The EMBO Journal vol.12 no.8 pp.3123–3132, 1993

The cdc2-related protein p40^{MO15} is the catalytic subunit of a protein kinase that can activate p33^{cdk2} and p34^{cdc2}

Randy Y.C.Poon, Katsumi Yamashita, Jörg P.Adamczewski, Tim Hunt[1] and John Shuttleworth[2]

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Potters Bar, Herts, EN6 3LD and [2]Department of Anatomy, The Medical School, University of Birmingham, Birmingham, B15 2TJ, UK

[1]Corresponding author

Communicated by T.Hunt

Activation of the cyclin-dependent protein kinases p34^{cdc2} and p33^{cdk2} requires binding with a cyclin partner and phosphorylation on the first threonine residue in the sequence THEVVTLWYRAPE. We present evidence that this threonine residue, number 160 in p33^{cdk2}, can be specifically phosphorylated by a cdc2-related protein kinase from *Xenopus* oocytes called p40^{MO15}. Binding to cyclin A and phosphorylation of this threonine are both required to activate fully the histone H1 kinase activity of p33^{cdk2}. In cell extracts, a portion of p40^{MO15} is found in a high molecular weight complex that is considerably more active than a lower molecular weight form. Wild-type MO15 protein expressed in bacteria does not possess kinase activity, but acquires p33^{cdk2}-T160 kinase activity after incubation with cell extract and ATP. We conclude that p40^{MO15} corresponds to CAK (cdc2/cdk2 activating kinase) and speculate that, like p33^{cdk2} and p34^{cdc2}, p40^{MO15} requires activation by phosphorylation and association with a companion subunit.

Key words: bacterial expression/CAK/cell cycle/cyclin/kinase cascade

Introduction

Cyclins and cyclin dependent protein kinases (cdk) are key components of the eukaryotic cell cycle engine (reviewed by Norbury and Nurse, 1992). In both fission and budding yeasts, the protein kinase activity of the product of the *cdc2/CDC28* gene is required for two key cell cycle transitions: passage through Start, and the $G_2 \rightarrow M$ transition. In budding yeast, a large number of cyclins are now known, which presumably produce protein kinases with different substrate specificity even though they share a common catalytic core (reviewed by Nasmyth, 1993). In higher eukaryotes, at least two cdc2-like catalytic subunits are known that appear to be specialized for mitotic and G_1/S-phase functions. In mammalian cells, the gene product corresponding to cdc2/CDC28 is p34^{cdc2} (Lee and Nurse, 1987), which is a component of maturation promoting factor (MPF) (Dunphy *et al.*, 1988; Gautier *et al.*, 1988) that plays an essential role in the entry to mitosis (Nurse, 1990). A closely related kinase subunit, p33^{cdk2}, is also found in vertebrates (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991; Hirai *et al.*, 1992), functions somewhat less well as a substitute for cdc2/CDC28 in yeast, and is thought to function earlier in the cell cycle than p34^{cdc2} (Koff *et al.*, 1991, 1992; Dulić *et al.*, 1992; Lees *et al.*, 1992). In mammalian cells, p33^{cdk2} associates with cyclin E in G_1-phase (Dulić *et al.*, 1992; Koff *et al.*, 1992) and cyclin A during S-phase (Rosenblatt *et al.*, 1992), whereas active forms of p34^{cdc2} in mitosis are principally associated with B-type cyclins (Labbé *et al.*, 1989; Pines and Hunter, 1989; Gautier *et al.*, 1990; Whitfield *et al.*, 1990).

The activity of p33^{cdk2} and p34^{cdc2} is regulated not only by association with cyclins, but also by an intricate network of protein kinases and phosphatases (reviewed by Fleig and Gould, 1991). Most recent genetic and biochemical work has focused on inhibitory phosphorylations at residues T14 and Y15, where the Wee1$^+$ protein kinase (Russell and Nurse, 1987; Featherstone and Russell, 1991; Parker *et al.*, 1992) and Cdc25 family of tyrosine phosphatases (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991; Lee *et al.*, 1992) have mutually antagonistic roles. Much less is known about the genes and enzymes that are responsible for the state of phosphorylation of T167/161 (in *Schizosaccharomyces pombe*/human p34^{cdc2}) or T160 in p33^{cdk2}. It is clear that this threonine residue is phosphorylated in active forms of p33^{cdk2} (Gu *et al.*, 1992) and p34^{cdc2} (Krek and Nigg, 1992; Solomon *et al.*, 1992); mutations at this site that produce non-phosphorylatable forms of p34^{cdc2} or p33^{cdk2} abolish their activity (Booher and Beach, 1986; Ducommun *et al.*, 1991; Gould *et al.*, 1991), and can give dominant-negative forms of the kinase (Fleig *et al.*, 1992). In the case of p34^{cdc2}, the logic of the phosphorylation states is clear: the only significantly active forms of the kinase have phosphorylated T161/167 and non-phosphorylated T14/Y15 residues (Solomon *et al.*, 1990; Norbury *et al.*, 1991). Therefore, the enzymes responsible for the activating phosphorylation, which has been partially purified and called CAK (cdc2 activating kinase) by Solomon *et al.* (1992) should, as they put it, be 'a central component in the [cell cycle] regulatory network'.

In this paper, we describe a biochemical assay for the p33^{cdk2} activating T160 kinase that differs in significant respects from the one developed by Solomon *et al.* (1992). In place of *in vitro* synthesized p34^{cdc2}, we used bacterially expressed glutathione-S-transferase (GST)–cdk2 and protein A–cyclin A (PA–cyclin A) as substrates. As purified from bacteria, GST–cdk2 is inactive as a protein kinase, but it can be activated by incubation with PA–cyclin A, ATP and a cellular factor. We were surprised to discover that the majority (>85%) of this activating factor could be depleted by antibodies raised against another member of the cdc2 family, known as p40^{MO15}, which was originally isolated from *Xenopus* oocytes during a search for homologues of frog p34^{cdc2} (Shuttleworth *et al.*, 1990). Furthermore, the

immunoprecipitates carrying $p40^{MO15}$ are able to phosphorylate wild-type GST−cdk2 on a threonine residue, and display no kinase activity towards the same substrate in which T160 is mutated to alanine. Although GST−MO15 fusion protein produced in bacteria is not active as a protein kinase, it acquires such activity after incubation with cell extracts in the presence of ATP. Two forms of $p40^{MO15}$ can be detected in cell extracts, one with an apparent molecular weight of 150−200 kDa on gel-filtration, the other of ~80 kDa. The larger form has considerably higher specific activity as an activator of cyclin A−$p33^{cdk2}$. Therefore, we strongly suspect that $p40^{MO15}$ requires an activating partner that may play the equivalent role as do cyclins for $p34^{cdc2}$. We find that immunoprecipitates containing $p40^{MO15}$ are capable of phosphorylating both $p33^{cdk2}$ and $p34^{cdc2}$, but do not phosphorylate GST−MO15 or a member of the PCTAIRE family of cdc2-related kinase subunits.

Essentially the same conclusion was reached independently at the same time using a different assay, a different approach (protein purification and peptide sequencing) and a different organism (starfish, rather than frogs), by the cell cycle group in Montpellier. Their results, which complement ours, are described in the accompanying paper (Fesquet *et al.*, 1993).

Results

Expression and purification of recombinant $p33^{cdk2}$ and cyclin A from bacteria

To develop a biochemical assay for the activation of cyclin−cdk complexes, we used *Escherichia coli* strain BL21(DE3) to overexpress recombinant proteins. A substantial portion of both GST−cdk2 (human) and PA−cyclin A (bovine) fusion proteins were soluble when their synthesis was induced at 23°C with IPTG. GST−cdk2 was purified by affinity chromatography on glutathione (GSH)−Sepharose (see Materials and methods). Cyclin A, tagged at its N-terminus with protein A (Bandara *et al.*, 1991; Kobayashi *et al.*, 1992) was further modified by the addition of 10 histidine residues to its N-terminus, which allowed it to be purified in one step on a nickel−agarose column (Hoffmann and Roeder, 1991).

Recombinant cyclin A can bind to and activate $p34^{cdc2}$ and $p33^{cdk2}$

To check that the recombinant PA−cyclin A was active, it was added to *Xenopus* egg extracts, which were then immunoprecipitated either with $p34^{cdc2}$ or $p33^{cdk2}$-specific antibodies. Figure 1 shows that both $p34^{cdc2}$ and $p33^{cdk2}$ kinases were rapidly activated by the recombinant cyclin A. The cdk-bound PA−cyclin A was detected on the immunoblots by virtue of its N-terminal protein A domain, which bound to the IgG used in the ECL detection system. The amount of PA−cyclin A binding to $p34^{cdc2}$ or $p33^{cdk2}$ was roughly proportional to the kinase activity of the complex (Figure 1, bottom panel). These results show that PA−cyclin A was active as a companion subunit for $p34^{cdc2}$ and $p33^{cdk2}$ kinase subunits. The rapid activation of the histone H1 kinase activity by PA−cyclin A suggested that it had a native configuration, and probably did not require additional protein folding factors in the *Xenopus* extract to assist its binding to the $p34^{cdc2}$ or $p33^{cdk2}$ subunit.

