The EMBO Journal Vol.16 No.2 pp.343–354, 1997

Dual phosphorylation of the T-loop in cdk7: its role in controlling cyclin H binding and CAK activity

Anne-Marie Martinez, Mohammad Afshar[1], François Martin, Jean-Claude Cavadore, Jean-Claude Labbé and Marcel Dorée[2]

Centre de Recherches de Biochimie Macromoléculaire, CNRS ERS155 and INSERM U 249, route de Mende, BP 5051, 34033 Montpellier Cedex, France and [1]Department of Chemistry, University of York, Heslington, York, UK

[2]Corresponding author

A cyclin-dependent kinase (cdk)-activating kinase (CAK) has been shown previously to catalyze T-loop phosphorylation of cdks in most eukaryotic cells. This enzyme exists in either of two forms: the major one contains cdk7, cyclin H and an assembly factor called MAT-1, whilst the minor one lacks MAT-1. Cdk7 is unusual among cdks because it contains not one but two residues (S170 and T176 in *Xenopus* cdk7) in its T-loop that are phosphorylated *in vivo*. We have investigated the role of S170 and T176 phosphorylation in the assembly and activity of cyclin H–cdk7 dimers. In the absence of MAT-1, phosphorylation of the T-loop appears to be required for cdk7 to bind cyclin H. Phosphorylation of both residues does not require cyclin H binding *in vitro*. Phosphorylation of S170 is sufficient for cdk7 to bind cyclin H with low affinity, but high affinity binding requires T176 phosphorylation. By mutational analysis, we demonstrate that in addition to its role in promotion of cyclin H binding, S170 phosphorylation plays a direct role in the control of CAK activity. Finally, we show that dual phosphorylation of S170 and T176, or substitution of both phosphorylatable residues by aspartic residues, is sufficient to generate CAK activity to one-third of its maximal value *in vitro*, even in the absence of cyclin H and MAT-1, and may thus provide further clues as to how cyclins activate cdk subunits.

Keywords: cdk7/cdk-activating kinase/cyclin H/MAT-1/T-loop phosphorylation

Introduction

Activation of cyclin-dependent kinases (cdks) requires not only association with a cyclin subunit, but also phosphorylation of a conserved threonine residue (T161 in human cdc2) located within the so-called T-loop of kinase subdomain VIII (for reviews, see Solomon, 1994; Jeffrey *et al.*, 1995; Morgan, 1995).

Two distinct protein kinases have so far been shown to catalyze T-loop phosphorylation of cdks in eukaryotic cells. The first one, purified from both vertebrate and invertebrate species (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993), is itself a member of the cdk family of protein kinases (cdk7), which associates with two further subunits to form CAK (cdk-activating kinase). The cyclin partner is cyclin H (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994). The third subunit is a RING finger protein, called MAT-1 ('menage-à-trois') because it was shown to function *in vitro* as an assembly factor, promoting a stable interaction between cdk7 and cyclin H (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995). The trimeric CAK complex also associates with 'core' $TF_{II}H$ to form a complex of at least nine subunits which is required for nucleotide excision repair of DNA and for transcription by RNA polymerase II (Drapkin and Reinberg, 1994; Seroz *et al.*, 1995). Potential homologues of cdk7 and cyclin H have been identified in yeasts. In fission yeast, a complex between Crk1/Mop1 (the putative cdk7 homologue) and Mcs2 (the putative cyclin H homologue) displays CAK activity *in vitro* (Buck *et al.*, 1995; Damagnez *et al.*, 1995). In budding yeast, in contrast, the complex between KIN 28 and CCL1 (the closest budding yeast structural homologues of cdk7 and cyclin H respectively) does not seem to display CAK activity, even though it readily associates with core $TF_{II}H$ and is able to phosphorylate the C-terminal domain of RNA polymerase II (Feaver *et al.*, 1994; Cismowski *et al.*, 1995; Valay *et al.*, 1995).

Recently, a novel CAK (CAK1/Civ1) has been identified in budding yeast (Kaldis *et al.*, 1996; Thuret *et al.*, 1996). Although CAK1/Civ1 is most similar in sequence to the cdk family of kinases, unlike the cdks, CAK1/Civ1 is active as a monomer and may thus be the founding member of a new family of protein kinases. Genetic data indicate that it is indeed a physiologically relevant CAK in budding yeast. To our knowledge however, no CAK1/Civ1 homologues have yet been found in other eukaryotes, and their potential role in controlling activation of cdk complexes remains elusive, except in budding yeast. As depletion of cdk7 from oocyte or egg homogenates has been shown to suppress their ability to support T161 phosphorylation of cdc2 and formation of active cdc2–cyclin A complexes (Fesquet *et al.*, 1993), whilst microinjection of antibodies directed against cdk7 severely impairs assembly of active complexes between cdc2 and cyclins in microinjected *Xenopus* oocytes (M.Dorée, unpublished results), it thus seems almost certain that a complex containing cdk7 is the physiologically relevant CAK, at least in oocytes and early embryos.

Whilst assembly of active trimeric CAK complexes does not require phosphorylation of cdk7 (Devault *et al.*, 1995; Fisher *et al.*, 1995), it has been reported that assembly of active cdk7–cyclin H dimers can occur through an alternative MAT-1-independent pathway that requires phosphorylation of cdk7 in its T-loop by a CAK-activating kinase (Fisher *et al.*, 1995). Whether cdk7–cyclin H complexes can actually assemble *in vivo* in the absence of MAT-1 was, however, not documented.

Here, we first provide evidence that, unlike cdk7 and cyclin H, the RING finger protein MAT-1 is not a universal component of CAK. Indeed, gel filtration of *Xenopus* egg extracts reproducibly yielded a peak of CAK activity with an apparent M_r of 75 kDa containing both cdk7 and cyclin H, but not MAT-1. As *Xenopus* cdk7 has been shown to be phosphorylated *in vivo* on two residues of the T-loop, S170 and T176 (Labbé *et al.*, 1994; Poon *et al.*, 1994), we further investigated the role of S170 and T176 phosphorylation of *Xenopus* cdk7 in the assembly and activity of cyclin H–cdk7 dimers. We show that, besides promoting association with cyclin H, both phosphorylations further increase the catalytic activity of the complex. In fact, dual phosphorylation of S170 and T176 appears to be sufficient to generate CAK activity *in vitro*, even in the absence of cyclin H and MAT-1.

Results

Xenopus oocytes contain an active heterodimeric cdk7–cyclin H complex

Analysis by gel filtration on a sizing Superdex 200 column of extracts prepared from unfertilized *Xenopus* eggs resolved from trimeric CAK complexes a minor peak eluting with an estimated apparent M_r of 75 kDa, that was shown by Western blot analysis of anti-cdk7 immunoprecipitates to contain cyclin H, but apparently not MAT-1 (Devault *et al.*, 1995). However, the MAT-1 antibody used in the previous work was directed against the N-terminal RING finger motif of MAT-1. Thus, we could not exclude the possibility that this minor CAK complex could have contained an N-terminally truncated form of the third subunit. This was not unlikely, as the N-terminal RING finger domain can be deleted from MAT-1 and is not required for CAK assembly (Tassan *et al.*, 1995).

For this reason, a mutant MAT-1 protein deleted of the N-terminal RING finger domain was expressed in *Escherichia coli* and used to immunize rats, then the resulting affinity-purified polyclonal antibodies were used to analyze, by Western blotting, materials immunoprecipitated with anti-cdk7 antibodies from the minor peak of CAK activity. Again, no MAT-1 protein was detected (Figure 1A). In contrast, antibodies directed against the RING finger or the N-terminally deleted protein readily detected MAT-1 in the major peak of CAK activity. Hence, *Xenopus* oocytes actually contain an active heterodimeric CAK complex. We previously showed that cdk7 is phosphorylated on both Ser170 and Thr176 in heterotrimeric CAK complexes (Labbé *et al.*, 1994). This appears also to be the case for heterodimeric CAKs, as they contain cdk7 of identical electrophoretic mobility after SDS–PAGE (Figure 1B). In the next experiments, we investigated how cdk7–cyclin H complexes can form through cdk7 phosphorylation in the absence of the MAT-1 assembly factor.

In vitro phosphorylation of cdk7 by cdk2–cyclin A or cdc2–cyclin B promotes cyclin H binding

In a previous study, we failed to co-precipitate cyclin H and cdk7 from a reticulocyte lysate lacking MAT-1, even though a strong CAK activity was measured in the absence of the RING finger protein (Devault *et al.*, 1995; see below Figure 4). We confirmed this result using a gel

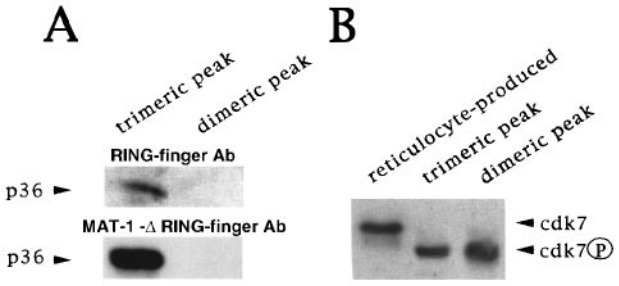

Fig. 1. Analysis by Western blotting of materials immunoprecipitated with anti-cdk7 antibodies from 160 (trimeric) and 75 kDa (dimeric) peaks of CAK activity, resolved by gel filtration from *Xenopus* egg extracts. (**A**) Immunoprecipitated materials were analysed by SDS–PAGE and Western blotting with antibodies directed against the N-terminal RING finger domain (upper panel) or the N-terminally deleted MAT-1 protein lacking the RING finger (lower panel). (**B**) Materials immunoprecipitated from the trimeric and dimeric peaks of CAK activity were analysed by SDS–PAGE and Western blotting with an antibody directed against the C-terminus of *Xenopus* cdk7: the same downward shift of cdk7 was observed in both cases (unphosphorylated ^{35}S-radiolabelled cdk7, translated in reticulocyte lysate and run on the same gel, is shown on the left).

filtration assay on a Superose12 SMART column: whilst a complex of MAT-1–cyclin H-associated cdk7 was clearly resolved as a peak of apparent M_r 200 kDa (fractions 12–15) when a mixture of lysates programmed with MAT-1, cyclin H and cdk7 mRNAs was loaded (Figure 2A), cdk7 eluted as a peak of apparent M_r not higher than 40 kDa (fractions 33–37) when it was mixed before loading with unprogrammed lysate (Figure 2B) or cyclin H-programmed lysate (Figure 2C). Thus, cdk7 did not appear to bind cyclin H in these experimental conditions.

