PN24 or 4711 followed by [^{125}I]protein A. Protein kinase assays of the immune complexes were performed in 20 μl HB buffer with 100 μM unlabelled ATP, 0.2 μCi [^{32}P]ATP (3000 Ci/mmol) and 10 μg histone H1 (Boehringer Mannheim) for 10 min at 30°C. Reactions were stopped by the addition of 2 × sample buffer and loaded onto SDS–15% polyacrylamide gels. Phosphorylated histone H1 was visualized by autoradiography.

Flow cytometry and microscopy

For flow cytometric analysis, cells were fixed in 70% ethanol, stained with propidium iodide, and analysed as detailed previously (Sazer and Sherwood, 1990) using a FACScan flow cytometer. 4',6-Diamidino-2-phenylindole (DAPI) staining was as described (Moreno *et al.*, 1991a). For indirect immunofluorescence, cells were fixed with paraformaldehyde and glutaraldehyde and stained with a monoclonal antibody raised against rat tubulin followed by Texas Red-conjugated goat anti-mouse IgG essentially as described (Moreno *et al.*, 1991a). Cells were viewed with a Zeiss Axioskop microscope and photographs were taken using XP-1 film (Ilford).

Phosphoamino acid analysis

Slices of immobilon-P membrane containing ^{32}P-labelled p34^{cdc2} were subjected to partial acid hydrolysis (Kamps and Sefton, 1989) and the phosphoamino acids were separated in two dimensions by thin-layer electrophoresis at pH 1.9 and pH 3.5 (Cooper *et al.*, 1983).

Phosphatase treatments

p34^{cdc2} was immunoprecipitated with 4711 as detailed above and resuspended in 20 μl protein-tyrosine phosphatase buffer (25 mM HEPES, pH 7.2, 0.1% β-mercaptoethanol, 1 mM PMSF and 5 mM EDTA) or PP2A buffer (10 mM Tris, pH 7.5, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM $MnCl_2$ and 1 mM PMSF). PP2A in 1 μl was added to a final concentration of 10 mU/ml. One μl of the truncated 37 kDa form of the T-cell PTPase purified from SF9 cells with an undetermined specific activity was used to remove phosphotyrosine from p34^{cdc2} as reported previously (Gould *et al.*, 1990). Incubations with the phosphatases were performed at 30°C for 30 min.

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