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Cyclin is a component of the sea urchin egg M-phase specific histone H_1 kinase

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A so-called 'growth-associated' or 'M-phase specific' histone H_1 kinase (H_1K) has been described in a wide variety of eukaryotic cell types; $p34^{cdc2}$ has previously been shown to be a catalytic subunit of this protein kinase. In fertilized sea urchin eggs the activity of H_1K oscillates during the cell division cycle and there is a striking temporal correlation between H_1K activation and the accumulation of a phosphorylated form of cyclin. H_1K activity declines in parallel with proteolytic cyclin destruction of the end of the first cell cycle. By virtue of the high affinity of the fission yeast $p13^{suc1}$ for the $p34^{cdc2}$ protein, H_1K strongly binds to p13-Sepharose beads. Cyclin, $p34^{cdc2}$ and H_1K co-purify on this affinity reagent as well as through several conventional chromatographic procedures. Anticyclin antibodies immunoprecipitate the M-phase specific H_1K in crude extracts or in purified fractions. Sea urchin eggs appear to contain much less cyclin than $p34^{cdc2}$, suggesting that $p34^{cdc2}$ may interact with other proteins. These results demonstrate that cyclin and $p34^{cdc2}$ are major components of the M-phase specific H_1K.

Key words: H_1 kinase/M phase/sea urchin

Introduction

Eukaryotic cells contain a histone H_1 kinase (H_1K) that is maximally active as cells enter into metaphase. This kinase is calcium and cyclic nucleotide independent and has been variously described as the 'growth associated' or the 'M-phase specific' H_1K (reviewed in Hohman, 1983; Matthews and Huebner, 1985; Wu *et al.*, 1986). Its transient activation at the G_2M transition has been found in a wide range of eukaryotic cell types, and occurs in both mitosis and meiosis (Lake and Salzman, 1972; Schlepper and Knippers, 1975; Bradbury *et al.*, 1974, Hardie *et al.*, 1976; Zeilig and Langan, 1980; Quirin-Stricker, 1984; Quirin-Stricker and Schmidt, 1984; Sano, 1985; Woodford and Pardee, 1986; Meijer *et al.*, 1987; Pelech *et al.*, 1987; Cicirelli *et al.*, 1988; Labbé *et al.*, 1988a,b; Meijer and Pondaven, 1988; Muller and Little, 1989).

One subunit of the starfish oocyte H_1K is a 34 kd protein related to the yeast *cdc2/CDC28* gene product (Arion *et al.*, 1988; Labbé *et al.*, 1988a). This protein is present in all actively proliferating eukaryotic cells investigated so far (Reed *et al.*, 1985; Simanis and Nurse, 1986; Draetta *et al.*, 1987; Lee and Nurse, 1987; Draetta and Beach, 1988) and its amino acid sequence clearly reveals it to be a member of the protein kinase family. In frog eggs, it has been found as one component of the M-phase promoting factor (MPF, Dunphy *et al.*, 1988; Gautier *et al.*, 1988), a non-species specific inducer of mitosis (reviewed in Kishimoto, 1988). Recent evidence further suggests that the histone H_1K and MPF are different manifestations of the same entity, which contains $p34^{cdc2}$ as a core kinase unit in association with other proteins.

The cdc2 protein kinase exists in association with at least two other polypeptides. One, of 13 kd, is the product of the fission yeast *suc1*$^+$ gene (Brizuela *et al.*, 1987) or its higher eukaryote counterpart (Draetta *et al.*, 1987; Draetta and Beach, 1988). The other is the *cdc13*$^+$ gene product (Booher and Beach, 1987; Booher and Beach, 1988; Booher *et al.*, 1989). In human cells a 62 kd cdc2-associated protein, that acts as a substrate in the protein kinase complex (Draetta and Beach, 1988), is probably an equivalent protein but this has yet to be formally established (Draetta *et al.*, 1989). The human p62-cdc2 complex is a potent cell-cycle regulated H_1K (Draetta *et al.*, 1988; Brizuela *et al.*, 1989).