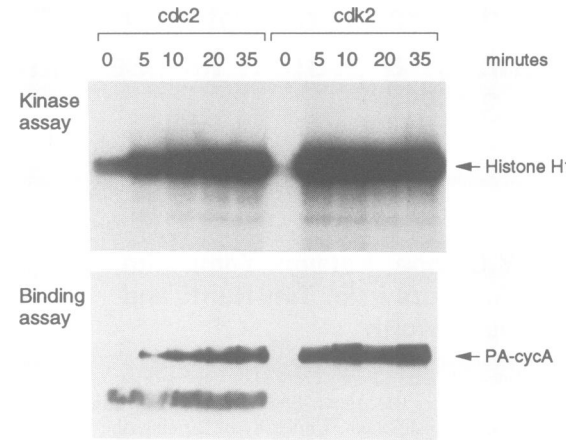

Fig. 1. PA−cyclin A binds to and activates both $p34^{cdc2}$ and $p33^{cdk2}$. *Xenopus* egg extract was incubated with 100 μg/ml PA−cyclin A protein. At the indicated time points, two aliquots of 10 μl each were frozen in liquid N_2. After thawing, one set of samples was immunoprecipitated with A17 monoclonal anti-$p34^{cdc2}$, and the other set with polyclonal rabbit anti-$p33^{cdk2}$ as indicated. The histone H1 kinase activities of the immunoprecipitates were assayed and analysed by SDS−PAGE (upper panel). The same samples were immunoblotted with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies (Dakopatts: 1:5000 dilution), and developed with ECL to visualize the PA−cyclin A (lower panel). The lower band present in the anti-$p34^{cdc2}$ lanes represents immunoglobulins from the mouse monoclonal anti-$p34^{cdc2}$, to which the probe antibody also bound.

Cyclin A−cdk2 can be activated by incubation with cell extracts

We next investigated whether recombinant GST−cdk2 and PA−cyclin A proteins would bind together to form a heterodimer with histone H1 kinase activity; and if not, what was required for their association and activation. GSH−Sepharose was used to recover the GST−cdk2 from extracts after various incubations, allowing us to distinguish between the activity of the added GST−cdk2 and endogenous $p34^{cdc2}$, $p33^{cdk2}$ and related kinases that were present in crude cell extracts.

As purified from bacteria, GST−cdk2 had negligible histone H1 kinase activity (Figure 2, lane 4). GST−cdk2 could bind to PA−cyclin A, however, and as previously found by Connell-Crowley *et al.* (1993), even without other additions, the kinase activity of this complex was always appreciably higher than that of GST−cdk2 alone (Figure 2, lane 3). But when GST−cdk2 and PA−cyclin A were incubated with cell extracts, and recovered by affinity chromatography on GSH−Sepharose, a 50-fold increase in histone H1 kinase activity occurred (Figure 2A, lane 1). Scanning densitometry showed that the amount of PA−cyclin A binding to GST−cdk2 was increased ~2-fold by this procedure (Figure 2B, comparing lanes 1 and 3), but this enhanced binding was insufficient to account for the increased kinase activity. The kinase activity did not appear to be due to non-specific binding of endogenous kinase to the beads, because in the absence of added GST−cdk2 or PA−cyclin A, no histone H1 kinase activity was detected (Figure 2A, lane 5). As a further control, we used a mutant construct of PA−cyclin A lacking 16 amino acid residues at its C-terminus (PA−cyclin A CΔ16), which binds GST−cdk2 very weakly, and did not significantly activate

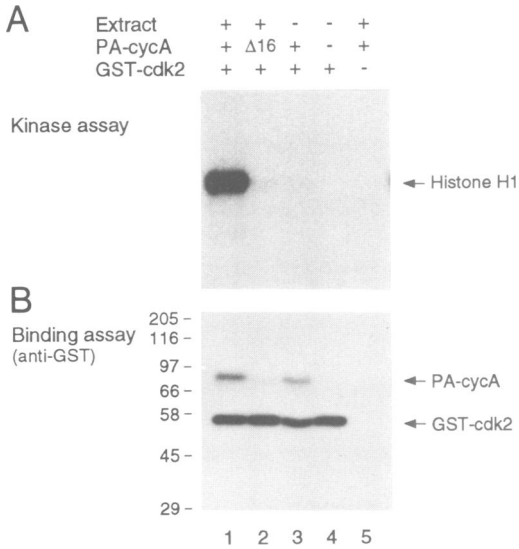

Fig. 2. Activation of cdk2 by cyclin A. (**A**) Different combinations of PA–cyclin A, GST–cdk2 and *Xenopus* interphase egg extract were mixed as indicated. After incubation for 1 h, GST–cdk2 (and proteins bound to it) was harvested with GSH–Sepharose (see Materials and methods). In cases where a component was omitted, it was replaced with an equivalent volume of buffer B. In lane 2, the mutant PA–cyclin A CΔ16 was used in place of the wild-type PA–cyclin A. The histone H1 kinase activity associated with the GSH–Sepharose was assayed and detected by SDS–PAGE and autoradiography. (**B**) The corresponding samples were immunoblotted with anti-GST antibody to visualize both the GST–cdk2 and the PA–cyclin A. The positions of molecular weight markers in kDa are shown on the left.

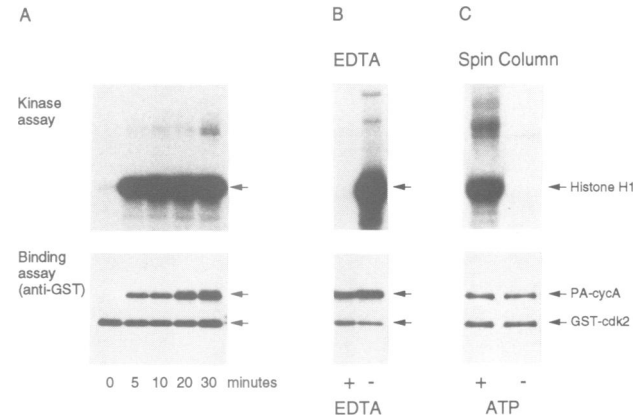

Fig. 3. Cyclin A–cdk2 activation is rapid and requires ATP. (**A**) GST–cdk2 and PA–cyclin A protein (5 μg each) were added to 100 μl of HeLa cell extract and incubated at 23°C. At the indicated times, 20 μl samples were removed and immediately diluted with 250 μl of ice cold bead buffer. After all the samples were taken, GST–cdk2 was recovered with GSH–Sepharose and the histone H1 kinase activity associated with the beads measured as before (upper panel). The samples were also immunoblotted with anti-GST antibody (lower panel). (**B**) EDTA inhibits the activation of cyclin A–cdk2 by cell extract. The cyclin A–cdk2 activator assay was performed with *Xenopus* CSF-arrested egg extract, PA–cyclin A and GST–cdk2 as described in Materials and methods. Where indicated, 10 mM (final) EDTA was included in the reaction during incubation with the egg extract. The top panel shows the phosphorylation of histone H1 kinase and the lower panel shows the corresponding lanes immunoblotted with anti-GST antibody. (**C**) Activation of cyclin A–cdk2 requires ATP. A spin column containing 1.5 ml of Sephadex G50 coarse (Pharmacia) was equilibrated with buffer A (10 mM HEPES-KOH pH 7.2, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 15 μg/ml benzamidine) and spun at 200 *g* for 2 min. 100 μl of HeLa cell extract was applied to the column and spun at 200 *g* for 2 min. The eluate was made 15 mM in $Mg(OAc)_2$ and assayed for cyclin A–cdk2 activator as described in Materials and methods. Where indicated, 1 mM ATP was added to the extract after spin column treatment.

the kinase activity of GST–cdk2 (Figure 2, lane 2). Immunoblotting with anti-GST antibody (Figure 2B) showed that the recovery of GST–cdk2 was the same in all cases where it was added. Hence, recombinant cyclin A and p33^{cdk2} could bind together to form a stable complex with low activity, and some factor in the cell extract was capable of activating p33^{cdk2} in a cyclin dependent manner. The activating factor was present in both *Xenopus* egg extract and human HeLa cell extracts. The activation of cyclin A–p33^{cdk2} by cell extracts occurred rapidly, without a detectable lag phase (Figure 3A). Fully activated PA–cyclin A–GST–cdk2 could transfer ~2 pmol $^{32}PO_4$ to histone H1 per min per pmol GST–cdk2 under standard assay conditions. This is at the low end of values for p33^{cdk2} reported by Connell-Crowley *et al.* (1993) and for p34^{cdc2} by Erikson and Maller (1989).

The amount of the cdk2 activator did not appear to vary during the cell cycle; its activity was the same in a *Xenopus* egg extract in M-phase (CSF-arrested) as in interphase [CSF-arrested extracts released by the addition of Ca^{2+} in the presence of cycloheximide (Shamu and Murray, 1992)]. The activator was also present in extracts of stage VI oocytes at essentially the same level as in extracts of mature eggs (data not shown).

Activation of cyclin A–GST–cdk2 requires ATP

We next asked whether the activation of cyclin A–p33^{cdk2} as a histone H1 kinase required Mg^{2+}-ATP. When 10 mM EDTA was added to the cell extract before incubation with GST–cdk2 and PA–cyclin A, the kinase activity of GST–cdk2 did not turn on (Figure 3B). If the cell extract was first passed through a G50 Sephadex spin column to remove low molecular weight compounds, the activation of cyclin A–p33^{cdk2} was lost, and could be restored by addition of Mg^{2+}-ATP (Figure 3C). Neither EDTA nor spin column treatment affected the binding of PA–cyclin A to GST–cdk2 (Figure 3B and C, lower panel), suggesting that while the activation of cyclin A–p33^{cdk2} required ATP, the binding of cyclin A to p33^{cdk2} did not.