The absence or presence of GST–cdk2–cyclin A, used as a CAK substrate, was the only parameter that differed between the previous immunoprecipitation experiments and the test of CAK activity. Even though unphosphorylated cdk2 gains only very low kinase activity when it binds cyclin A (Connell-Cowley *et al.*, 1993), this could perhaps have been sufficient to catalyse initial phosphorylation of cdk7, thereby promoting cyclin H binding.

A downwards shift of ^{35}S-labelled cdk7, characteristic of T-loop phosphorylation in the cdk family (Lorca *et al.*, 1992b; Rosenblatt *et al.*, 1992), was observed when cdk7–cyclin H co-translation reactions were incubated in the presence of ATP and both recombinant GST–cdk2 and cyclin A (Figure 3, left panel). In the presence of GST–cdk2 alone, which lacks basal protein kinase activity, no shift was observed. The downwards shift was again observed when cdc2–cyclin B kinase, purified from starfish oocytes, was used instead of GST–cdk2–cyclin A.

It has been shown that phosphorylation by cdk2 or cdc2 kinase can bypass the requirement for MAT-1 for formation of stable complexes between cdk7 and cyclin H (Fisher *et al.*, 1995). Indeed, we observed that antibodies against the C-terminus of cdk7 co-precipitated cyclin H from a reticulocyte lysate lacking MAT-1 if cdk7 was phosphorylated previously by either GST–cdk2–cyclin A (Figure 3, right panel) or cdc2–cyclin B (not shown). In contrast, cyclin H did not co-immunoprecipitate with cdk7 when the reticulocyte lysate was not incubated with either of these kinases, indicating that no contaminant such as MAT-1 or an active cdk is present in a significant amount

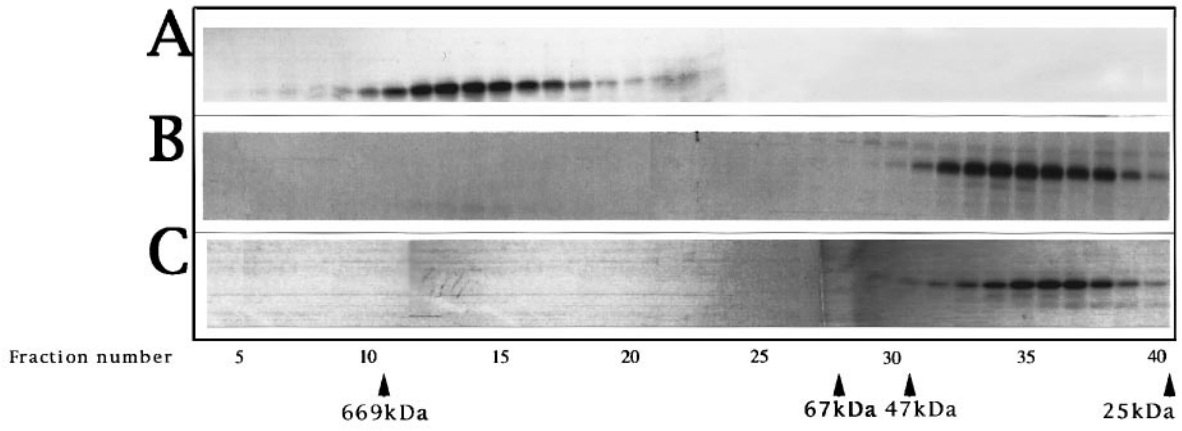

Fig. 2. *In vitro* translated [^{35}S]cdk7 does not bind cyclin H in the absence of MAT-1 if not phosphorylated. A clarified reticulocyte lysate (20 µl) containing [^{35}S]methionine and programmed with cdk7 mRNA only was mixed with 1 vol of unprogrammed lysate (**C**), 1 vol of unlabelled lysate programmed with cyclin H mRNA (**B**) or 1 vol of unlabelled lysate programmed with both cyclin H and MAT-1 mRNAs (**A**), submitted to gel filtration on a Superose 12 column and fractions (20 µl) analysed by SDS–PAGE and fluorography. No free cdk7 is detected in (A) because cyclin H and MAT-1 were translated in excess.

in the reticulocyte lysate. Hence, phosphorylation of the T-loop seems to be required for cdk7 to bind cyclin H in the absence of MAT-1.

To confirm the above experiments *in vitro*, we took advantage of our previous observation that microinjection of mRNAs encoding cdk7 can generate CAK activity only if cdk7 is allowed to translocate in the nucleus (Labbé *et al.*, 1994). As shown in Figure 4A, the downwards shift of cdk7 characteristic of T-loop phosphorylation was observed readily in manually isolated nuclei but not in the cytoplasm of enucleated oocytes.

Next, both cdk7 and cyclin H mRNAs were microinjected into intact oocytes and, after 8 h expression, homogenates prepared from the cytoplasm of enucleated oocytes, or from the same number of manually isolated nuclei, were immunoprecipitated with anti-cdk7 antibodies. As shown in Figure 4B (lane 3), cyclin H was detected in equivalent amounts to the downward-shifted and thus phosphorylated form of cdk7 in nuclear immunoprecipitates. In contrast, neither cyclin H nor the downward-shifted form of cdk7 were detected in cytoplasmic immunoprecipitates (lane 1). The absence of cyclin H was not due to failure of cyclin H to accumulate in cytoplasm, as cyclin H was readily recovered together with phosphorylated cdk7 in anti-cdk7 immunoprecipitates (lane 2) when enucleated oocytes were microinjected with starfish cyclin B–cdc2 kinase 30 min before immunoprecipitation. These results validate the *in vitro* assay that was used in the next experiments to analyse further the role of cdk7 phosphorylation in assembly of active cdk7–cyclin H complexes.

Cdk7 can undergo phosphorylation on both Ser170 and Thr176 in the absence of cyclin H expression

We confirmed in the previous section that formation of a stable dimeric complex with cyclin H in the absence of MAT-1 requires phosphorylation of cdk7. We next wondered if the catalytic subunit could be phosphorylated *in vitro* by cdk2 or cdc2 kinase, even in the absence of an associated cyclin subunit. Indeed, cdk7, translated in reticulocyte lysate in the absence of cyclin H, was found to shift following incubation with either GST–cdk2–cyclin

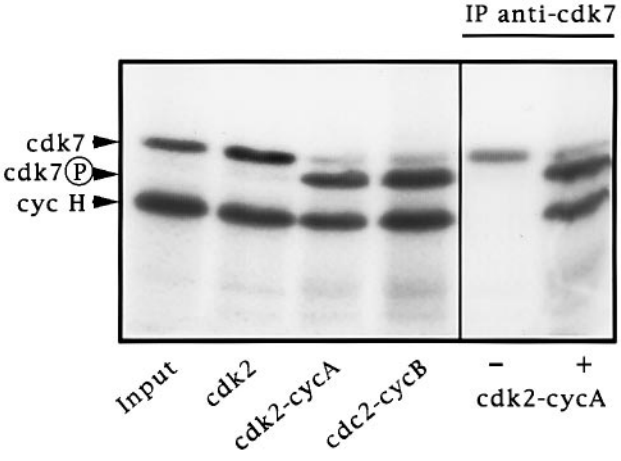

Fig. 3. Phosphorylation of cdk7 by cdk2–cyclin A or cdc2–cyclin B promotes cyclin H binding in reticulocyte lysate. Left panel: a mixture of wild-type cdk7 and cyclin H, both translated in reticulocyte lysate containing [^{35}S]methionine, was supplemented with either GST–cdk2, GST–cdk2 plus recombinant cyclin A or cdc2–cyclin B purified from starfish oocytes, and analysed by fluorography after SDS–PAGE. The positions of unphosphorylated cdk7, phosphorylated cdk7 and cyclin H are indicated by arrowheads. Right panel: proteins immunoprecipitated with an antibody directed against the C-terminus of cdk7 were analysed as above: cyclin H is recovered, in a stoichiometric amount with the phosphorylated form of cdk7, after (+) but not before (–) incubation with cdk2–cyclin A.

A or cdc2–cyclin B, as already observed when cyclin H was present (Figure 5A); its electrophoretic mobility became identical to that of cdk7 from *in vivo* assembled complexes (not shown), which have been shown previously to be phosphorylated on both Ser170 and Thr176. This is not the case for cdk2, however: using *in vitro* translated *Xenopus* cdk2, we found in parallel experiments that CAK induces the downward shift of cdk2 characteristic of T160 phosphorylation exclusively when a cdk2 cyclin partner is co-translated or added in the reticulocyte lysate (not shown).