The fission yeast *cdc13*$^+$ gene product shares sequence homology with a class of proteins known as mitotic cyclins (Solomon *et al.*, 1988; Goebl and Byers, 1988). Cyclins were first identified as the most abundantly synthesized polypeptides in cleaving embryos of invertebrates such as sea urchin, starfish and clams (Rosenthal *et al.*, 1980; Evans *et al.*, 1983; Swenson *et al.*, 1986; Pines and Hunt, 1987; Standart *et al.*, 1987; Westendorf *et al.*, 1989). Although the synthesis of cyclins is continuous, these proteins derive their name from their property of selective, abrupt and essentially complete proteolytic degradation at the end of each metaphase. This property generates a striking cell-cycle oscillation in the abundance of cyclins, which is maximal at metaphase and minimal within minutes of the metaphase-anaphase transition (reviewed in Minshull *et al.*, 1988; Ruderman, 1988).

The possible involvement of cyclins in controlling entry into M phase has been investigated by microinjection of clam (Swenson *et al.*, 1986; Westendorf, 1989) and sea urchin (Pines and Hunt, 1987) cyclin mRNA into *Xenopus* oocytes. In each case oocyte maturation occurs. Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis (Minshull *et al.*, 1989; Murray and Kirschner, 1989). In the fission yeasts *cdc13*$^+$ is essential for the initiation of mitosis and interacts both genetically (Booher and Beach, 1987) and physically (Booher *et al.*, 1989) with *cdc2*$^+$. In the clam, there are two cyclin proteins (A and B), both of which have been found in physical association with the cdc2 protein kinase (Draetta *et al.*, 1989). The cyclin A-$p34^{cdc2}$ and cyclin B-$p34^{cdc2}$ complexes each display H_1K activity *in vitro*. Here we have investigated the relationship between $p34^{cdc2}$, cyclins and

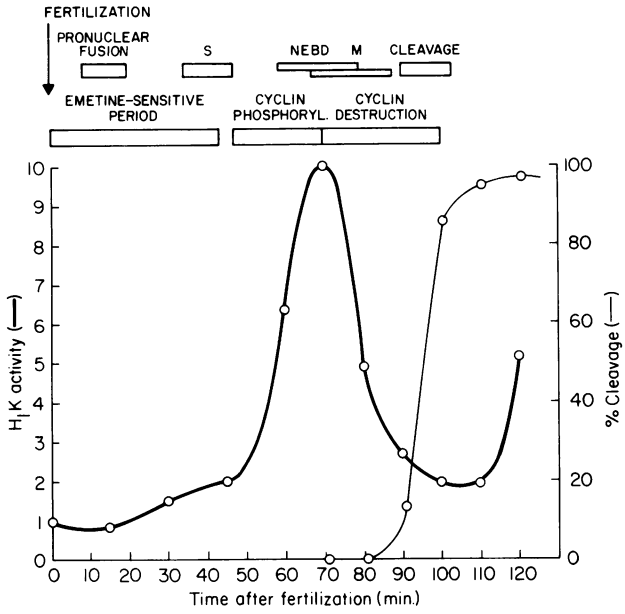

Fig. 1. Summary of post-fertilization cell cycle events in relation to activation of H_1K. M-phase-specific histone H_1K assayed in crude cell lysate undergoes a transient activation at the G_2-M transition (expressed here in fold stimulation over the unfertilized egg H_1K). Cyclin is continuously synthesized but undergoes phosphorylation that begins before nuclear membrane breakdown (NEBD) and proteolysis as the cells exit from metaphase (M). The emetine-sensitive period is that during which protein synthesis is required for H_1K activation and also for the first mitotic cycle to occur.

histone H_1 kinase activity in cleaving sea urchin eggs and find that $p34^{cdc2}$ and a phosphorylated form of cyclin are essential components of the M-phase specific H_1K.

Results

Assay of mitotic histone H_1K on $p13^{suc1}$–Sepharose

Sea urchin eggs are naturally arrested in post-meiotic interphase with a 1N female pronucleus. Fertilization activates protein synthesis and induces a series of rapid and highly synchronous mitotic divisions. Figure 1 shows that the G_2-M transition of these cell cycles is accompanied by a large (10- to 30-fold) increase in the activity of histone H_1K, which declines rapidly at the end of mitosis, well before the eggs undergo cytokinesis (see Meijer and Pondaven, 1988). This enzyme activity can be measured in crude cell extracts by using histone H_1 as substrate and by inhibiting calcium-dependent kinases by addition of EGTA and cAMP-dependent protein kinase by a synthetic peptide corresponding to the active region of the heat-stable inhibitor of this enzyme (Meijer *et al.*, 1987; Cicirelli *et al.*, 1988; see Materials and methods).