Preliminary attempts to purify the activator from extracts of HeLa cells were partially successful, in that activity could be detected after several steps of conventional purification, but even the most highly fractionated samples contained many polypeptide bands.

What is the substrate for p40^{MO15}?

The idea of testing p40^{MO15} for its ability to activate cyclin A–p33^{cdk2} was suggested by the following observations. No protein kinase activity could be detected in immunoprecipitates containing p40^{MO15}, or in acrylamide gels following electrophoresis of immunoprecipitated p40^{MO15}, when conventional protein kinase substrates such as histone H1, casein or myelin basic protein were used (see Figures 4A, comparing lanes 1 and 5, and 5, lanes 3 and 7). Moreover, the immunoprecipitates showed no significant self-phosphorylation. Therefore, since p40^{MO15} is largely localized in the germinal vesicle of stage VI *Xenopus*

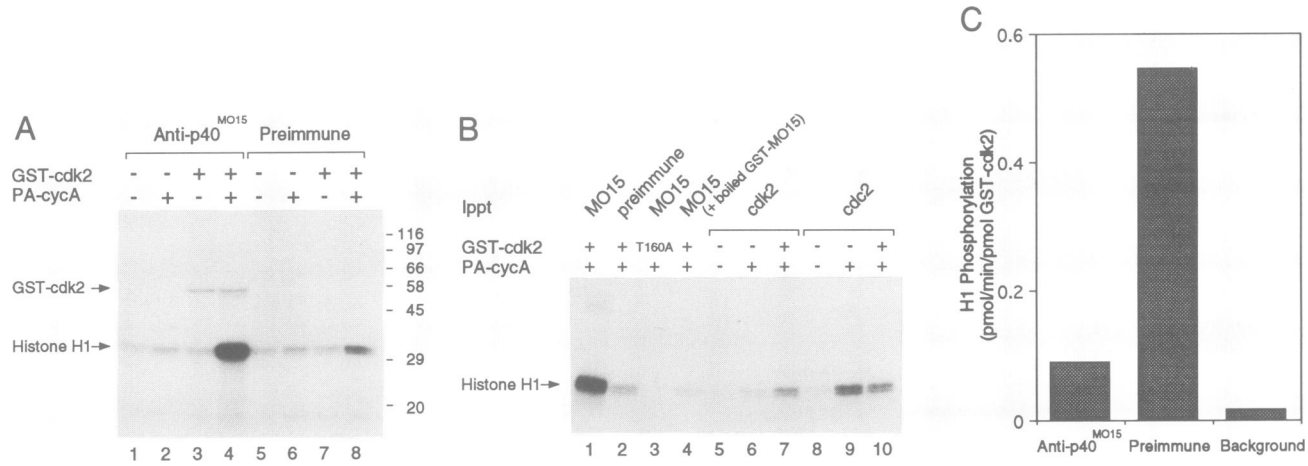

Fig. 4. Activation of cyclin A−cdk2 by $p40^{MO15}$ immunoprecipitates. **(A)** Immunoprecipitates with anti-$p40^{MO15}$ (lanes 1−4) or preimmune serum (lanes 5−8) were incubated alone (lanes 1 and 5), with 100 μg/ml PA−cyclin A (lanes 2 and 6), 100 μg/ml GST−cdk2 (lanes 3 and 7) or with PA−cyclin A and GST−cdk2 together (lanes 4 and 8). After incubation at 23°C for 20 min, the histone H1 kinase activity was assayed. **(B)** Immunoprecipitates with anti-$p40^{MO15}$ serum (lanes 1, 3 and 4), preimmune serum (lane 2), anti-$p33^{cdk2}$ (lanes 5−7) or anti-$p34^{cdc2}$ (lanes 8−10) were incubated with or without PA−cyclin A and GST−cdk2 as indicated, and then assayed for histone H1 kinase activity. Mutant GST−cdk2 T160A replaced wild-type GST−cdk2 in lane 3, and boiled GST−MO15 was used to block the anti-$p40^{MO15}$ in lane 4. **(C)** Immunodepletion of $p40^{MO15}$ from cell extract. *Xenopus* egg extracts were subjected to two rounds of immunoprecipitation with anti-$p40^{MO15}$ antiserum (left column) or preimmune serum (middle column), and the CAK activity of the supernatants measured. An activation assay containing buffer instead of cell extract provided the background level shown in the right-hand column.

oocytes (J.Shuttleworth, unpublished data), we sought candidate substrates in crude extracts of manually dissected germinal vesicles. These extracts were first depleted of endogenous $p40^{MO15}$ by immunoprecipitation, and the resulting mixture of proteins were incubated with $p40^{MO15}$ immune complexes bound to protein A−Sepharose, together with [γ-^{32}P]ATP. These immune complexes efficiently phosphorylated a set of polypeptides with molecular weights in the 32−34 kDa range, whereas preimmune complexes had no such activity (data not shown). The molecular weight of these apparent target proteins suggested that they might be members of the cdc2 family.

Activation of cyclin A−$p33^{cdk2}$ by $p40^{MO15}$

We therefore tested $p40^{MO15}$ immunoprecipitates from *Xenopus* egg extracts for their ability to serve as the activator for PA−cyclin A−GST−cdk2 as shown in Figure 4A. The immunoprecipitates containing $p40^{MO15}$ did not show significant histone H1 kinase activity above the background given by preimmune serum. Addition of PA−cyclin A or GST−cdk2 singly to the immunoprecipitates produced little or no increase in histone H1 kinase activity, but when all three components: $p40^{MO15}$ immunoprecipitate, PA−cyclin A and GST−cdk2 were incubated together in the presence of ATP, histone H1 kinase activity was strongly activated. Preimmune complexes gave no significant stimulation of histone H1 kinase above the level seen with PA−cyclin A and GST−cdk2 alone. Very significantly, lanes 3 and 4 of Figure 4 show that a polypeptide with the same gel mobility as GST−cdk2 was phosphorylated by the $p40^{MO15}$ immunoprecipitate when GST−cdk2 was present, whether or not PA−cyclin A was added. No labelling of GST−cdk2 occurred in the presence of the control immunoprecipitate. Figure 4B shows a series of control incubations. Thus, substitution of GST−cdk2 by the mutant, GST−cdk2 T160A, caused complete loss of activity, and immunoprecipitation of the cdk2 activator by the anti-$p40^{MO15}$ antibody was blocked by pre-incubation with denatured GST−MO15 fusion protein (Figure 4B, lane 4).

Since the sequences of $p34^{cdc2}$ and $p33^{cdk2}$ are very similar to that of $p40^{MO15}$, we checked whether immunoprecipitates made with anti-$p34^{cdc2}$ or anti-$p33^{cdk2}$ immune sera could activate the PA−cyclin A−GST−cdk2 complex. Figure 4B shows that neither of these immunoprecipitates showed significant activity in the assay.

To test if $p40^{MO15}$ represented the major component of the cdk2 activator in *Xenopus* cell extracts, two rounds of immunodepletion of frog egg extracts were performed, using either preimmune or immune anti-$p40^{MO15}$ antisera. Figure 4C shows that the cdk2 activator was depleted by >85% by the anti-$p40^{MO15}$ antibody compared with preimmuine serum. Taken together, these data strongly suggested that $p40^{MO15}$, or a protein tightly associated with $p40^{MO15}$, was capable of activating the cyclin A−cdk2 complex, and that it accounted for the majority of the activity in crude extracts of *Xenopus* oocytes and eggs.

Immunoprecipitates of $p40^{MO15}$ can activate the histone H1 kinase activity of cyclin A−$p34^{cdc2}$ complexes

We have been unable to produce $p34^{cdc2}$ as an active GST fusion protein in bacteria, even though *Xenopus* GST−cdc2 is soluble (whereas for some reason, the equivalent human construct is not). This soluble GST−$cdc2^{Xe}$ can, however, be activated by incubation in crude cell extracts in the presence of added PA−cyclin A. To test whether $p34^{cdc2}$ could serve as a substrate for the $p40^{MO15}$ immunoprecipitates, bacterially expressed GST−$cdc2^{Xe}$ was incubated with *Xenopus* egg extracts, which appears to promote its correct folding. The GST−$cdc2^{Xe}$ was then harvested on GSH−Sepharose, and the $p34^{cdc2}$ released from the GST by digestion with thrombin (see Materials and methods). We prepared $p33^{cdk2}$ in parallel in a similar manner. The released *Xenopus* $p34^{cdc2}$ and human $p33^{cdk2}$ were then tested for their ability to be activated by the $p40^{MO15}$ immunoprecipitate and PA−cyclin A. As shown in Figure 5, both $p34^{cdc2}$ and $p33^{cdk2}$ were strongly