To confirm that both residues are phosphorylated by cdc2–cyclin B kinase, in the absence of cyclin H expression, a recombinant wild-type GST–cdk7 and an inactive kinase mutant GST–cdk7-K47R were used as substrates

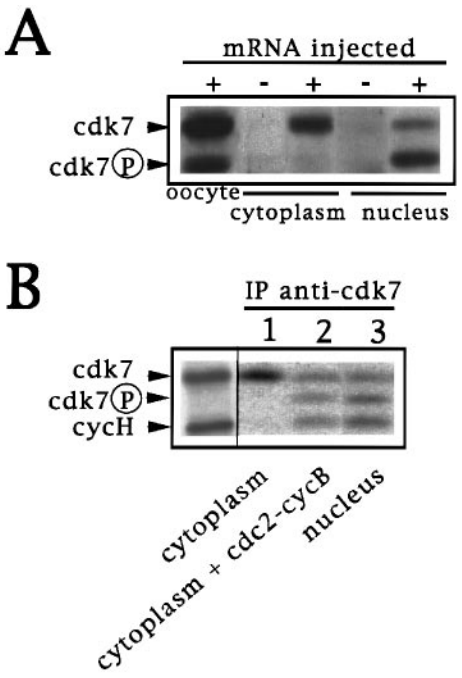

Fig. 4. *In vivo* phosphorylation by microinjected cdc2–cyclin B allows overexpressed cdk7 to bind cyclin H in cytoplasm. *Xenopus* oocytes were microinjected, or not, with mRNAs encoding cdk7 alone (A) or both cdk7 and cyclin H (B) and allowed to express the corresponding proteins for 8 h in the absence (A) or presence (B) of [^{35}S]methionine. (**A**) Homogenates were prepared from 10 oocytes (oocyte), 10 manually isolated germinal vesicles (nucleus) or cytoplasm from 10 enucleated oocytes (cytoplasm) microinjected (+) or not (–) with cdk7 mRNAs. Aliquots (equivalent to 0.5 oocyte) were analysed by SDS–PAGE and Western blot with anti-cdk7 antibodies. The downward-shifted form of cdk7, not observed in cytoplasm, corresponds to phosphorylated cdk7 [cdk7 (P)]. (**B**) Homogenates, prepared from 10 enucleated oocytes (cytoplasm) or the corresponding manually isolated germinal vesicles (nucleus) were first immunodepleted of MAT-1, then immunoprecipitated with anti-cdk7 antibodies and analysed by SDS–PAGE and fluorography (mobility of unphosphorylated cdk7 and cyclin H, both translated in reticulocyte lysate containing [^{35}S]methionine, is shown on the left). Lanes 1 and 3: homogenates were prepared directly 8 h after mRNAs microinjection. Lane 2: highly purified starfish cyclin B–cdc2 kinase was microinjected into enucleated oocytes 8 h after mRNA microinjection and allowed to phosphorylate cdk7 for 30 min before homogenization.

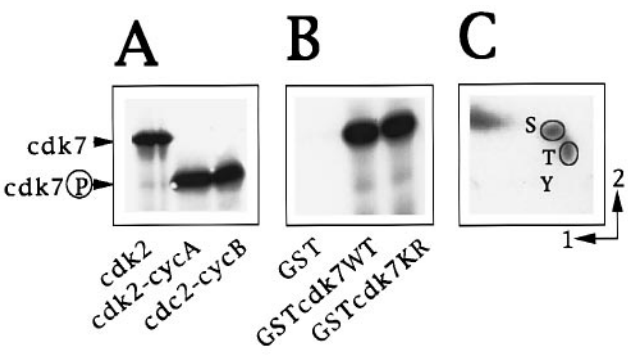

Fig. 5. cdk7 undergoes double phosphorylation on serine and threonine residues even in the absence of cyclin H. (**A**) *In vitro* translated ^{35}S-labelled cdk7 was incubated either with cdk2 alone, cdk2–cyclin A or cdc2–cyclin B and analysed by fluorography after SDS–PAGE. (**B**) Wild-type GST–cdk7, a GST–cdk7-K47R mutant or a GST protein (control) were phosphorylated by cdc2–cyclin B in the presence of [γ-^{32}P]ATP. Proteins were recovered on glutathione–Sepharose beads, subjected to SDS–PAGE and analysed by fluorography. (**C**) Phosphoamino acids released by acid hydrolysis of the ^{32}P-labelled GST–cdk7-K33R mutant were identified by bidirectional analysis. Positions of unlabelled phosphoserine (S) and phosphothreonine (T) markers are circled.

(GST was used as a control). Following incubation with [γ-^{32}P]ATP, proteins were recovered on glutathione–Sepharose beads, separated by SDS–PAGE and transferred to an Immobilon membrane. The membrane was submitted to autoradiography and blotted with an anti-cdk7 antibody. Immunoreactivity co-localized with the autoradiographic signal (not shown). Both the wild-type and the inactive mutant were phosphorylated equally, incorporating [γ-^{32}P]ATP with a ratio >1 mol of phosphate/mol of protein (Figure 5B). Following phosphorylation by the cdc2–cyclin B kinase, the phosphoamino acid composition was determined for the K47R mutant. The cdk7-dead enzyme was found to contain both ^{32}P-labelled phosphoserine and phosphothreonine (Figure 5C) as *in vivo* phosphorylated cdk7. Accordingly, kinase-dead cdk7 mutant translated in a reticulocyte lysate programmed with the corresponding mRNA underwent a downward shift equivalent to that of wild-type cdk7 when phosphorylated by cdk2 or cdc2 kinase (data not shown). The above results ruled out the possibility that phosphorylation of cdk7 on either Ser170 or Thr176 might be due to a cyclin B–cdc2-activated autophosphorylation reaction.

Phosphorylation confers significant CAK activity on cdk7 in the absence of cyclin H translation

To quantify CAK activity generated in reticulocyte lysate by expressing *Xenopus* mRNAs, we used the classical two-step assay (increase in the rate of H1 phosphorylation after CAK-dependent activation of GST–cdk2–cyclin A due to expression of *Xenopus* mRNAs) which is more sensitive by ~2 orders of magnitude than direct measurement of GST–cdk2 phosphorylation, due to the final amplification step. As expected, CAK activity was generated readily in reticulocyte lysate expressing both cdk7 and cyclin H, whilst no CAK activity was detected in the control (lysate programmed with cyclin H only). However, surprisingly, wild-type cdk7 alone was found consistently to generate significant CAK activity, representing from one-third to one-half (depending on the experiments) of that generated in lysates expressing both cdk7 and cyclin H (Figure 6). At this point, it was tempting to speculate that cyclin-free cdk7 may have CAK activity if doubly phosphorylated in its T-loop by cdk2 or cdc2 kinase.

We were concerned, however, that recombinant cyclin A, added in the second step of the above experiment, could possibly have substituted for cyclin H and activated phosphorylated cdk7 to some extent. To circumvent this difficulty, starfish cyclin B–cdc2 kinase was used in the next experiment in place of recombinant cyclin A–cdk2. This preparation, purified by affinity chromatography on p13^{suc1}, does not contain any free cyclin (Labbé *et al.*, 1991) and does not phosphorylate GST–cdk2. We first phosphorylated cdk7 with the starfish kinase, then recombinant GST–cdk2 was allowed to marinate in the lysate. GST–cdk2 was next recovered on glutathione–Sepharose beads, which were washed extensively under stringent conditions to remove both cdk7 and cyclin B–cdc2 kinase. The starfish kinase was completely removed from the

beads, as no H1 kinase was detected on the beads in the absence of added cyclin A, as shown in Figure 7A, lane 1. In contrast, extensive phosphorylation of H1 histone was observed when cyclin A was added subsequent to the washing step (lane 2). Only a much lower phosphorylation of H1 histone, due to basal activation of unphosphorylated GST–cdk2 by cyclin A, was detected when GST–cdk2 was incubated in unprogrammed lysate (lane 3), in lysate programmed with cyclin H mRNA instead of cdk7 mRNA (lane 4) or in lysate programmed with cdk7 mRNA only (lane 5) or both cdk7 and cyclin H mRNAs (lane 6) in the absence of starfish cyclin B–cdc2. Again, the CAK activity of the presumably cyclin-free cdk7 was found to be about one-half that of the cdk7–cyclin H complex (Figure 7B).

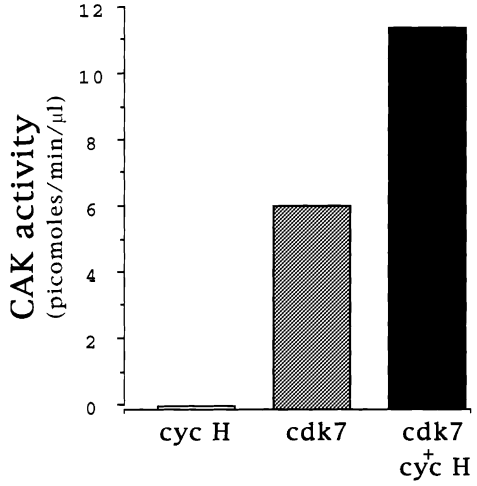

Fig. 6. CAK activities of reticulocyte-translated cdk7 in the absence or presence of co-translated cyclin H. CAK activities were measured as picomoles of phosphate transferred from ATP to H1 histone per min and microlitre of lysate, using the two-step assay (increase in the rate of H1 phosphorylation after CAK-dependent activation of GST–cdk2–cyclin A) in reticulocyte lysate programmed with cdk7 mRNA only and mixed with one volume of unprogrammed lysate in the presence of GST–cdk2–cyclin A (grey box) or in the same volume of the mixed reticulocyte lysates programmed with cyclin H and cdk7, still in the presence of GST–cdk2–cyclin A (solid box). No CAK activity was detected by the differential assay in lysate programmed with cyclin H only (open box).

Cyclin-free, doubly phosphorylated cdk7 with CAK activity is eluted from a gel filtration column as a monomeric 40 kDa protein

A trivial explanation for the above observations could have been that non-programmed reticulocyte lysate contains a small amount of endogenous cyclin H that associates with *in vitro* translated cdk7. This seemed unlikely, as we failed to detect endogenous cyclin H in lysates using polyclonal antibodies raised against bacterially produced *Xenopus* or human cyclin H, that both readily cross-react with rabbit cyclin H (data not shown). Moreover, CAK activity could still be generated by further translating cdk7 after the putative endogenous cyclin H had been first co-immuno-depleted with newly synthesized cdk7 using an antibody directed against the C-terminus of *Xenopus* cdk7 (data not shown).