The M-phase activated H_1K of starfish has previously been assayed using $p13^{suc1}$–Sepharose beads (Arion *et al.*, 1988). Since this reagent provides a rapid and convenient method to purify $p34^{cdc2}$-containing complexes from any eukaryotic cell type (Arion *et al.*, 1988; Dunphy *et al.*, 1988; Brizuela *et al.*, 1989; Draetta *et al.*, 1989), we have used it to investigate the H_1K in cleaving sea urchin eggs. The time-course of H_1K activation was determined both in crude extracts prepared throughout the cell cycle or after incubation of these extracts with p13–Sepharose, followed by assay of the material attached to the beads (see experimental procedures). H_1K binds effectively to the beads and the

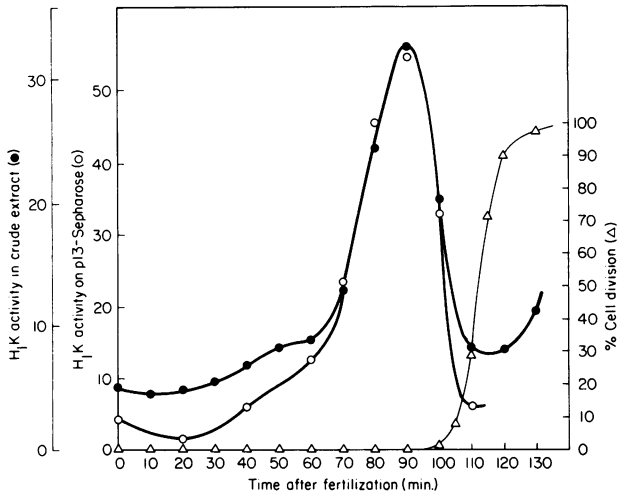

Fig. 2. H_1K binds to p13-Sepharose beads. At given intervals after fertilization egg extracts were prepared. They were assayed for H_1K activity either in crude lysates (●, activity expressed in pmol/10 min/5 μl extract) or following binding to p13-Sepharose beads (○, activity in pmol/10 min/10 μl of beads). A representative of three separate experiments is shown.

time course of activation using this reagent is identical to that assayed in the crude cell lysate (Figure 2), although the full activity of the crude lysate is not recovered on the p13–Sepharose (~60% of the H_1K binds to p13–Sepharose). No H_1K activity was detected using control Sepharose beads (CNBr-activated sepharose which had been reacted with ethanolamine) (data not shown).

Examination by SDS–PAGE of the proteins bound to the p13–Sepharose at different stages of the cell cycle revealed major silver-staining polypeptide species of 34 and 42 kd (Figure 3). The 34 kd protein co-migrated with authentic $p34^{cdc2}$ and was formally identified by immunoblotting and is therefore taken to be $p34^{cdc2}$ (not shown). A 42 kd protein of unknown identity also binds to the p13-Sepharose in addition to numerous other minor proteins. None of the silver stained proteins bound to p13-Sepharose changed in abundance during the cell-cycle dependent oscillation of H_1K activity. Nor did any significantly decline after exposure of fertilized eggs to the protein synthesis inhibitor emetine (not shown), which prevents activation of the H_1K (Meijer and Pondaven, 1988; Arion and Meijer, 1989; Figure 3).

Cyclin–$p34^{cdc2}$ complex

Although no cyclin-like protein was detected by silver staining of material eluted from p13-Sepharose, the synthesis of cyclin correlates with activation of the H_1K. To investigate the link between cyclin and H_1K, [^{35}S]methionine labelled whole cell extracts were analysed by SDS–PAGE and H_1K activity was measured in the same batch of eggs throughout the cell cycle. Cyclin was clearly visible as a strongly labelled pair of polypeptides, with the smaller giving rise to the larger as the cells entered mitosis (Figure 4, upper panel). We believe that the upper band represents a phosphorylated form of the lower one, since the upper band can be converted to the lower one by phosphatase treatment. Furthermore the lower band can be converted *in vitro* to the upper band in the presence of ATP and NaF (Golsteyn and Hunt, data not shown). When the activity of histone H_1K was correlated with the abundance of the cyclin bands (deter-