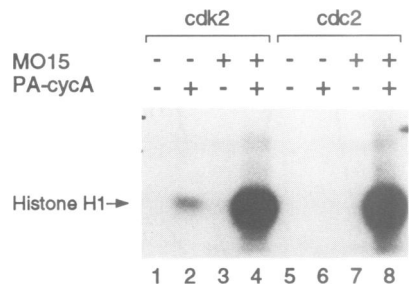

Fig. 5. Activation of p34^{cdc2} and p33^{cdk2} by p40^{MO15} immunoprecipitates. GST–cdk2 of GST–cdc2 were incubated at a concentration of 100 μg/ml with *Xenopus* egg extract at 23°C for 30 min to obtain correct folding of GST–cdc2. GST fusion proteins were then isolated on GSH–Sepharose beads and digested with thrombin. The supernatant containing p33^{cdk2} (lanes 1–4) and p34^{cdc2} (lanes 5–8) released by thrombin digestion were incubated with PA–cyclin A (even numbered lanes) and p40^{MO15} immunoprecipitates (lanes 3, 4, 7 and 8), and the histone H1 kinase activity measured.

activated in a p40^{MO15} and cyclin A dependent manner by this procedure, suggesting that, like CAK, p40^{MO15} is capable of activating both p33^{cdk2} and p34^{cdc2} (see Connell-Crowley *et al.*, 1993). It should be noted that a certain amount of activation of p33^{cdk2} by cyclin A occurred when p40^{MO15} was omitted (Figure 5, lane 2), which did not occur in the case of p34^{cdc2}.

p40^{MO15} phosphorylates p33^{cdk2} exclusively on threonine 160

As discussed in the introduction, p33^{cdk2} is normally phosphorylated on threonine 160 *in vivo* (Gu *et al.*, 1992) and phosphorylation of the equivalent site (T161) in p34^{cdc2} is required for the kinase activity of p34^{cdc2} (Gould *et al.*, 1991; Desai *et al.*, 1992; Solomon *et al.*, 1992). To test whether p40^{MO15} was acting as a specific T160 kinase of p33^{cdk2}, various forms of GST–cdk2 were used as a kinase substrate for p40^{MO15} immunoprecipitates (see also Figure 4B). In order to preclude the possibility of autophosphorylation by p33^{cdk2}, we used a kinase-inactive mutant of GST–cdk2 in which K33 was replaced by an arginine (K33R). [The equivalent of this lysine residue is conserved in all protein kinases and is required for transfer of phosphate from ATP to the protein substrate (Hanks *et al.*, 1988).] Figure 6 shows that when bacterially expressed GST–cdk2 K33R was incubated with immunoprecipitates of p40^{MO15} and [γ-^{32}P]ATP, it became strongly labelled. On the other hand, a mutant of GST–cdk2 in which threonine 160 was altered to alanine (GST–cdk2 T160A) was not detectably phosphorylated by the p40^{MO15} immunoprecipitate (lanes 7 and 8). As was seen in the experiment shown in Figure 4 for wild-type GST–cdk2 (compare lanes 3 and 4), phosphorylation of GST–cdk2 K33R by the p40^{MO15} immunoprecipitate occurred equally well whether or not cyclin A was present (Figure 6, lanes 3 and 4). Phosphoamino acid analysis of the phosphorylated GST–cdk2 K33R confirmed that phosphorylation occurred exclusively on threonine (Figure 6B). Neither the K33R nor the T160A mutants of GST–cdk2 could be activated as a protein kinase, in contrast to wild-type GST–cdk2 or the T14A+Y15F mutant (Figure 7), although these mutant constructs of p33^{cdk2} all bound cyclin A to the same extent as wild-type GST–cdk2 (Figure 7), suggesting that none of these point mutations caused serious misfolding of the p33^{cdk2} moiety.

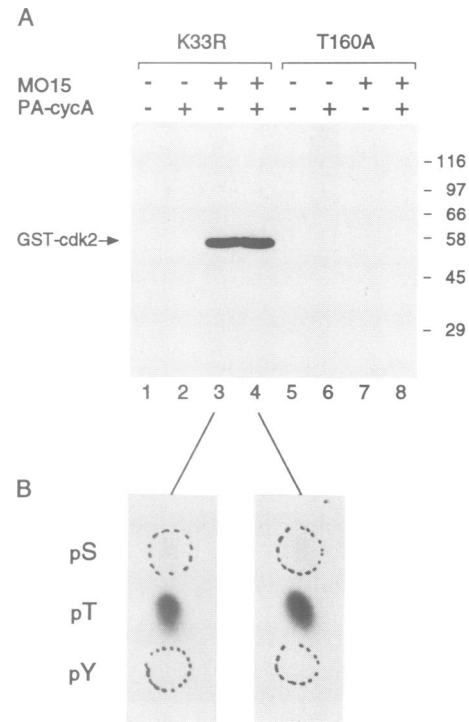

Fig. 6. p40^{MO15} phosphorylates Thr160 of cdk2. (**A**) Kinase inactive mutant GST–cdk2 K33R (lanes 1–4), or GST–cdk2 T160A (lanes 5–8) were incubated with anti-p40^{MO15} immunoprecipitates (lanes 3–4 and 7–8) and [γ-^{32}P]ATP either in the presence (even numbered lanes) or absence (odd numbered lanes) of PA–cyclin A. (**B**) One dimensional phosphoamino-acid analysis of the radioactively labelled bands from Figure 5A, lanes 3 and 4. The positions of internal marker phosphoserine (pS) phosphothreonine (pT) and phosphotyrosine (pY) are indicated.

Hence, p40^{MO15} appears to be able to act as a very specific protein kinase that phosphorylates T160 of p33^{cdk2} [we have not checked its ability to phosphorylate T161 of cdc2, but the accompanying paper by Fesquet *et al.* (1993) confirms this point]. The GST–cdk2 construct contains 22 other threonine, and 24 serine residues, none of which were phosphorylated under the assay conditions we used. It thus seems appropriate to consider the cdk2 activator as p33^{cdk2} T160 kinase, or CAK (cdk2 activating kinase). Solomon *et al.* (1992) called the p34^{cdc2} activator CAK, standing for cdc2 activating kinase, but since it seems that the same enzyme can also phosphorylate p33^{cdk2}, CAK should stand for cdc2/cdk2 activating kinase; it would be premature to consider it as an activator of all cyclin dependent protein kinases before further tests of specificity have been conducted.

p40^{MO15} probably forms a complex with another protein(s)

Using the activation of PA–cyclin A–GST–cdk2 dependent histone H1 kinase activity in parallel with the phosphorylation of GST–cdk2 K33R as assays, we have partially purified CAK from *Xenopus* oocytes, using sequential chromatography on DEAE–Sepharose, ammonium sulfate precipitation, hydroxyapatite, threonine Sepharose,. Mono Q and gel filtration. The cyclin A–cdk2 kinase activator and the protein kinase activity towards GST–cdk2 T160 co-migrated on all columns (a tyrosine kinase of GST–cdk2 present in the cell extract was removed by the DEAE step). During the early stages of purification (up to

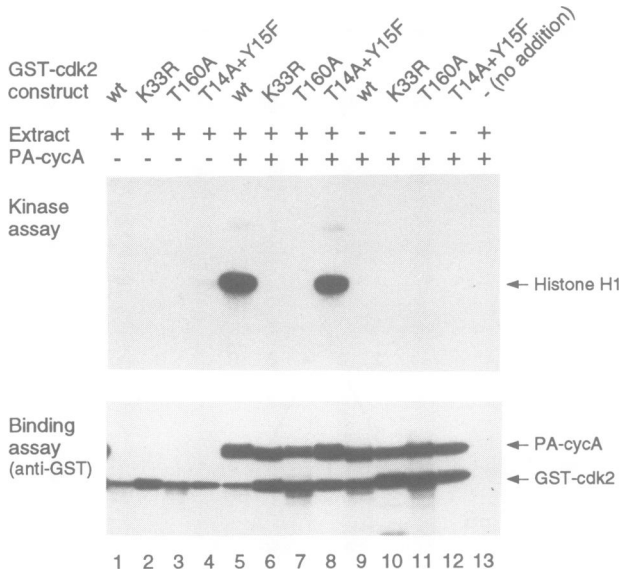

Fig. 7. K33R and T160A mutations abolish the cyclin dependent kinase activity of GST−cdk2. Upper panel: equal amount of wild-type GST-cdk2 (lanes 1, 5 and 9), GST−cdk2 K33R mutant (lanes 2, 6 and 10), GST−cdk2 T160A mutant (lanes 3, 7 and 11) or GST−cdk2 T14A/Y15F mutant (lanes 4, 8 and 12) were incubated with *Xenopus* CSF-arrested extracts (lanes 1−8) and PA−cyclin A (lanes 5−12) as indicated; the GST−cdk2 proteins were harvested with GSH−Sepharose and the histone H1 kinase activities associated with the beads were measured as described in Materials and methods. Lower panel: the amount of GST−cdk2 and associated PA−cyclin A bound to the GSH-beads was visualized by immunoblotting with anti-GST antibody. A control without addition of GST−cdk2 had no histone H1 kinase activity and bound no PA−cyclin A (lane 13).