None the less, to rule out this possibility unambigously, a Superose 12 SMART column was used to fractionate a cdk7-programmed reticulocyte lysate, after its phosphorylation by cdk2–cyclin A and control of its CAK activity. Okadaic acid was added at a final concentration of 1 µM before loading the Superose column, in order to inhibit phosphoprotein phosphatases 1 and 2A and to avoid dephosphorylation of cdk7, as gel filtration segregates cdk7 from both ATP and cdk2–cyclin A. A single peak of downward-shifted cdk7 (fractions 32–36) was resolved

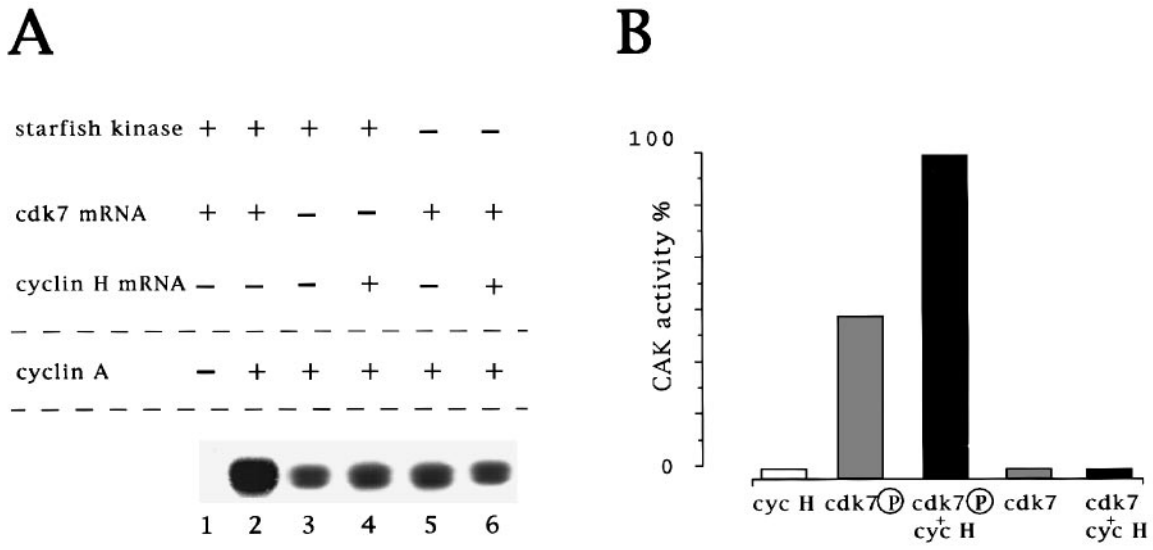

Fig. 7. Activation of GST–cdk2–cyclin A by *in vitro* translated cdk7 phosphorylated by starfish cdc2–cyclin B in the absence of cyclin H translation. (**A**) Recombinant GST–cdk2 was allowed to incubate in reticulocyte lysate with the indicated components (except cyclin A), recovered on glutathione–Sepharose beads and assayed for its ability to phosphorylate H1 histones with or without addition of cyclin A. Bottom: an autoradiogram of phosphorylated H1 histones after SDS–PAGE. (**B**) Histogram showing CAK activities generated in reticulocyte lysate by translating cyclin H only (open box), cdk7 only (grey boxes: with or without starfish cdc2–cyclin B) or both cdk7 and cyclin H (black boxes: with or without starfish cdc2–cyclin B) in the above experimental conditions (100%: CAK activity of lysate translating both cdk7 and cyclin H in the presence of starfish cdc2–cyclin B). For each set of experimental conditions, activities were measured as differences of H1 histone phosphorylation catalysed by GST–cdk2–cyclin A after incubating GST–cdk2 either in the programmed or the corresponding non-programmed lysate.

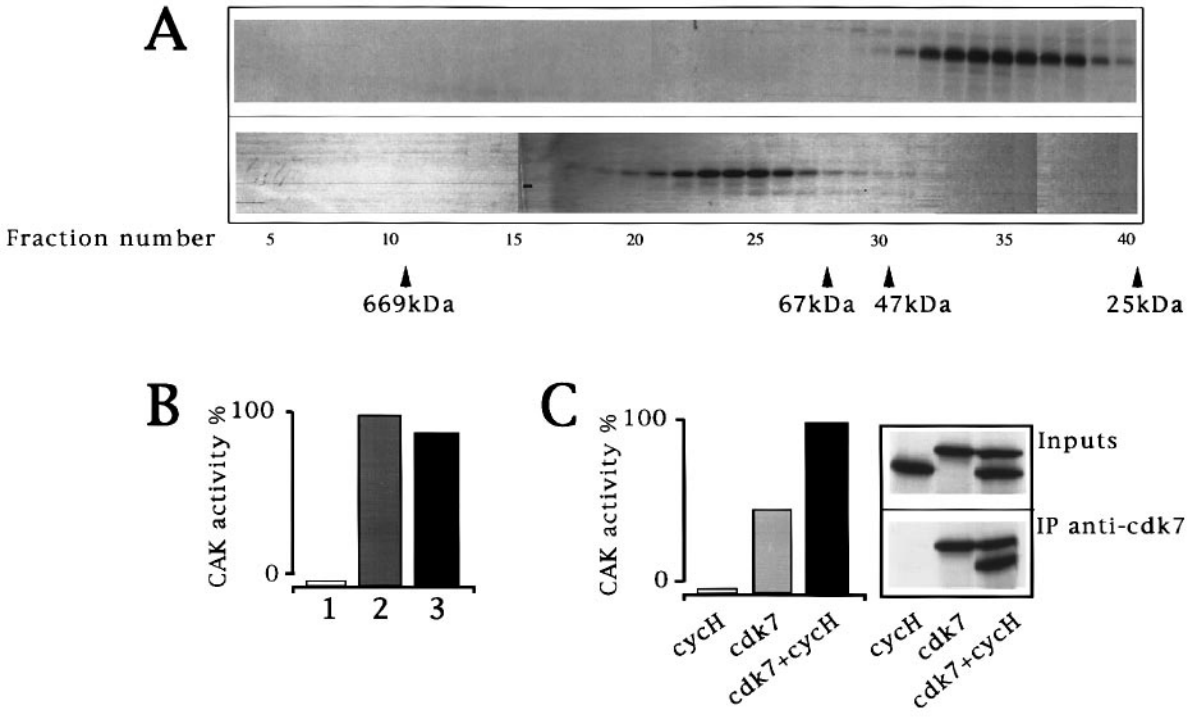

Fig. 8. Cyclin-free, doubly phosphorylated cdk7 with CAK activity is eluted from a gel filtration column as a monomeric 40 kDa protein. (**A**) Analysis by gel filtration on a Superose 12 column of a reticulocyte lysate programmed with cdk7 only (upper panel) or both cdk7 and cyclin H (lower panel; only cdk7 is radiolabelled) after phosphorylation of cdk7 by cdk2–cyclin A. ^{35}S-Labelled cdk7 is eluted as a 40 kDa monomeric component in the first case or as a component of an 80 kDa complex in the second case. (**B**) CAK activity of cyclin-free phosphorylated cdk7 (fraction 34 of the above gel filtration). Phosphorylation was performed before loading the column (box 2) or in fraction 34 after running the column (box 3). No CAK activity was detected in fraction 34 originating from a lysate programmed with cyclin H only (box 1). (**C**) Cyclin H stably binds phosphorylated and purified cdk7 (fraction 34) and further increases its CAK activity. Left: histogram showing CAK activities of cdk7 alone (grey box) and cdk7 supplemented with purified cyclin H (black box). The open box is a control with fraction 34 derived from a lysate programmed with cyclin H only. Right: proteins immunoprecipitated with an antibody directed against the C-terminus of cdk7 from fraction 34 alone (cdk7), from fraction 34 supplemented with purified, radiolabelled cyclin H (cyclin H + cdk7) or from purified cyclin H alone (cyclin H) were analysed by fluorography after SDS–PAGE (lower panel). The upper panel shows the input materials which were subjected to immunoprecipitation.

on the sizing column, corresponding to an apparent M_r of ~40 kDa (Figure 8A, upper panel). This peak was discriminated clearly from a peak of cyclin H-associated cdk7 eluting with an apparent M_r of 80 kDa (fractions 23–26) when cyclin H-programmed reticulocyte lysate (cyclin H in excess) was mixed with phosphorylated cdk7 before loading the column (Figure 8A, lower panel).

Next, peak fraction 34 was assayed for CAK activity. As shown in Figure 8B, CAK activity, not detected in fraction 34 originating from a lysate containing cdk2–cyclin A and programmed with cyclin H mRNA only (box 1), was measured readily in fraction 34 originating from a lysate containing cdk2–cyclin A and programmed with cdk7 mRNA only (box 2). An almost identical CAK activity was measured in fraction 34 when cdk7 was phosphorylated by cdk2–cyclin A in fraction 34 only after running the sizing column (box 3).

Finally, samples containing either phosphorylated and purified cdk7 (fraction 34) alone, cyclin H purified by gel filtration from the lysate programmed with the corresponding mRNA or a mixture of both were assayed for CAK activity (Figure 8C). As expected, CAK activity increased by ~2-fold in samples containing both cyclin H and cdk7 as compared with samples containing only cdk7, and a stable complex of cyclin H and cdk7 could be immunoprecipitated using antibodies directed against the C-terminus of cdk7.

Taken together, the above results are consistent with the view that phosphorylated cdk7 has significant CAK activity in the absence of an associated cyclin H subunit. Furthermore, cyclin H binding can occur after cdk7 phosphorylation and it increases its CAK activity.

Phosphorylation of Thr176 in cdk7 is required and phosphorylation of Ser170 is dispensable for cdk7 to bind cyclin H stably in vitro

We first investigated the ability of either the S170A or the T176A cdk7 mutant (in which the phosphorylatable hydroxyl amino acid has been substituted by a non-phosphorylatable alanine residue) to bind cyclin H in the absence or presence of GST–cdk2–cyclin A, using gel filtration on a Superose 12 SMART column to assay the formation of complexes that do not dissociate when subjected to substantial dilution during the column run (Figure 9).