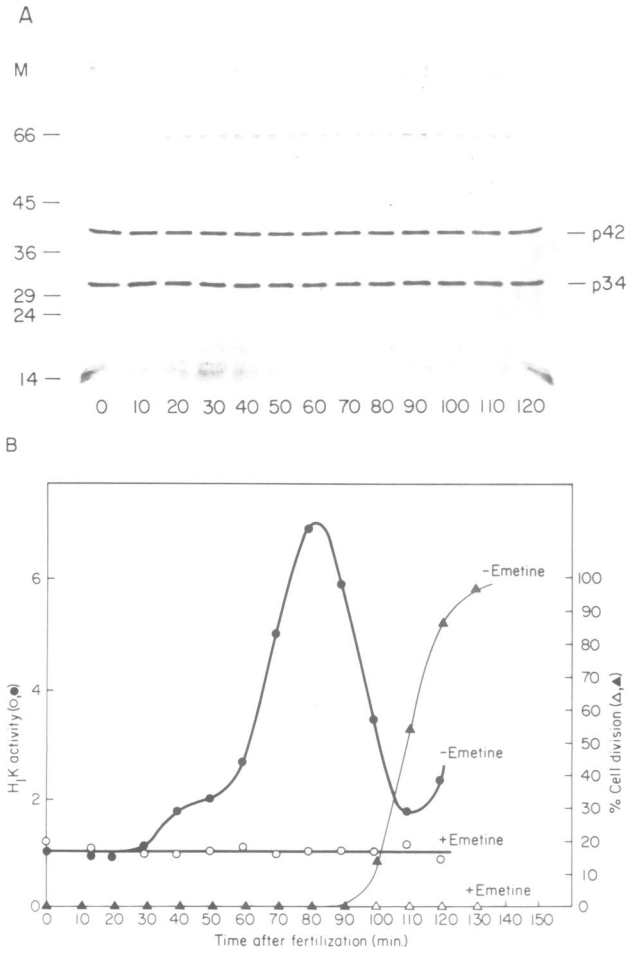

Fig. 3. Patterns of proteins bound to p13-Sepharose during the cell cycle. Eggs were fertilized and divided into two batches; one was left untreated and the other was exposed to 100 μM emetine added at 9 min. Samples were taken every 10 min, and extracts of the cells were incubated with p13−Sepharose or assayed for H_1K. **Panel A** shows the proteins bound to p13−Sepharose beads, analysed by SDS−PAGE. **Panel B** shows the H_1K activity of the crude cell extracts (pmol phospate/min/0.5 μl). Closed symbols, control culture; open symbols, emetine-treated culture. A representative of six independent experiments is shown.