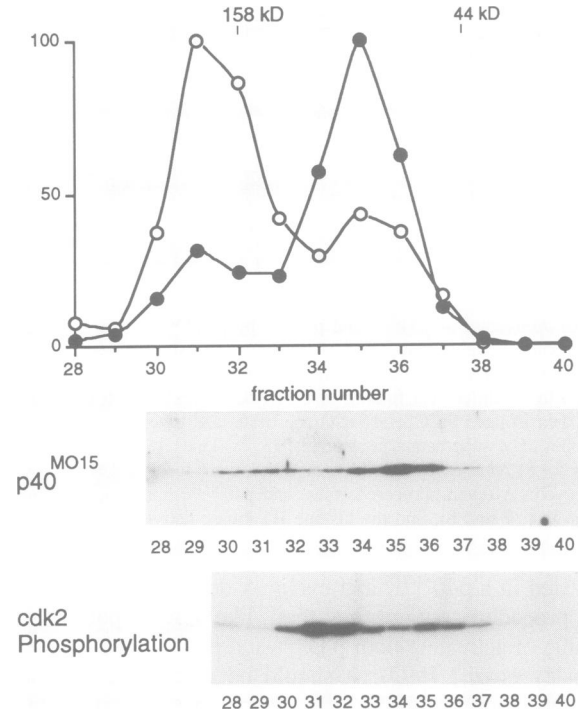

Fig. 8. *Xenopus* egg extract contained two populations of $p40^{MO15}$. A *Xenopus* CSF-arrested extract (300 μl) was subjected to gel filtration chromatography on a Superdex 200 column equilibrated with buffer B (flow rate 0.4 ml/min, 1 fraction/min). Fractions were immunoblotted with anti-$p40^{MO15}$ (●), and also immunoprecipitated with anti-$p40^{MO15}$ antibody and assayed for kinase activity towards the GST−cdk2 K33R (○). The top panel shows the quantitation of the lower panels, which show the $p40^{MO15}$ immunoblot and the autoradiogram of the GST−cdk2 K33R kinase assay as indicated.

threonine Sepharose), fractions containing CAK or GST−cdk2 K33R T160 kinase activity also contained $p40^{MO15}$, but the peaks of CAK activity and $p40^{MO15}$ level did not co-migrate exactly. After the threonine Sepharose step, however, there was accurate correspondence between $p40^{MO15}$ and CAK.

The imprecise correspondence between $p40^{MO15}$ and CAK appears to reflect genuine heterogeneity of the former, reminiscent of $p34^{cdc2}$, which exists as both monomeric and cyclin-complexed forms (Brizuela *et al.*, 1989). Figure 8 shows that fractionation of *Xenopus* egg extract on Superdex 200 gel filtration chromatography resolved two peaks of $p40^{MO15}$ and CAK activity. The apparent sizes of the two peaks were ~80 and 180 kDa. The 180 kDa form of $p40^{MO15}$ had considerably higher activity than the 80 kDa form, though the higher molecular weight fractions contained less $p40^{MO15}$. Thus, all three assays (CAK, GST−cdk2 K33R kinase and $p40^{MO15}$ immunoblotting) showed the existence of two separable forms of CAK and $p40^{MO15}$. The relative amount of $p40^{MO15}$ in the 80 and 180 kDa peaks varied depending on the batch of extract used, but there was usually considerably less $p40^{MO15}$ in the larger form.

The CAK and GST-cdk2 K33R kinase activities have proven unstable during purification, however, and our most highly purified preparations still contain several bands as revealed by silver staining. But as yet, we have not found active CAK fractions that lacked an immunoreactive $p40^{MO15}$ band.

Cyclin A−$p33^{cdk2}$ can be activated by recombinant MO15 protein

The evidence presented so far that $p40^{MO15}$ is a $p33^{cdk2}$ T160 kinase relies very heavily on the specificity of the antiserum against $p40^{MO15}$, which reacted with at least one other high molecular weight polypeptide on immunoblots (but unlike $p40^{MO15}$, this protein did not co-fractionate with CAK during purification). It could be argued that the real activity is provided by a protein kinase that cross-reacts with the anti-$p40^{MO15}$ antiserum. We therefore took an alternative approach, and tested whether bacterially expressed $p40^{MO15}$ could activate the histone kinase activity of the cyclin A−cdk2 complex in the presence of ATP. A GST−MO15 fusion protein was expressed and purified from bacteria in the same way as GST−cdk2 (see Materials and methods). Incubation of GST−MO15 with GST−cdk2, PA−cyclin A and ATP did not lead to the appearance of histone H1 kinase activity (Figure 9A, lane 7), but when GST−MO15 was first pre-incubated with cell extract, and then harvested on GSH−Sepharose, the GST−MO15 was able to activate the kinase activity of PA−cyclin A−GST−cdk2 (Figure 9A, lane 10) and to phosphorylate GST−cdk2 K33R (Figure 9B, lane 6). One could still argue, however, that this activity was due to an unidentified kinase from the extract binding to $p40^{MO15}$. To test whether the kinase activity of $p40^{MO15}$ was required for its ability to phosphorylate T160 and activate PA−cyclin A−GST−

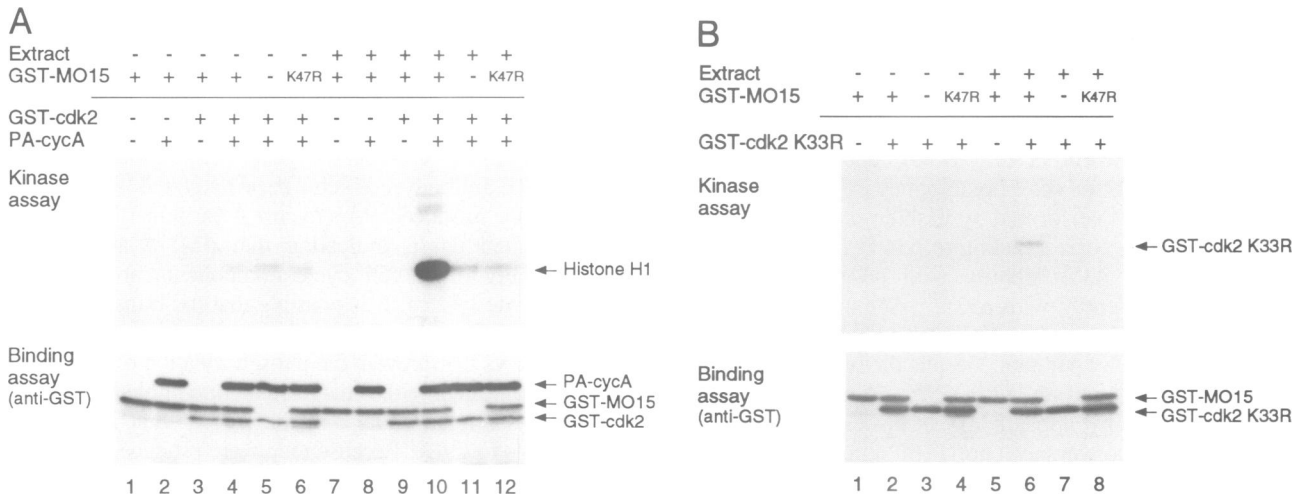

Fig. 9. Activation of cdk2 by recombinant p40^{MO15}. (**A**) PA–cyclin A–GST–cdk2 dependent kinase activity. Buffer (lanes 1–6) or *Xenopus* egg extract (lanes 7–12) were incubated with 100 μg/ml of GST–MO15 (lanes 1–4 and 7–10), kinase-inactive mutant GST–MO15 K47R (lanes 6 and 12) or buffer (lanes 5 and 11). After 30 min at 23°C, the GST–MO15 and K47R mutant were harvested on GSH–Sepharose. The activation of PA–cyclin A–GST–cdk2 was measured by addition of GST–cdk2 (lanes 3–6 and 9–12) and PA–cyclin A (lanes 2, 4–6, 8 and 10–12) as described in Materials and methods. Samples were analysed with SDS–PAGE and autoradiography to detect histone H1 phosphorylation (upper panel), and corresponding samples immunoblotted with anti-GST antibody (lower panel). (**B**) Phosphorylation of GST–cdk2 by GST–MO15. Buffer (lanes 1–4) or *Xenopus* egg extract (lanes 5–8) was incubated with 100 μg/ml of GST–MO15 (lanes 1, 2, 5 and 6), kinase-inactive mutant GST–MO15 K47R (lanes 4 and 8) or buffer (lanes 3 and 7). After 30 min at 23°C, GST–MO15 and the GST–MO15 K47R mutant were immobilized with GSH–Sepharose, washed and assayed for their ability to phosphorylate the kinase-inactive mutant GST–cdk2 K33R (lanes 2–4 and 6–8) as described in Materials and methods. Lanes 1 and 5 were controls without GST–cdk2 K33R. Samples were analysed with SDS–PAGE and autoradiography to detect GST–cdk2 phosphorylation (upper panel), and the corresponding samples were immunoblotted with anti-GST antibody (lower panel).

cdk2, we constructed a kinase-inactive mutant of GST–MO15 with lysine 47 (equivalent to lysine 33 in p33^{cdk2}) changed to arginine (GST–MO15 K47R). This construct was purified and tested exactly as for wild-type GST–MO15, and even after pre-incubation with cell extract it did not phosphorylate or activate GST–cdk2 (Figure 9A, lane 12 and 9B lane 8). This result strongly suggested that the phosphorylation and activation of p33^{cdk2} depended on the activity of GST–MO15 itself, rather than some other kinase that GST–MO15 picked up from the cell extract.