In the absence of GST–cdk2–cyclin A, neither the S170A nor the T176A cdk7 mutants were found to form stable complexes with cyclin H. Thus they behaved just like wild-type cdk7. In the presence of GST–cdk2–cyclin A, the two mutants were found to behave differently, which implies that each one was phosphorylated on its single phosphorylatable residue. Whilst the S170A mutant was found to form a stable complex with cyclin H (peak in 80 kDa fractions), only part of the T176A mutant was

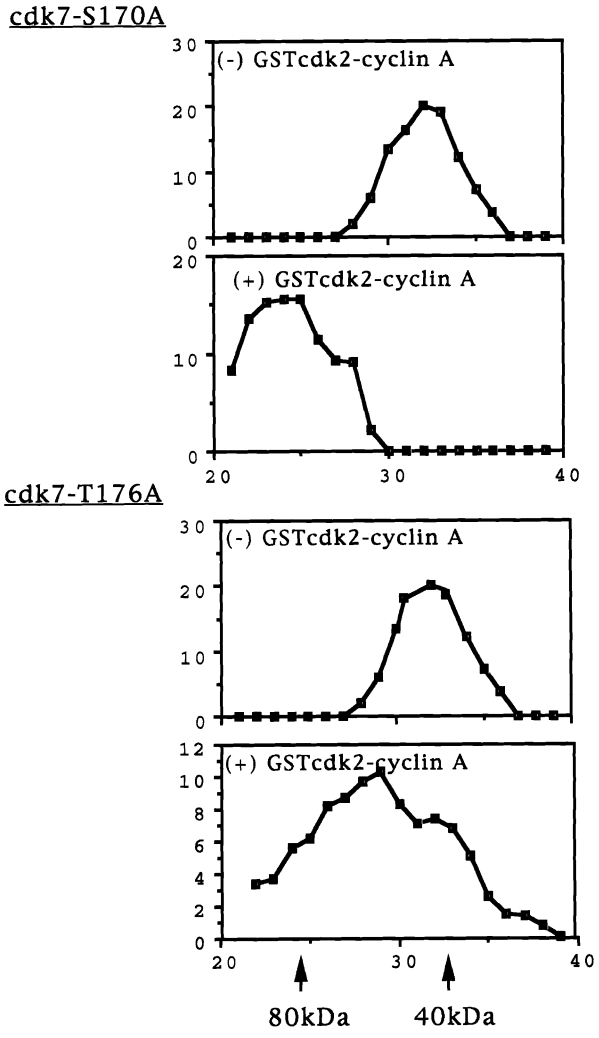

Fig. 9. Interaction with cyclin H of cdk7-S170A and cdk7-T176A mutants, both translated in reticulocyte lysate lysate containing [^{35}S]methionine after (+) or without (–) phosphorylation by GST–cdk2–cyclin A. The phosphorylated or non-phosphorylated ^{35}S-labelled cdk7 mutants were mixed with unlabelled cyclin H in excess, loaded on a Superose 12 column and fractions analysed by SDS–PAGE and fluorography after the run (see Figures 1 and 6). The fluorograms were scanned by densitometry and the profile of optical densities plotted (arbitrary units).

found to be shifted from its monomeric position towards a larger size. In fact, the cdk7-T176A mutant did not shift to the expected dimeric position, but rather to an intermediate one, suggesting progressive dissociation of the dimeric complex during gel filtration and thus a shift of the elution profile. These results suggested that phosphorylation of S170 is neither required nor sufficient for cdk7 to form a stable complex with cyclin H. In contrast, cdk7 and cyclin H do not associate with high affinity if cdk7 is not phosphorylated at T176.

To confirm the above results, we used a more stringent assay, immunoprecipitation with an antibody directed against the C-terminus of cdk7 after dilution of the lysate in a buffer containing 1% of the non-ionic NP-40 detergent. As shown in Figure 10, cyclin H was recovered in anti-cdk7 immunoprecipitates when the S170A mutant was incubated (+) in the presence of GST–cdk2–cyclin A. In contrast, no cyclin H was recovered in anti-cdk7

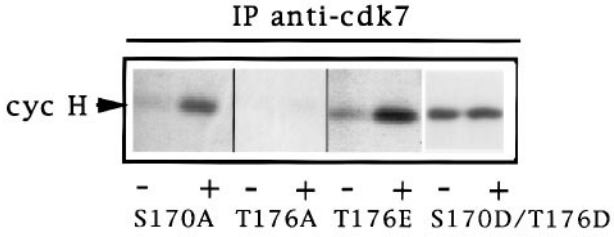

Fig. 10. Ability of various *in vitro* translated cdk7 mutants to bind ^{35}S-labelled cyclin H stably following (+) or without (–) phosphorylation by cdk2–cyclin A. Lysates containing ^{35}S-labelled cyclin H and each cdk7 mutant were diluted 50-fold in a buffer containing 1% NP-40 and immunoprecipitated with an antibody directed against the C-terminus of cdk7. The immunoprecipitated proteins were analysed by SDS–PAGE and fluorography. The arrowhead points to cyclin H, co-immunoprecipitated with cdk7 in some, but not all, conditions.

immunoprecipitates when the T176A mutant was incubated in the same conditions, confirming that T176 phosphorylation is essential for cdk7 to bind the regulatory subunit stably.

Next, two cdk7 mutants in which either T176 alone (T176E) or both S170 and T176 (S170D/T176D) were substituted by acidic residues were constructed. The acidic residues were expected to mimic phosphorylated residues and to allow mutant cdk7 to bind cyclin H stably in the absence of phosphorylation. Indeed, the S170D/T176D double mutant was found to co-immunoprecipitate with cyclin H, even in the absence of GST–cdk2–cyclin A (Figure 10). Some cyclin H was found to co-immunoprecipitate with the T176E mutant in the absence of GST–cdk2–cyclin A, but recovery of cyclin H was higher in its presence, suggesting that S170 phosphorylation, even though not strictly required for cyclin H binding, stabilizes its association with cdk7.

Evidence for a direct role of S170 phosphorylation in CAK activation

In the previous section, we established that S170A and T176A cdk7 mutants can be phosphorylated singly. We next evaluated the effect of these unique phosphorylations on CAK activation, in the absence or presence of the cyclin H subunit (Figure 11). In the absence of cyclin H, neither the S170A nor the T176A cdk7 mutants were found to have any significant CAK activity, even when GST–cdk2–cyclin A was present in the reticulocyte lysate. Thus, phosphorylation of neither S170 nor T176 alone is sufficient to confer CAK activity to monomeric cdk7, and phosphorylation of both residues is required. In agreement with this view, the double mutant S170D/T176D was found to have significant CAK activity in the absence of cyclin H. As expected, this held true in the absence of GST–cdk2–cyclin A (not shown). The cyclin H-free T176E mutant also had CAK activity, but only in the presence of GST–cdk2–cyclin A, indicating that prior phosphorylation of S170 is required for this mutant to become active.

In the presence of cyclin H, CAK activity was increased in all cdk7 mutants. Cyclin H conferred on the T176A mutant CAK activity almost comparable with that of wild-type cdk7. In contrast, only a low CAK activity was generated when cyclin H was added to the S170A mutant, demonstrating that phosphorylation of S170 is required for cdk7 to generate maximal CAK activity. The lower

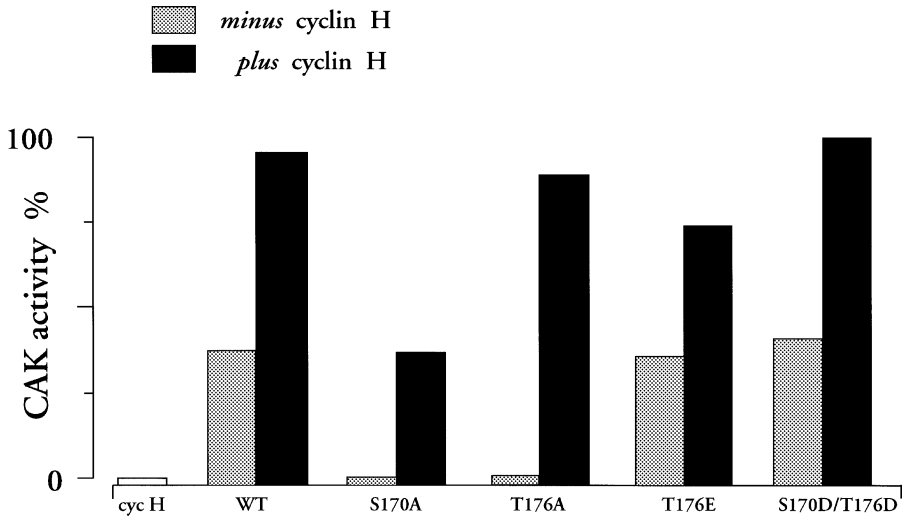

Fig. 11. CAK activities of phosphorylation site mutants in the absence or presence of cyclin H. CAK activities were measured as a percentage of that of the wild-type in the presence of cyclin H, as described in the legend to Figure 4. Identical amounts of each mutant were used in all experiments, as standardized by monitoring the incorporation of [^{35}S]methionine into *in vitro* translation products.

CAK activity of S170A is not due merely to reduced binding of cyclin H, since the S170A mutant binds cyclin H more efficiently than does the T176A mutant.

Discussion

Whilst activity of most cdks is positively controlled by association with cyclins and phosphorylation of a conserved threonine residue in the T-loop of the catalytic subunit, cdk7, the catalytic subunit of CAK, appeared to be an exception among the cdk family, as it was shown to require association with a RING finger protein, MAT-1, to form a detectable complex *in vitro* with cyclin H in the absence of T-loop phosphorylation (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995). The requirement for MAT-1 was, however, reported to be suppressed if mammalian cdk7 is phosphorylated *in vitro* by cdk2–cyclin A or cdc2–cyclin B (Fisher *et al.*, 1995). In the present work, we provided direct experimental evidence that heterodimeric complexes comprising cyclin H and T-loop-phosphorylated cdk7 actually exist as a minor form of CAK in *Xenopus* oocytes, and further investigated the role of T-loop phosphorylation in the assembly and activity of cdk7–cyclin H complexes.