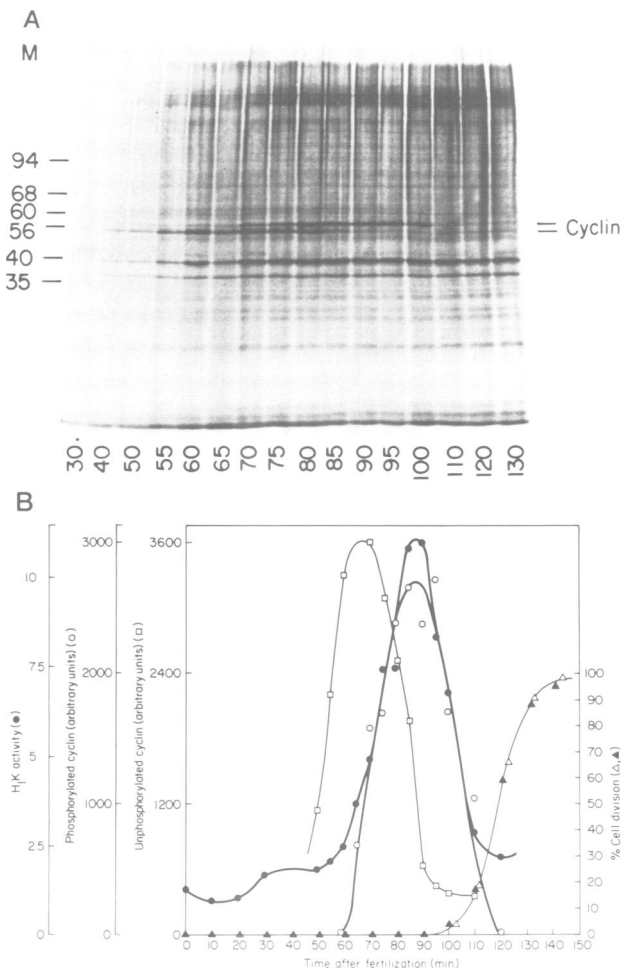

Fig. 4. Time-course of H_1K activation in relation to cyclin synthesis, phosphorylation and degradation. Eggs were fertilized and divided into two batches; one was exposed to [^{35}S]methionine at 10−15 min post-fertilization and used to detect cyclin by SDS−PAGE and autoradiography (**A**). The other batch was used to prepare extracts for assay of H_1K activity in crude cell lysates [**B** (●), activity expressed in pmol/10 min/1 μl)]. The time-course of cleavage in each batch (△,▲) shows that the two kept in exact synchrony. Quantification of unphosphorylated (□) and phosphorylated (○) cyclin by scanning of the autoradiograph is shown.

mined by densitometry), a striking correlation emerged (Figure 4, lower panel). There was essentially no correlation between the total cyclin, or the lower cyclin band and the kinase activity. However, the appearance of phosphocyclin showed a precise temporal correlation with H_1K activity (Figure 4, lower panel) and a plot of phosphorylated cyclin against H_1K activity gave an excellent straight line, including points on both the up- and down-swings of the time course.

Although no cyclin is visible in silver-stained gels of p13-Sepharose precipitates we directly examined the possibility that cyclin was bound on the matrix. Fertilized eggs were labelled with [^{35}S]methionine, extracts were prepared throughout the cell cycle and incubated with p13−Sepharose beads. The p13-associated material was analysed by SDS−PAGE and autoradiography. Figure 5A shows that a pair of labelled bands with the mobilities of cyclin and its phosphorylated form were bound to the resin. The p13 beads showed excellent selectivity for cyclin in that very few of the many other labelled proteins bound to the resin (compare the autoradiographs in Figures 4A and 5A). Plotting the abundance of the slower migrating species against the activation of H_1K, that was assayed in the same batch of fertilized eggs, revealed a close temporal correlation between the presence of the phosphorylated form of cyclin and active H_1K (Figure 5B and inset). This doublet of proteins was totally absent in emetine-treated cells (data not shown).

To confirm that these bands indeed corresponded to cyclin, the experiment was repeated with no labelled methionine present and the p13-Sepharose-bound proteins were transferred to nitrocellulose after PAGE separation and probed with an anti-sea urchin cyclin antibody (see Materials and methods). As shown in Figure 6A, the two bands reacted with the antibody, confirming their identity as alternative forms of sea urchin B-type cyclin. This experiment shows another example of the excellent correlation between the level of the upper cyclin band and histone H_1K activity (Figure 6B). It is noteworthy that in both experiments, the two forms of cyclin are bound equally well by the $p13^{suc1}$ beads.

In *Xenopus*, a 45 kd protein and in humans a 62 kd protein are found in association with $p34^{cdc2}$ (Gautier *et al.*, 1988;