We also tested whether immunoprecipitates of p40^{MO15} from *Xenopus* extracts were capable of phosphorylating bacterially expressed MO15 protein itself, but it was not a substrate (data not shown). We tested another member of the cdc2 family called PCTAIRE-1 that we have cloned from *Xenopus* and expressed in *E.coli*. PCTAIRE-1 was not detectably phosphorylated by the p40^{MO15} immunoprecipitates (R.Y.C.Poon, unpublished data). It would thus appear that p40^{MO15}, together with its presumed partner, may be restricted in its specificity towards p34^{cdc2} and p33^{cdk2} as substrates.

Discussion

In this paper, we show that immunoprecipitates of p40^{MO15} from *Xenopus* egg extracts, or recombinant GST–MO15 preincubated in cell extracts, can phosphorylate T160 of bacterially expressed GST–cdk2 and turn on the histone H1 kinase activity of cyclin A–p33^{cdk2} and cyclin A–p34^{cdc2} complexes in an ATP dependent reaction. Moreover, anti-p40^{MO15} antiserum depletes crude frog egg extract of >85% of its CAK. Thus, p40^{MO15} appears to be a protein kinase that is capable of phosphorylating T160 of p33^{cdk2}. As shown in the accompanying paper from the Montpellier group, T161 of p34^{cdc2} is likewise an excellent target for this kinase. It will be interesting to learn what other substrates p40^{MO15} can phosphorylate; we found it unable to phosphorylate itself or *Xenopus* PCTAIRE-1, but there are several other members of the cdc2 family that should be tested.

Using purified bacterially expressed substrate, p40^{MO15} can phosphorylate GST–cdk2 whether cyclin A is present or not, but the kinase activity of GST–cdk2 was only manifest when the phosphorylated GST–cdk2 was mixed with cyclin A. These findings show very clearly, at least for these fusion proteins, (i) that cyclin is not absolutely required for the phosphorylation of T160 in p33^{cdk2} and (ii) that p33^{cdk2} phosphorylated on T160 has little or no intrinsic protein kinase activity. The association with cyclin thus appears to be essential for protein kinase activity.

The original results on the possible role of p40^{MO15} obtained by Shuttleworth *et al.* (1990) were based on antisense ablation of its mRNA, and suggested that p40^{MO15} might negatively regulate meiosis. It is unlikely that such an effect would be exerted by the cdk2/cdc2 activating property of p40^{MO15} demonstrated here. Moreover, given the large maternal stockpile of the MO15 polypeptide, it is unlikely that depletion of its mRNA in stage VI oocytes would have much effect. Nevertheless, it would be premature to exclude the possibility that p40^{MO15} performs multiple functions, especially in the absence of data on the identity of the active complex, its regulation and subcellular localization. Furthermore, it is possible that MO15-related kinases may exist, with different cell-cycle regulatory roles. More data are needed to reconcile these issues.

Neither these old results nor the sequence of $p40^{MO15}$ encouraged any thoughts that it might encode the long-sought T160/161 kinase of $p33^{cdk2}/p34^{cdc2}$. For $p40^{MO15}$ is a good member of the cdc2/cdk2 family of protein kinases, and was indeed originally isolated during a search for *Xenopus* $p34^{cdc2}$ (Shuttleworth *et al.*, 1990), and recently found again in a search for cdc2 homologues in mouse haematopoietic stem cells performed by Ershler *et al.* (1993). Over the 300 residues corresponding to $p34^{cdc2}$, the sequence of $p40^{MO15}$ shows 43% identity with $p34^{cdc2}$ and slightly higher (49%) identity with $p33^{cdk2}$. We are surprised and intrigued by the incestuous relationships between these close relatives implied by our data. Despite the sequence similarity between $p40^{MO15}$ and $p34^{cdc2}/p33^{cdk2}$, no significant histone H1 kinase activity appears to be associated with active $p40^{MO15}$, and conversely, no T160 kinase activity was found to be associated with immunoprecipitates of $p34^{cdc2}$ or $p33^{cdk2}$ that exhibit very high levels of histone H1 kinase activity. We also note that the sequence phosphorylated by CAK does not contain a proline following the threonine, although at least one other member of the cdc2 family, known as PSSALRE, has been shown to be a 'proline directed' protein kinase (Lew *et al.*, 1992).

Given the existence of lower (80 kDa) and higher (180 kDa) molecular weight forms of $p40^{MO15}$ in cell extracts (possibly monomeric and complexed forms of $p40^{MO15}$ respectively, although 80 kDa seems much too large for a simple 40 kDa monomer), it is likely that $p40^{MO15}$ binds to a protein that acts like a cyclin in terms of its ability to serve as a kinase activator, reminiscent of other members of this protein kinase family. The 180 kDa form of $p40^{MO15}$ has higher specific activity towards $p33^{cdk2}$ than does the 80 kDa form, but it is unclear why the lower molecular weight form of $p40^{MO15}$ still has some activity towards $p33^{cdk2}$. It is noteworthy that $p40^{MO15}$ contains a threonine residue (T176) at the equivalent position of T160 of cdk2, and the sequence around T176 is similar to but not the same as those of cdc2 and cdk2. It is very likely that $p40^{MO15}$ also requires phosphorylation on T176 to be active, and identification of the protein kinase responsible for this reaction may reveal further surprises. In any case, the requirement for a putative partner and an activating protein kinase probably explains why bacterially expressed GST–MO15 is inactive unless it is first incubated with cell extract; we imagine that GST–MO15 picks up a partner and gets properly phosphorylated during the pre-incubation. If the GST–MO15 does indeed require a partner for function, it would imply that the putative partner is in excess in the cell, or that the partner is exchangeable between endogenous $p40^{MO15}$ and the added GST–MO15.

The results presented here for the activation of $p33^{cdk2}$ are very similar to those previously reported for the activation of $p34^{cdc2}$. Phosphorylation of T161 is required for cyclin–$p34^{cdc2}$ activation (Lorca *et al.*, 1992; Solomon *et al.*, 1992), and the cyclin B–$p34^{cdc2}$ activator was shown to be a protein kinase that phosphorylates T161 (Solomon *et al.*, 1992). Our results differ from those of Fesquet *et al.* (1993) and Solomon *et al.* (1992) for $p34^{cdc2}$ activation mainly in respect of their finding that $p34^{cdc2}$ was not phosphorylated unless cyclin was also present. The difference could either be due to a real difference in the mechanism of activation of $p33^{cdk2}$ and $p34^{cdc2}$, which we consider unlikely, or to the difference in assay conditions. Whereas we used purified substrates made in bacteria in our assays, Solomon *et al.* (1992) and the Montpellier group used $p34^{cdc2}$ produced by cell-free translation in rabbit reticulocyte lysates. Although reticulocytes are terminally differentiated, non-dividing cells, they do contain $p34^{cdc2}$ (or very similar proteins that react with a monoclonal anti-PSTAIRE antibody), which can be activated by addition of bacterially produced PA–cyclin A protein (R.Y.C.Poon, unpublished data), indicating that $p34^{cdc2}$ activator and presumably other cell cycle components are present in reticulocyte lysates. It is possible that the translated $p34^{cdc2}$ may associate with other, as yet unidentified cellular components that prevent the phosphorylation of $p34^{cdc2}$ until cyclins bind, and either displace them completely, or cause a conformational change in the $p34^{cdc2}$ that makes T161 accessible to CAK. Because they were produced in bacteria, the GST–cdk2 proteins we used would not have acquired this hypothetical extracellular component. An alternative explanation could invoke regulation by protein phosphatases: whereas T160 in free $p33^{cdk2}$ would be readily accessible to the phosphatase, binding to cyclin would impair the ability of the phosphatase to act. Preliminary experiments (R.Y.C.Poon, unpublished data) disfavour this idea, and support the 'additional component' model.

Given the existence of a large family of cyclins (Xiong and Beach, 1991) and protein kinases related in sequence to $p34^{cdc2}$ (Meyerson *et al.*, 1992), an interesting question is whether $p40^{MO15}$ represents the sole protein kinase that can activate all combinations of cyclin X–cdkY, or whether some of the other members of the family of cdc2-like kinases may function as $p40^{MO15}$-like activators, with specificity towards particular cyclin–cdk complexes. The possibilities for recursive networks of control suggested by these thoughts are daunting. Construction of a phylogenetic tree based on sequence comparisons suggests that a cdc2 homologue from rice (Hata, 1991), and the *Saccharomyces cerevisiae* protein kinase subunit specified by the *KIN28* gene (Simon *et al.*, 1986) are the best candidates for the $p40^{MO15}$ equivalents in plants and budding yeast respectively. The *KIN28* gene is essential for viability, but apparently has not been further studied since its original isolation (Simon *et al.*, 1986). It is noteworthy that these potential cdk activator protein kinases are slightly different from $p34^{cdc2}$ and $p33^{cdk2}$ in the surroundings of T160/161; a Q replaces an E and an R replaces an L in the sequence VYTHEVVTLWYRAPE (*Xenopus* cdc2 and cdk2) which is IYTHQVVTRWYRSPE in MO15. The E in THE and the L before the W are conserved in all known members of the cdc2 and cdk2 family.

The bacterially expressed fusion proteins used in the present study provide a simple yet powerful system to study the biochemistry of cell cycle regulators. Although we are aware of the possibility that the size and nature of the extra domains present in our cyclin and cdk constructs mean that some aspects of their behaviour might differ from the native proteins, as yet we have not found aspects of their behaviour that clearly diverge from their 'natural' counterparts. We consider it a significant practical and theoretical advantage that these proteins are produced in bacterial cells, which are easy to handle and make much less extensive use of serine/threonine phosphorylation than do eukaryotic cells.