Xenopus cdk7 has been shown to be phosphorylated *in vivo* on two residues of the T-loop, S170 and T176 (Labbé *et al.*, 1994; Poon *et al.*, 1994), corresponding to S164 and T170 in mammalian cdk7. Whilst S164 phosphorylation was due to cyclin A–cdk2 or cdc2–cyclin B in experiments by Fisher and colleagues, it remained unclear whether or not T170 phosphorylation was an autophosphorylation reaction. The same authors reported that S164 phosphorylation is not sufficient for mammalian cdk7 to bind cyclin H, as a S164-phosphorylated cdk7-T170A mutant was not found to form a detectable complex with cyclin H in the absence of MAT-1, and no CAK activity was detected in the corresponding anti-cdk7 immunoprecipitates. In contrast, phosphorylation of T170 was reported to be absolutely required for cdk7 to bind cyclin H stably *in vitro* in the absence of MAT-1 and, accordingly, a cdk7-T170A mutant was found to lack CAK activity in the presence of cyclin H, even after S164 phosphorylation.

In the present work, we first confirmed for *Xenopus* cdk7 that *in vitro* phosphorylation by cdk2–cyclin A or cdc2–cyclin B suppresses the requirement for MAT-1, strongly suggesting that in the absence of the assembly factor, phosphorylation of the T-loop is a pre-requisite for cdk7 to bind cyclin H. We further established that, even in these conditions, T176 phosphorylation is not an autophosphorylation reaction, as a kinase-dead mutant of cdk7 was found to undergo both serine and threonine phosphorylation in the presence of cdc2–cyclin B. We previously reported that this kinase-dead mutant also undergoes phosphorylation on S170 and T176 *in vivo* when overexpressed in *Xenopus* oocytes (Labbé *et al.*, 1994).

The finding that phosphorylation of the cdk7 T-loop does not require and occurs prior to binding of the cyclin partner contrasts with a current model based on crystal structures of cdk2 and cdk2–cyclin A (De Bondt *et al.*, 1993; Jeffrey *et al.*, 1995). In this model, cyclin binding first allows the T-loop to point away from the active site, thereby conferring basal catalytic activity on the cdk. In the next step, CAK-mediated phosphorylation of the T-loop would occur, stabilizing the T-loop and conferring maximal activity on the cyclin–cdk complex. Our molecular model of the cdk7 T-loop shown in Figure 12 highlights considerable differences in the electrostatic environment of T176 in cdk7 as compared with T160 in cdk2. Whilst there is an extensive negatively charged surface formed by E12, E162 and D38 surrounding the hydroxyl group of T160, the electrostatic environment of the corresponding phosphorylation site in our model of cdk7 is much less negatively charged. E26 (corresponding to cdk2 E12) is present but, in cdk7, N178 substitutes cdk2 E162 and G52 substitutes cdk2 D38. This dramatic change in the electrostatic environment of the cdk7 T176 phosphorylation site is consistent with its capacity to be phosphorylated more readily in the absence of a cyclin. In cdk2, this region undergoes considerable changes in conformation

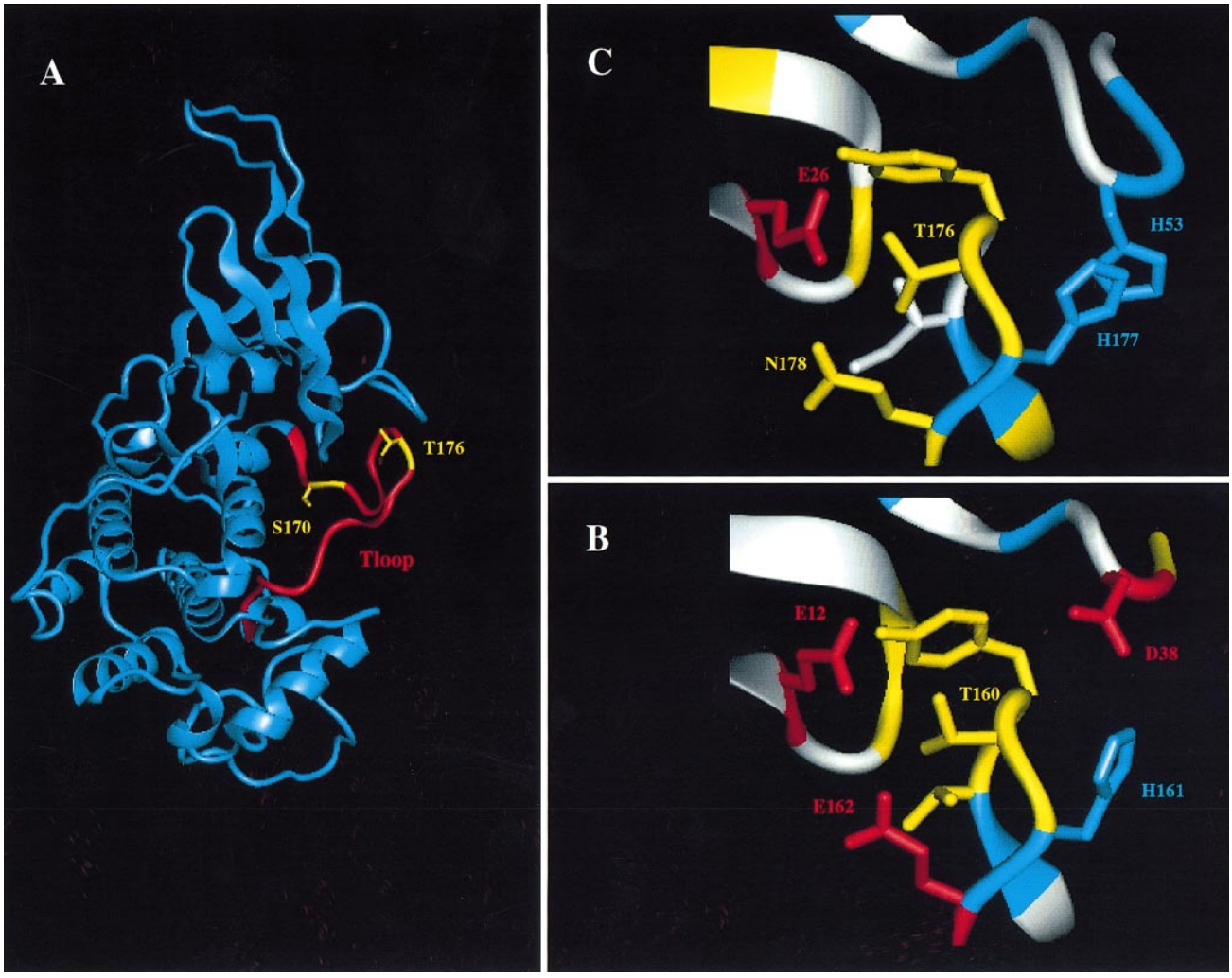

Fig. 12. A molecular model of cdk7 core (residues 17–314) based on the crystal structure of the cdk2–ATP–Mg complex (De Bondt *et al.*, 1993). The sequences were aligned using Quanta (Molecular Simulations Inc.) and the cdk7 model was constructed and optimized as described in Sali and Blundell (1993) using the program Modeler. (**A**) Ribbon diagram of the cdk7 core highlighting the T-loop residues (F168–S186) coloured in red and the phosphorylation sites (S170 and T176) coloured in yellow. (**B**) A close-up view of the T160 phosphorylation site of cdk2 (De Bondt *et al.*, 1993): a portion of the T-loop and all side chains that fall within 5 Å of T160 are shown. Acidic residues are shown in red, basic residues in blue, other polar residues in yellow and non-polar residues in white. The atoms in this region generally have high *B*-values in the crystal structure (De Bondt *et al.*, 1993), suggesting conformational mobility. It is possible, nevertheless, to characterize the electrostatic environment of this site. There is an extensively negatively charged surface formed by E12, E162 and D38 surrounding the hydroxyl group of T160. (**C**) The close-up view of the corresponding T176 phosphorylation site in our model of cdk7: this site displays a very different electrostatic environment to that seen in cdk2. E26 (corresponding to cdk2 E12) is present but, in cdk7, N178 substitutes cdk2 E162 and G52 substitutes cdk2 D38. In addition, H53 (adjacent to R54) is recruited into the 5 Å contact volume.

on binding to cyclin A (Jeffrey *et al.*, 1995) which are possibly necessary for phosphorylation to occur. Through structural comparison with the cyclic AMP-dependent protein kinase catalytic subunit (PKA) structure (Knighton *et al.*, 1991), it has been speculated that, when phosphorylated, cdk2 T160 (in the cdk2–cyclin A complex) could interact with an electrostatic pocket formed by residues R50, R126 and R150 (De Bondt *et al.*, 1993; Jeffrey *et al.*, 1995). We note that the polarity of these residues is well preserved in cdk7 (R67, R142 and K166 respectively) and that the phosphorylated T176 of cdk7 potentially could interact with a similar negatively charged pocket through the same conformation as seen in PKA.

We also confirmed that, even after phosphorylation with cdk2–cyclin A, the *Xenopus* cdk7-T176A mutant does not co-immunoprecipitate with cyclin H in the absence of MAT-1, as does its mammalian equivalent and, accordingly, no CAK activity was detected in anti-cdk7 immunoprecipitates. However, we found that *in vitro* translated cdk7-T176A gains CAK activity almost comparable with wild-type cdk7 (upon phosphorylation by cdk2–cyclin A), if measured directly in cyclin H-containing reticulocyte lysate, without immunoprecipitation. This suggested that, even if not resistant to immunoprecipitation conditions, a complex could form between cdk7-T176A and cyclin H in reticulocyte lysate. We were able to confirm this view using gel filtration to assay the formation of complexes that dissociate only progressively when subjected to dilution during the column run. Phosphorylation of S170 is thus sufficient for *Xenopus* cdk7 to bind cyclin H with low affinity.