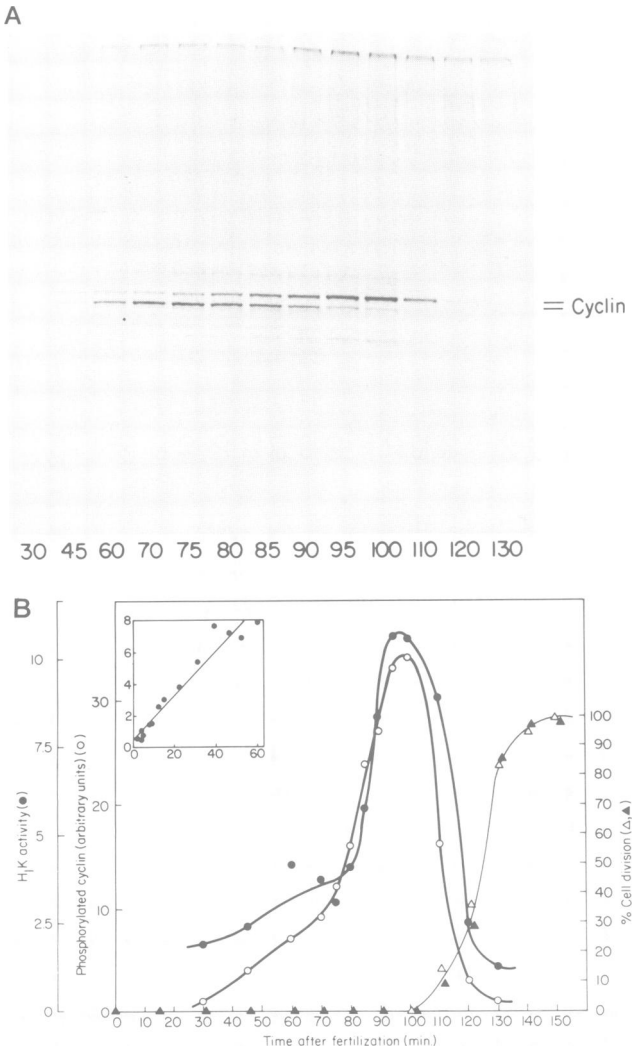

Fig. 5. Cyclin binds to p13-Sepharose beads. Eggs were fertilized and divided in two batches. One was exposed to [^{35}S]methionine at 8 min post-fertilization and extracts made at given intervals after fertilization and incubated with p13-Sepharose. The ^{35}S-labelled material was analysed by SDS−PAGE and autoradiography (**A**). The other batch was used to prepare extracts for H_1K activity determination [**B**, (●), activity expressed in pmol/10 min/μl]. The time-course of cleavage in each batch (△,▲) shows that the two batches kept synchronized. Quantification of the phosphorylated cyclin on the autoradiograph (○) shows superimposability of H_1K activation and phosphorylated cyclin. The inset represents M-phase-specific H_1K activity as a function of phosphorylated cyclin amount. A representative of three independent experiments is shown.

Draetta and Beach, 1988). This subunit appears to be a target for the p34-associated kinase activity, and can be phosphorylated with [γ-^{32}P]ATP and there are indications that these subunits correspond to cyclin. To test whether this was true of sea urchin cyclin, p13 beads loaded with extracts from sea urchin eggs taken throughout the cell cycle were extensively washed, incubated with [γ-^{32}P]ATP, and analysed by SDS−PAGE. Several proteins were labelled by this procedure, among them one that migrated with precisely the same mobility as the upper cyclin band (Figure 7A). An unidentified high molecular weight protein showed intense mitotic phosphorylation. When the same procedure was applied to emetine-treated eggs, phosphorylated proteins were not detected (data not shown). Quantitation of the ^{32}P-labelled cyclin shows that its transient appearance