It will be of great interest to learn more about the substrate specificity and the regulation of $p40^{MO15}$ activity.

Materials and methods

Construction and mutagenesis of recombinant cyclin A, p34^{cdc2} and p33^{cdk2}

PA–cyclin A and PA–cyclin A CΔ16 were described by Kobayashi *et al.* (1992). We also constructed a version of PA–cyclin A with 10 histidine residues at its N-terminus, using PCR with the oligonucleotides: 5′-GGAGGTCATATGGAACAACGCATAA-3′ (a 5′ primer introducing a *Nde*I site) and 5′-AAAACCTAAGATCTAATCTGTACAC-3′ (3′ primer introducing a *Bgl*II site). The PCR product was cut with *Nde*I and *Bgl*II, and ligated with *Nde*I/*Bam*HI cut pET16b vector (Novagen). GST–cdk2 in pGEX-2T was the generous gift of Li-Huei Tsai and Ed Harlow (see Tsai *et al.*, 1991), and all mutants of cdk2 were derived from this clone. Construction of K33R, T160A and T14A+Y15F mutants of cdk2 was carried out with a PCR method essentially as described by Horton and Pease (1991).

GST–cdc2 in pGEX-2T was constructed by first placing the *Nco*I–*Eco*RI fragment of *Xenopus* cdc2 (Milarski *et al.*, 1991) into *Nco*I/*Eco*RI cut GST–cdk2 in pGEX-2T, which was then cut with *Nco*I to receive the small 5′ *Nco*I fragment of cdc2. In order to transfer the cdc2 ORF from pGEX-2T into pGEX-KG, the pGEX-2T version of cdc2 was cut with *Bam*HI and *Eco*RI, blunt-ended with Klenow and ligated into *Xba*I cut, blunt-ended pGEX-KG. To obtain in-frame translation, the large partial *Nco*I fragment from this construct was ligated with *Nco*I cut pGEX-KG. Six histidine residues were then added to the C-terminus of GST–cdc2 using PCR with the 3′ oligonucleotide 5′-CTTGAGCTCGAGTCGTTAGTGATGGTGATGGTGATGGTTTCTAATCTGATTGTC-3′ and a 5′ primer from within the coding region. The PCR product was digested with *Bsm*I and *Xho*I and inserted into *Bsm*I/*Xho*I cut GST–cdc2 in pGEX-KG.

p40^{MO15} constructs

GST–MO15 was constructed by subcloning fragments amplified by PCR using the oligonucleotides 5′-CCGGGGATCCATGGAAGGTATAGCGGCGAG-3′ (introducing a *Bam*HI site upstream of the second ATG of the original p40^{MO15} clone) and oligonucleotide 15-1 (Shuttleworth *et al.*, 1990). The 838 bp *Bam*HI–*Eco*RI fragment of MO15 was first subcloned into the plasmid pGEX-2T to form pGEX15/BR. Next, the 431 bp *Bam*HI fragment containing 5′ coding sequence generated by PCR was cloned into the *Bam*HI site of pGEX15/BR, and its orientation checked by PCR and sequencing. The K47R mutant was constructed by site-directed mutagenesis using a 'Transformer' site-directed mutagenesis kit from Clontech.

Expression and purification of recombinant proteins from bacteria

Wild-type or mutant clones of GST–cdk2, GST–cdc2 and GST–MO15 were transformed into *E. coli* strain BL21(DE3), and 400 ml cultures were grown to an A_{600} of ~1.0 at 30°C. Synthesis of the recombinant proteins was induced with 100 μM isopropyl-β-D galactoside (IPTG) at 23°C for 16 h. The cells were harvested and lysed with 25 ml of 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 1 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 2 mg/ml lysozyme at 4°C for 15 min. All subsequent steps were carried out at 4°C. The lysate was sonicated and centrifuged at 18 000 *g* for 30 min and the supernatant was filtered through a 0.45 μm filter (Millipore) and applied onto a 0.5 ml column of GSH–Sepharose (Pharmacia), equilibrated with PBS (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) supplemented with 0.25 M KCl, 0.1% Tween-20, 1 mM DTT, 0.25 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin and 15 μg/ml benzamidine. After loading, the column was washed with 10 vol of the same buffer, followed by 3 vol of PBS containing 1 mM DTT. GST–cdk2 was eluted with 5 mM reduced glutathione (Sigma) in 50 mM Tris–HCl pH 8.0, 1 mM DTT.

His-tagged PA–cyclin A was purified by a modification of the procedures described by Hoffmann and Roeder (1991). Overexpression and cell lysis followed the same procedure as described above, but after filtration through 0.45 μm filter (Millipore) the supernatant was applied onto a Ni-NTA–agarose (Qiagen) column. The column was washed with 5 column volumes of 20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, followed by 5 column volumes of 20 mM Tris–HCl pH 8.0, 1.0 M NaCl, 25 mM imidazole, 0.5% Triton X-100, 0.5% Tween-20, and finally 5 column volumes of 20 mM Tris–HCl pH 8.0, 0.5 NaCl, 50 mM imidazole. Histidine-tagged proteins were eluted with 20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 150 mM imidazole. Purified proteins were dialysed against buffer B (20 mM Tris–HCl pH 8.0, 20 mM NaCl, 0.5 mM EDTA, 1 mM DTT).

To obtain correctly folded GST–cdc2 protein, purified GST–cdc2 (and in parallel GST–cdk2) was incubated at 100 μg/ml (final concentration) in *Xenopus* interphase extract. After 30 min at 23°C, the GST fusion proteins were recovered on GSH–Sepharose and washed with bead buffer.

For thrombin digestion of GST–cdk2 and GST–cdc2, ~1 μg of the fusion protein bound to GSH–Sepharose was washed with thrombin buffer (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$), and digested with 10 μl of 10 U/ml thrombin (Sigma) in thrombin buffer at 23°C for 30 min. The supernatant containing the released cdc2 or cdk2 proteins was used without further purification in activation assays.

Cyclin A – cdk2 activator assay

Samples to be assayed for their CAK activity were mixed with 100 μg/ml GST–cdk2 and 100 μg/ml PA–cyclin A, made to 15 mM $Mg(OAc)_2$, 1 mM ATP and incubated at 23°C for 30 min. The reaction was stopped by adding ice-cold bead buffer (Kobayashi *et al.*, 1992), and the GST–cdk2 was recovered with 15 μl of GSH–Sepharose with end-over-end rotation for 30 min at 4°C. The GSH–Sepharose was then washed three times with bead buffer, twice with H1 kinase buffer [80 mM Na-β-glycerophosphate pH 7.4, 20 mM EGTA, 15 mM $Mg(OAc)_2$ and 1 mM DTT], and the kinase activity towards histone H1 was measured by adding 7.5 μl of H1 kinase buffer supplemented with 30 μM ATP and 2.5 μl of H1 mix [containing 1 μg histone H1, 1.25 μCi [γ-^{32}P]ATP, 15 mM $Mg(OAc)_2$, 30 μM ATP and 1 mM DTT], followed by incubation at 23°C for 20 min. The reaction was terminated by addition of 25 μl of SDS–PAGE sample buffer and heated to 100°C. The samples were applied on 15% SDS–PAGE and detected by autoradiography. For assaying immobilized samples, 7.5 μl solution containing 1 μg PA–cyclin A, 1 μg GST–cdk2, 30 μM ATP and 15 mM $Mg(OAc)_2$ was added, incubated at 23°C for 30 min before the histone H1 kinase activity and measured by addition of 2.5 μl H1 assay mix as described above.

Phosphorylation of cdk2

Kinase activity towards cdk2 was measured by incubating test fractions with 100 μg/ml GST–cdk2 K33R mutant, 15 mM $Mg(OAc)_2$, 30 μM ATP and 1.5 μCi [γ-^{32}P]ATP in the presence or absence of PA–cyclin A. The reaction was carried out at 23°C for 45 min.

Phosphoamino-acid analysis

One dimensional phosphoamino-acid analysis after partial acid hydrolysis was performed on $^{32}PO_4$-labelled polypeptides after transfer to Immobilon (Millipore) as described by Kamps (1991). Polygram TLC plates (Macherey-Nagel) were used to separate phosphoamino acids by electrophoresis at 1000 V for 30 min with pH 3.5 buffer.

Preparation of Xenopus egg extracts

Xenopus CSF-arrested extracts were prepared as decribed by Murray (1991). Interphase extracts were prepared by adding 0.4 mM of $CaCl_2$ and 100 μg/ml of cycloheximide to CSF-arrested extracts, followed by incubation at 23°C for 10 min.

Immunoprecipitation and immunoblotting

Immunoprecipitations wth anti-p40^{MO15} (Shuttleworth *et al.*, 1990), anti-p33^{cdk2} or anti-p34^{cdc2} antibody (Kobayashi *et al.*, 1991) were carried out as described by Kobayashi *et al.* (1992). Immunoprecipitation with anti-p40^{MO15} antibody was blocked with 5 μg of GST–MO15 protein boiled in 1% SDS, and diluted to 0.1% SDS final in the immunoprecipitation.