No specific role could be assigned to S164 and T170

phosphorylation of mammalian cdk7 besides favouring association with cyclin H, in the work of Fisher and colleagues. In contrast, we were able to analyse further the role of S170 and T176 phosphorylation in *Xenopus* CAK. We found that, even though it binds cyclin H with higher affinity than the cdk7-T176A mutant, cdk7-S170A does not gain full CAK activity upon phosphorylation by cdk2–cyclin A or cdc2–cyclin B, in contrast to cdk7-T176A. This indicates that, besides its role in promoting cyclin H binding, S170 phosphorylation plays a more direct role in the control of CAK activity.

We previously reported that expression from microinjected mRNAs of a cdk7-S170A mutant allows production of CAK activity in the recipient oocyte, in contrast to that of a cdk7-T176A mutant (Labbé *et al.*, 1994). This suggests that efficient binding of cyclin H, mediated by phosphorylation of T176, though not so efficiently by phosphorylation of S170, is essential *in vivo* to assemble CAK complexes, even though in the absence of S170 phosphorylation the newly assembled kinase cannot undergo full activation.

As the acronym indicates, binding of a cyclin is widely believed to be absolutely required for a cdk to express catalytic activity. This may be due partly to the fact that T-loop-phosphorylated cyclin-free cdks are highly unstable and undergo rapid dephosphorylation *in vivo*, as shown for example after degradation of mitotic cyclins at exit from mitosis (Gould and Nurse, 1989; Gould *et al.*, 1991; Lorca *et al.*, 1992b; Lee *et al.*, 1994). Yet, even *in vitro*, kinase activity of a cyclin-free cdk has never been demonstrated. For example, GST–cdk2, even containing a phosphorylated T160 residue in its T-loop, has no detectable kinase activity, and kinase activity readily appears upon cyclin A addition (Poon *et al.*, 1993). This also appears to hold true for monomeric cdc2 (Desai *et al.*, 1995).

Cdk7 is peculiar among cdks because it contains two residues in its T-loop that are phosphorylated both *in vivo* and *in vitro*. In this respect, cdk7 is similar to another family of protein kinases, MAP kinases, that require for catalytic activity dual phosphorylation of the T-loop but no interaction with a companion protein (Hanks and Hunter, 1995). In the present work, we found that, even in the absence of cyclin H or any other cyclin, *Xenopus* cdk7 acquires *in vitro* CAK activity of one-third of its maximal value if phosphorylated by cdk2–cyclin A or cdc2–cyclin B. Double phosphorylation of S170 and T176 is required for this cyclin-free activity of cdk7. The unphosphorylatable double mutant with S170 and T176 replaced by aspartic residues has significant CAK activity in the absence of a cyclin, as does the T176E mutant if further phosphorylated on S170. In all cases, cyclin H further enhances CAK activity.

The conclusions of the present work, as well as those of previous investigators, are derived from *in vitro* experiments. In fact, we do not know how CAK subunits assemble *in vivo* and how CAK interacts with ERCC2, ERCC3 and at least three other subunits (Fisher *et al.*, 1992; Humbert *et al.*, 1994) to form the basal transcription factor $TF_{II}H$. Even though *in vitro* studies support the view that T-loop phosphorylation of cdk7 is dispensable for its assembly with cyclin H and MAT-1 and for CAK activity of the trimeric complex, it readily occurs *in vivo*. T-loop phosphorylation of cdk7 was detected first when tryptic peptides derived from the purified trimeric complex were microsequenced (Fesquet *et al.*, 1993). In addition, cdk7 exhibits on SDS–PAGE the same characteristic downwards shift in both the major trimeric form and the minor dimeric form of CAK in *Xenopus* oocytes (Devault *et al.*, 1995). We do not know whether T-loop phosphorylation occurs *in vivo* after or before assembly of trimeric CAK. If it occurs before, MAT-1 cannot be required *in vivo* as an assembly factor for CAK, and must fulfill another role, perhaps that of a junction subunit with 'core' $TF_{II}H$. MAT-1 might interact with core $TF_{II}H$ through its RING finger domain, which is not required for MAT-1 to assemble cyclin H and cdk7 *in vitro* (Devault *et al.*, 1995; Tassan *et al.*, 1995). Reports that assembly factors at first sight functionally related to MAT-1 may exist for other cdk–cyclin complexes (Kato *et al.*, 1994; Gerber *et al.*, 1995) are also ambiguous and require further investigation.

The kinase responsible for *in vivo* phosphorylation of cdk7 has not yet been identified. Even though both cdk2–cyclin A and cdc2–cyclin B readily catalyse dual phosphorylation of cdk7 T-loop *in vitro*, suggesting that 'cdk activation could be governed by a positive feedback loop in which the targets of CAK, the cdks, are also its activators' (Fisher *et al.*, 1995), it seems unlikely that any of these kinases may fulfill this role *in vivo*, at least in *Xenopus* and starfish oocytes. Indeed fully grown, G_2-arrested oocytes have never been shown to contain any active cdk, in spite of extensive investigation. None the less, they contain cdk7 doubly phosphorylated in its T-loop. Moreover, cdk7 expressed from microinjected mRNAs undergoes dual phosphorylation on S170 and T176 in G_2-arrested *Xenopus* oocytes. As nuclear translocation is required for newly synthesized cdk7 to gain CAK activity (Labbé *et al.*, 1994), the CAK-activating kinase (CAKAK) may be nuclear. Work is in progress to characterize this key enzyme.

Materials and methods

Mutagenesis and protein expression

A PRSET-B vector (In Vitrogen) carrying a *Xenopus* cdk7 wild-type gene was used for mutagenesis. Single and double mutations of S170 and T176 were introduced by oligonucleotide-directed mutagenesis of a uracil-containing single-stranded Kunkel template (Kunkel *et al.*, 1987). All constructs were checked by direct sequencing.

Capped mRNAs, transcribed by T7 polymerase, were *in vitro* translated in reticulocyte lysates in the presence or absence of [^{35}S]methionine. The amount of protein produced was monitored by autoradiography and/or Western blotting.

The GST fusion proteins (GST–cdk2, GST–cdk7 and GST–cdk7-K47R) were expressed in *E.coli* and purified by affinity chromatography on glutathione–Sepharose. Recombinant cyclin A was produced and purified as previously described (Lorca *et al.*, 1992a).

Two oligonucleotides were used to create a 779 bp fragment of *Xenopus* MAT-1 cDNA truncated of its RING finger domain: 5′-ggAATTCATgAAAAgCAACTTTAAAgTCCAgCTC-3′ and 3′-ggAATTCCTAATgTgTCTgCCAgAATAgTCC-5′. The 5′-oligonucleotide contained an ATG in-frame codon allowing the expression of the N-terminally truncated MAT-1 protein (MAT-1-ΔRING). The PCR fragment was cloned into a pRSET B vector. Recombinant Mat-1-ΔRING was expressed in *E.coli* BL 21 (DE3) after induction with IPTG and purified. Protein (100 μg) was mixed with an equivalent volume of complete Freund adjuvant (Difco) and injected into rats for immunization.

Antibodies and immunoprecipitations

Cdk7 and cdk7-containing complexes were immunoprecipitated using a polyclonal antibody (C-ter antibody) raised in rabbits against a 15

amino acid peptide corresponding to the C-terminus of *Xenopus* cdk7 (Shuttleworth *et al.*, 1990). Samples were diluted with 1 ml of buffer containing 40 mM Tris pH 7.5, 150 nM NaCl and 0.5% NP-40. Immunoprecipitates were recovered by standard methods using protein A–Sepharose.

CAK activities

Two procedures were used to measure CAK activities of *Xenopus* cdk7, both based on the two-step assay previously described (Labbé *et al.*, 1994). In the first, samples (2 µl of mRNA-programmed reticulocyte lysate) were added to 3 µl of a mixture containing 0.33 mM ATP, 16.6 mM $MgCl_2$, 1 µg of GST–cdk2 and 1 µg of cyclin A. After 30 min at 25°C, samples were diluted by adding 25 µl of distilled water and assayed for H1 histone kinase activity by adding 10 µl of a mixture containing 4 mg/ml histone H1, 80 mM HEPES pH 7.4, 40 mM $MgCl_2$ and 0.8 mM [γ-^{32}P]ATP (100 c.p.m./pmol). The same assay was run in parallel using 2 µl of non-programmed reticulocyte lysate. CAK activity generated by translation of *Xenopus* mRNAs in reticulocyte lysate was deduced from the difference between H1 histone kinase activities measured in the programmed and the non-programmed lysate.

In the second assay, samples were added to 3 µl of a mixture containing 0.33 mM ATP, 16.6 mM $MgCl_2$ and 1 µg of GST–cdk2, but no cyclin A. After 30 min at 25°C, samples were diluted in 1 ml of buffer containing 40 mM Tris pH 7.5, 150 mM NaCl and 0.5% NP-40, and GST–cdk2 was recovered by affinity on glutathione–Sepharose 4B. The beads were washed twice with 1 ml of the same buffer, once with 1 ml of 40 mM Tris pH 7.5, resuspended in 30 µl of the latter buffer containing 1 µg of cyclin A, then H1 histone kinase activity was assayed as above. CAK activities were deduced from the differences in H1 histone phosphorylation catalysed by GST–cdk2–cyclin A after incubating GST–cdk2 in the programmed or the corresponding non-programmed lysate.

Phosphoamino acid analysis

Affinity-purified ^{32}P-labelled GST–cdk7-K47R was solubilized in Laemmli buffer, transferred to an Immobilon membrane (Millipore) after SDS–PAGE, and submitted to autoradiography. The labelled GST–cdk7 spot was hydrolysed in HCl and the released phosphoamino acids analysed by thin-layer electrophoresis in the first and thin-layer chromatography in the second direction, according to Cooper *et al.* (1993).