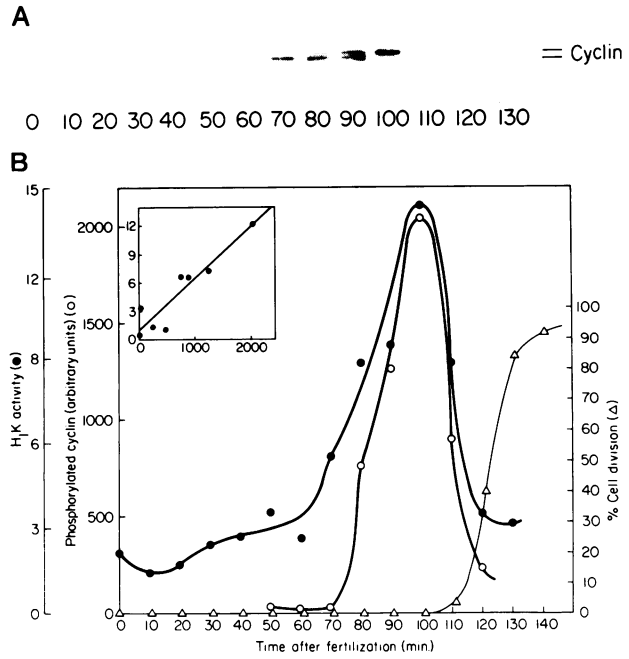

Fig. 6. Anti-cyclin immunoblotting of p13-Sepharose bound proteins. Eggs were fertilized and extracts were prepared at intervals after fertilization. They were assayed for H_1K activity (●, **panel B**, activity expressed in pmol/10 min/μl) or loaded on p13-Sepharose beads. The bound proteins were resolved by SDS−PAGE, transferred to nitrocellulose and blotted with anti-cyclin serum. The blotted proteins were revealed with [^{125}I]protein A (**A**). Quantification of the phosphorylated cyclin on the blots by direct counting of the radioactive spots (○) is shown in panel B. The inset represents M-phase-specific H_1K activity as a function of abundance of phosphorylated cyclin.

is superimposable with the transient activation of H_1K (Figure 7B and inset).

Cyclin co-purifies with p34^{cdc2} and H_1K

To investigate the relationship between histone H_1K and the cyclins we used standard chromatographic procedures to purify the kinase, and assayed the presence of cyclin and p34^{cdc2} by quantitative immunoblotting. After chromatography on hydroxylapatite, phenyl−Sepharose, reactive yellow 86 agarose, DEAE−Sepharose and polylysine−agarose the kinase was purified about 1000-fold. The fractions of the DEAE−Sepharose column were subjected to SDS−PAGE and the abundance of cyclin and p34^{cdc2} was assayed by quantitative immunoblotting. Cyclin and p34^{cdc2} both co-eluted exactly with H_1K (Figure 8A). Moreover, immunoprecipitates made with the anti-cyclin antibodies on the most active H_1K fraction of each chromatographic step contained histone H_1K activity (up to 50% of H_1K could be immunoprecipitated with the less diluted serum) (Figure 8B). Pre-immune serum from the same rabbit did not display any such activity. We also tested the activity of anti-cyclin immunoprecipitates from extracts prepared throughout the cell cycle. Figure 9 shows that they had identical activity to the activity of crude fractions. Neither pre-immune serum nor immunoprecipitates of emetine-treated activated eggs displayed histone kinase activity.

Discussion

In this paper we demonstrate a clear temporal relationship between the activity of histone H_1K and the presence of a