Immunoblotting was performed by separation of samples on 15% SDS–PAGE and transferred to Immobilon (Millipore). The membrane was blocked with TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 4% dry milk for 30 min at 23°C. The membrane was incubated with the primary antibody in TBST 2% dry milk (rabbit anti-GST antibody, 1000-fold dilution) either overnight at 4°C or for 2 h at 23°C. The membrane was washed three times, 10 min each time, with TBST 2% dry milk, incubated with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin (5000-fold dilution, Dakopatts) at 23°C for 2 h. The membrane was then washed six times with TBST and developed using an ECL chemiluminescence kit (Amersham).

Acknowledgements

We are particularly grateful to Marcel Dorée and Mark Solomon for exchanges of information and reassuring discussions. We thank Julian Gannon for antibodies, Ian Goldsmith and his staff for oligonucleotide synthesis, Gary Martin for care of the frogs, Li-Huei Tsai for the GST–cdk2 construct, and Jack Dixon for pGEX-KG. Alison Brown and Terry Jones gave expert help in preliminary experiments and in generating the GST-MO15 constructs. K.Y. was supported by a Fellowship from the Japanese Society for the Promotion of Science.

References

Bandara,L.R., Adamczewski,J.P., Hunt,T. and La Thangue,N.B. (1991) *Nature*, **352**, 249–251.

Booher,R. and Beach,D. (1986) *Mol. Cell. Biol.*, **6**, 3523–3530.

Brizuela,L., Draetta,G. and Beach,D. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 4362–4366.

Connell-Crowley,L., Solomon,M.J., Wei,N. and Harper,J.W. (1993) *Mol. Biol. Cell*, **4**, 79–92.

Desai,D., Gu,Y. and Morgan,D.O. (1992) *Mol. Biol. Cell*, **3**, 571–582.

Ducommun,B., Brambilla,P., Félix,M.A., Franza,B.R.J., Karsenti,E. and Draetta,G. (1991) *EMBO J.*, **10**, 3311–3319.

Dulić,V., Lees,E. and Reed,S.I. (1992) *Science*, **257**, 1958–1961.

Dunphy,W.G. and Kumagai,A. (1991) *Cell*, **67**, 189–196.

Dunphy,W.G., Brizuela,L., Beach,D. and Newport,J. (1988) *Cell*, **54**, 423–431.

Elledge,S.J. and Spottswood,M.R. (1991) *EMBO J.*, **10**, 2653–2660.

Erikson,E. and Maller,J. (1989) *J. Biol. Chem.*, **264**, 19577–19582.

Ershler,M.A., Nagorskaya,T.V., Visser,J.W.M. and Belyavsky,A.V. (1993) *Gene*, **124**, 305–306.

Featherstone,C. and Russell,P. (1991) *Nature*, **349**, 808–811.

Fesquet,D., Labbé,J.-C., Derancourt,J., Capony,J.-P., Galas,S., Girard,F., Lorca,T., Shuttleworth,J., Dorée,M. and Cavadore,J.-C. (1993) *EMBO J.*, **12**, 3111–3121.

Fleig,U.N. and Gould,K.L. (1991) *Semin. Cell Biol.*, **2**, 195–204.

Fleig,U.N., Gould,K.L. and Nurse,P. (1992) *Mol. Cell. Biol.*, **12**, 2295–2301.

Gautier,J., Norbury,C., Lohka,M., Nurse,P. and Maller,J. (1988) *Cell*, **54**, 433–439.

Gautier,J., Minshull,J., Lohka,M., Glotzer,M., Hunt,T. and Maller,J.L. (1990) *Cell*, **60**, 487–494.

Gautier,J., Solomon,M.J., Booher,R.N., Bazan,J.F. and Kirschner,M.W. (1991) *Cell*, **67**, 197–211.

Gould,K.L., Moreno,S., Owen,D.J., Sazer,S. and Nurse,P. (1991) *EMBO J.*, **10**, 3297–3309.

Gu,Y., Rosenblatt,K. and Morgan,D.O. (1992) *EMBO J.*, **11**, 3995–4005.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42–52.

Hata,S. (1991) *FEBS Lett.*, **279**, 149–152.

Hirai,T., Yamashita,M., Yoshikuni,M., Tokumoto,T., Kajiura,H., Sakai,N. and Nagahama,Y. (1992) *Dev. Biol.*, **152**, 113–120.

Hoffmann,A. and Roeder,R.G. (1991) *Nucleic Acids Res.*, **19**, 6337–6338.

Horton,R.M. and Pease,L.R. (1991) In McPherson,M.J. (ed.), *PCR, A Practical Approach*. IRL Press, Oxford, pp. 217–247.

Kamps,M.P. (1991) *Methods Enzymol.*, **201**, 21–27.

Kobayashi,H., Golsteyn,R., Poon,R., Stewart,E., Gannon,J., Minshull,J., Smith,R. and Hunt,T. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 437–447.

Kobayashi,H., Steward,E., Poon,R., Adamczewski,J.P., Gannon,J. and Hunt,T. (1992) *Mol. Biol. Cell*, **3**, 1279–1294.

Koff,A., Cross,F., Fisher,A., Schumacher,J., Leguellec,K., Philippe,M. and Roberts,J.M. (1991) *Cell*, **66**, 1217–1228.

Koff,A., Giodano,A., Desai,D., Yamashita,K., Harper,J.W., Elledge,S., Nishimoto,T., Morgan,D.O., Franza,B.R. and Roberts,J.M. (1992) *Science*, **257**, 1689–1694.

Krek,W. and Nigg,E.A. (1992) *New Biol.*, **4**, 323–329.

Labbé,J.-C., Capony,J.-P., Caput,D., Cavadore,J.-C., Derancourt,J., Kaghdad,M., Lelias,J.-M., Picard,A. and Dorée,M. (1989) *EMBO J.*, **8**, 3033–3058.

Lee,M.G. and Nurse,P. (1987) *Nature*, **327**, 31–35.

Lee,M.S., Ogg,S., Xu,M., Parker,L.L., Donoghue,D.J., Maller,J.L. and Piwnica-Worms,H. (1992) *Mol. Biol. Cell*, **3**, 73–84.

Lees,E., Faha,B., Dulić,V., Reed,S.I. and Harlow,E. (1992) *Genes Dev.*, **6**, 1874–1885.

Lew,J., Winkfein,R.J., Paudel,H.K. and Wang,J.H. (1992) *J. Biol. Chem.*, **267**, 25922–25926.

Lorca,T., Labbé,J.-C., Devault,A., Fesquet,D., Capony,J.-P., Cavadore,J.-C., Le Bouffant,F. and Dorée,M. (1992) *EMBO J.*, **11**, 2381–2390.

Meyerson,M., Ender,H.H., Wu,C.-L., Su,L.-K., Gorka,C., Nelson,C., Harlow,E. and Tsai,L.-H. (1992) *EMBO J.*, **11**, 2909–2917.

Milarski,K.L., Dunphy,W.G., Russell,P., Gould,S.J. and Newport,J.W. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 377–384.

Millar,J.B.A., McGowan,C.H., Lenaers,G., Jones,R. and Russell,P. (1991) *EMBO J.*, **10**, 4301–4309.

Murray,A.W., (1991) In Kay,B.K. and Peng,H.B. (eds), *Xenopus laevis Practical Uses in Cell and Molecular Biology*. Vol. **36**, Academic Press, pp. 573–597.

Nasmyth,K. (1993) *Curr. Biol.*, **5**, 166–179.

Ninomiya-Tsuji,J., Nomoto,S., Yasuda,H., Reed,S.I. and Matsumoto,K. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9006–9010.

Norbury,C. and Nurse,P. (1992) *Annu. Rev. Biochem.*, **61**, 441–470.

Norbury,C., Blow,J. and Nurse,P. (1991) *EMBO J.*, **10**, 3321–3329.

Nurse,P. (1990) *Nature*, **344**, 503–508.

Paris,J., Le Guellec,R., Couturier,A., Le Guellec,K., Omilli,F., Camonis,J., MacNeill,S. and Philippe,M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 1039–1043.

Parker,L.L., Atherton-Fessler,S. and Piwnica-Worms,H. (1992) *Proc. Natl Acad. Sci. USA.*, **89**, 2917–2921.

Pines,J. and Hunter,T. (1989) *Cell*, **58**, 833–846.

Rosenblatt,J., Gu,Y. and Morgan,D.O. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2824–2828.

Russell,P. and Nurse,P. (1987) *Cell*, **49**, 559–567.

Shamu,C.E. and Murray,A.W. (1992) *J. Cell Biol.*, **117**, 921–934.

Shuttleworth,J., Godfrey,R. and Colman,A. (1990) *EMBO J.*, **9**, 3233–3240.

Simon,M., Seraphin,B. and Faye,G. (1986) *EMBO J.*, **5**, 2697–2701.

Solomon,M., Glotzer,M., Lee,T., Philippe,M. and Kirschner,M. (1990) *Cell*, **63**, 1013–1024.

Solomon,M.J., Lee,T. and Kirschner,M.W. (1992) *Mol. Biol. Cell*, **3**, 13–27.

Tsai,L.-H., Harlow,E. and Meyerson,M. (1991) *Nature*, **353**, 174–177.

Whitfield,W.G.F., Gonzalez,C., Maldonado-Codina,G. and Glover,D.M. (1990) *EMBO J.*, **9**, 2563–2572.

Xiong,Y. and Beach,D. (1991) *Current Biol.*, **1**, 362–364.

Received on March 25, 1993; revised on April 30, 1993