Gel filtration

Association between ^{35}S-labelled cdk7 and unlabelled, reticulocyte-translated cyclin H (or cyclin H plus MA-1) was assessed by gel filtration. Samples, containing 1 mg/ml each of leupeptin and aprotinin, were centrifuged for 15 min at 100 000 *g* and injected onto a Superose 12 column equilibrated with 50 mM Na_2HPO_4, 1 mM EGTA, 1 mM dithiothreitol and 100 mM NaCl, and monitored by a Pharmacia SMART system. Fractions (20 µl) were collected and analysed by SDS–PAGE and fluorography.

Acknowledgements

We thank Dr Hubbard (University of York) for his help in modelling cdk7, Suzanne Vigneron for invaluable technical support and Jean-Paul Capony for performing phosphamino acid analysis. We would like to acknowledge Dr Kim for providing the cdk2 coordinates, Daniel Fisher for reading the manuscript and Daniel Bellenoue for iconography. This work was supported by grants from the Association pour la Recherche contre le Cancer (ARC) and the Ligue contre le Cancer.

References

Buck,V., Russell,P. and Millar,J.B. (1995) Identification of a cdk-activating kinase in fission yeast. *EMBO J.*, **14**, 6173–6183.

Cismowski,M.J., Laff,G.M., Solomon,M.J. and Reed,S.I. (1995) Kin 28 encodes a C-terminal domain kinase that controls mRNA transcription in *Saccharomyces cerevisiae* but lacks cyclin-dependent kinase-activating kinase (CAK) activity. *Mol. Cell. Biol.*, **15**, 2983–2992.

Connell-Cowley,L., Solomon,M.J., Wei,N. and Harper,J.W. (1993) Phosphorylation-independent activation of human cyclin-dependent kinase 2 by cyclin A *in vitro*. *Mol. Biol. Cell*, **4**, 79–92.

Cooper,J.A., Sefton,B.M. and Hunter,T. (1993) Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.*, **99**, 387–405.

Damagnez,V., Mäkela,T.P. and Cottarel,G. (1995) *Schizosaccharomyces pombe* Mop1-Mcs2 is related to mammalian CAK. *EMBO J.*, **14**, 6164–6172.

De Bondt,H.L., Rosenblatt,J., Jancarik,J., Jones,H.D., Morgan,D.O. and Kim,S.H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature*, **363**, 595–602.

Desai,D., Wessling,H.C., Fisher,R.P. and Morgan,D.O. (1995) Effects of phosphorylation by CAK on cyclin binding by CDC2 and CDK2. *Mol. Cell. Biol.*, **15**, 345–350.

Devault,A., Martinez,A.M., Fesquet,D., Labbé,J.C., Morin,N., Tassan,J.P., Nigg,E.A., Cavadore,J.C. and Dorée,M. (1995) MAT-1 ('menage à trois') a new RING finger protein subunit stabilizing cyclin H–cdk7 complexes in starfish and *Xenopus* CAK. *EMBO J.*, **14**, 5027–5036.

Drapkin,R. and Reinberg,D. (1994) The multifunctional $TF_{II}H$ complex and transcriptional control. *Trends Biochem. Sci.*, **19**, 504–508.

Feaver,W.J., Svejstrup,J.Q., Henry,N.L. and Kornberg,R.D. (1994) Relationship of CDK-activating kinase and RNA polymerase II CTD kinase $TF_{II}H/TF_{II}K$. *Cell*, **79**, 1103–1109.

Fesquet,D. *et al.* (1993) The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr 161 and its homologues. *EMBO J.*, **12**, 3111–3121.

Fisher,L., Gerard,M., Chalut,C., Lutz,Y., Humbert,S., Kanno,M., Chambon,P. and Egly,J.M. (1992) Cloning of the 62-kilodalton component of basic transcription factor-BTF2. *Science*, **257**, 1392–1395.

Fisher,R.P. and Morgan,D.O. (1994) A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell*, **78**, 712–724.

Fisher,R.P., Jin,P., Chamberlin,H.M. and Morgan,D.O. (1995) Alternative mechanisms of CAK assembly require an assembly factor or an activating kinase. *Cell*, **83**, 47–57.

Gerber,M.R., Farrell,A., Deshaies,R.J., Herskowitz,I. and Morgan,D.O. (1995) cdc 37 is required for association of the protein kinase cdc 28 with G1 and mitotic cyclins. *Proc. Natl Acad. Sci. USA*, **92**, 4651–4655.

Gould,K. and Nurse,P. (1989) Tyrosine dephosphorylation of the fission yeast cdc2+ protein kinase regulates into mitosis. *Nature*, **39**, 39–45.

Gould,K.L., Moreno,S., Owen,D.J., Sazar,S. and Nurse,P. (1991) Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34cdc2 function. *EMBO J.*, **10**, 3297–3309.

Hanks,S.E. and Hunter,T. (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.*, **9**, 576–596.

Humbert,S., Vanrunhen,H., Lutz,Y., Hoeijmakers,J., Egly,J.M. and Montcollin,V. (1994) p44 and p34 subunits of the $BTF2/TF_{II}H$ transcription factor have homologies with ssl1, a yeast protein involved in DNA-repair. *EMBO J.*, **13**, 2393–2398.

Jeffrey,P.D., Russo,A.A., Polyak,K., Gibbs,E., Hurwitz,J., Massagué,J. and Pavletitch,N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclin A–cdk2 complex. *Nature*, **376**, 313–320.

Kaldis,P., Sutton,A. and Solomon,M. (1996) The Cdk-activating kinase (CAK) from budding yeast. *Cell*, **86**, 553–564.

Kato,J.Y., Matsuoka,M., Strom,D.K. and Sherr,C.J. (1994) Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol. Cell. Biol.*, **14**, 2713–2721.

Knighton,D.R. *et al.* (1991) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase. *Science*, **253**, 407–414.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.

Labbé,J.C., Cavadore,J.C. and Dorée,M. (1991) M phase-specific cdc2 kinase: preparation from starfish oocytes and properties. *Methods Enzymol.*, **200**, 291–300.

Labbé,J.C. *et al.* (1994) $p40^{Mo15}$ associates with a p36 subunit and requires both nuclear translocation and Thr 176 phosphorylation to generate cdk-activating kinase in *Xenopus* oocytes. *EMBO J.*, **13**, 5155–5164.

Lee,T.H., Turck,C. and Kirschner,M.W. (1994) Inhibition of cdc2 activation by INH/PP2A. *Mol. Biol. Cell*, **5**, 323–338.

Lorca,T. *et al.* (1992a) Cyclin A–cdc2 kinase does not trigger but delays cyclin degradation in interphase extracts of amphibian eggs. *J. Cell Sci.*, **102**, 55–62.

Lorca,T., Labbé,J.C., Devault,A., Fesquet,D., Capony,J.P., Cavadore,J.C., Le Bouffant,F. and Doree,M. (1992b) Dephosphorylation of cdc2 on Thr 161 is required for cdc2 kinase inactivation and normal anaphase. *EMBO J.*, **11**, 2381–2390.

Mäkela,T.P., Tassan,J.P., Nigg,E.A., Frutiger,S., Hughes,G.J. and Weinberg,R. (1994) A cyclin associated with the CDK-activating kinase MO15. *Nature*, **371**, 254–257.

Morgan,D.O. (1995) Principles of CDK regulation. *Nature*, **374**, 131–134.

Poon,R.Y.C., Yamashita,K., Adamczewski,J.P., Hunt,T. and Shuttleworth,J. (1993) The cdc2-related protein $p40^{Mo15}$ is the catalytic subunit of a protein kinase that can activate p33cdk2 and $p34^{cdc2}$. *EMBO J.*, **12**, 3123–3132.

Poon,R.Y.C, Yamashita,K., Howell,M., Ershler,M.A., Belyavsky,A. and Hunt,T. (1994) Cell cycle regulation of the $p34^{cdc2}$/$p33^{cdk2}$-activating kinase $p40^{Mo15}$. *J. Cell Sci.*, **107**, 2789–2799.

Rosenblatt,J., Gu,Y. and Morgan,D.O. (1992) Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. *Proc. Natl Acad. Sci. USA*, **89**, 2824–2828.

Sali,A. and Blundell,T.L. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol.*, **234**, 779–815.

Seroz,T., Hwang,J., Montcollin,V. and Egly,J. (1995) $TF_{II}H$: a link between transcription, DNA repair and cell cycle regulation. *Curr. Opin. Genet. Dev.*, **5**, 217–221.

Shuttleworth,J., Godfrey,R. and Colman,A. (1990) $p40^{Mo15}$, a cdc2-related protein kinase involved in negative regulation of meiotic maturation in *Xenopus* oocytes. *EMBO J.*, **9**, 3233–3240.

Solomon,M.J. (1994) The function(s) of CAK, the $p34^{cdc2}$-activating kinase. *Trends Biochem. Sci.*, **19**, 496–500.

Solomon,M.J., Harper,J.W. and Shuttleworth,J. (1993) CAK, the $p34^{cdc2}$ activating kinase contains a protein identical or closely related to $p40^{Mo15}$. *EMBO J.*, **12**, 3133–3142.

Tassan,J.P., Jacquenoud,M., Fry,A.M., Frutiger,S., Hughes,G.J. and Nigg,E.A. (1995) *In vitro* assembly of a functional human CDK7–cyclin H complex requires MAT-1, a novel 36 kDa RING finger protein. *EMBO J.*, **14**, 5608–5617.

Thuret,J.Y., Valay,J.G., Faye,G. and Mann,C. (1996) Civ1 (CAK *in vivo*), a novel Cdk-activating kinase. *Cell*, **86**, 565–576.

Valay,J.G., Simon,M., Dubois,M.F., Bensaude,O., Facca,C. and Faye,G. (1995) The *KIN28* gene is required both for RNA polymerase II mediated transcription and phosphorylation of the Rpb1p CTD. *J. Mol. Biol.*, **249**, 535–544.

Received on July 26, 1996; revised on September 30, 1996