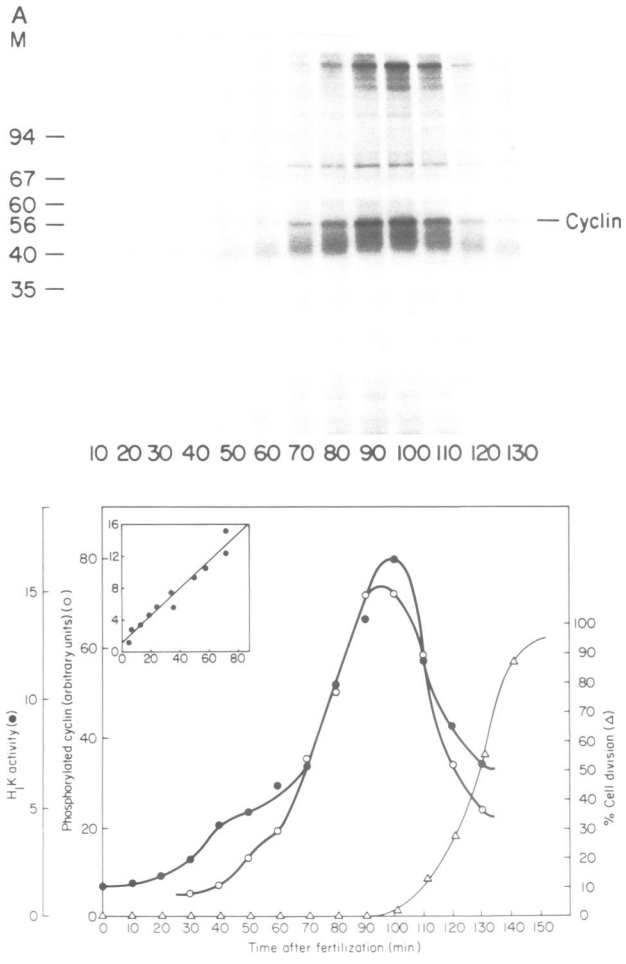

Fig. 7. *In vitro* phosphorylation of p13-bound cyclin. At intervals after fertilization extracts were prepared for H_1K activity determination (●, **panel B**, activity expressed in pmol/10 min/μl) or loaded onto p13-Sepharose. After extensive washing the beads were incubated at 30°C for 10 min with [^{32}P]ATP. The reaction was stopped by addition of Laemmli sample buffer and the phosphorylated proteins were analysed by SDS−PAGE and autoradiography (**A**). Quantification of ^{32}P-labelled cyclin from the autoradiograph is shown (○, panel B). The inset represents M-phase-specific H_1K activity as a function of phosphorylated cyclin amount. The result shown is a representative of three independent experiments.

phosphorylated form of cyclin during the first mitotic cell cycle of fertilized sea urchin eggs. We further show that cyclins are virtually the only newly synthesized proteins which bind to p13^{suc1}−Sepharose beads, and that cyclin and p34^{cdc2} co-purify through four steps of conventional purification. Moreover, immunoprecipitates using an anti-sea urchin cyclin B antibody possess histone H_1K activity. The present results are fully consistent with the recent finding that p34^{cdc2} exists in cyclin A and cyclin B complexes in clam embryos (Draetta *et al.*, 1989) and also the previously described genetic (Booher and Beach, 1987) and physical (Booher *et al.*, 1989) interactions between the *cdc2*$^+$ and *cdc13*$^+$ gene products of fission yeast. Also, M-phase p34^{cdc2} from human cells is a highly active H_1K and is complexed with a 62 kd endogenous substrate that is presumably a cyclin (Draetta and Beach, 1988; Brizuela *et al.*, 1989). Finally the 45 kd subunit of *Xenopus* MPF, which is tightly associated with p34^{cdc2} (Gautier *et al.*, 1988) probably corresponds to cyclin; this complex displays H_1K activity. All these cumulative results support the idea that the sea urchin mitotic cyclin, along with p34^{cdc2}, is a major component of the M-phase specific H_1K.

p34^{cdc2} − cyclin ratio

In the present experiments, it is striking that the cyclin was readily detectable in p13−Sepharose precipitates only if [^{35}S]methionine labelling or immunoblotting were used. It was quite undetectable by silver staining which readily revealed p34^{cdc2}. Since cyclin does not react particularly poorly with the silver staining reagent, this suggests that p34^{cdc2} is a very much more abundant protein even at its highest abundance during mitosis. According to our estimates, the concentration of p34^{cdc2} in sea urchin eggs is between 2 and 5 μM (68−170 μg/ml), whereas the concentration of cyclin is at most 0.25 μM (11 μg/ml). This implies that the majority of p34^{cdc2} is not complexed with cyclin. It further suggests that p34^{cdc2} may interact with other proteins; the 42 kd protein—which is not a cyclin—is one such candidate. It is thus possible that p34^{cdc2} forms other cell-cycle-dependent protein kinases. The use of myelin basic protein as a substrate has recently shown that kinases, different from H_1K, are activated during starfish oocyte maturation and sea urchin egg mitosis (Pelech *et al.*, 1989); they may however share the same p34^{cdc2} catalytic subunit. Although we favour the hypothesis that cyclin and p34^{cdc2} interact stoichiometrically, the possibility that cyclin acts catalytically to activate all the available p34^{cdc2} has not been completely ruled out.

Cyclin phosphorylation

The other interesting feature of these results is the excellent correlation between the level of 'shifted' cyclin and histone H_1 kinase activity. At first sight, the obvious interpretation of this result is that cyclin is a substrate for p34^{cdc2} kinase, and becomes phosphorylated and shifted when the cells enter M-phase. This view appears to be supported by the data of Figure 7, which show that cyclin is a substrate for the mitotic H_1K bound to p13 beads. It is not clear, however, that the phosphorylation due to the activity of the H_1K causes the shift in cyclin mobility, and preliminary results (Golsteyn, unpublished data) indicate that it probably does not. The identification of protein kinases that act on cyclin and the physiological consequences of these modifications require further investigation.

Activation and inactivation of H_1K

The abundance of p34^{cdc2} does not appear to be a regulatory factor in the activation of H_1K. Its concentration does not vary substantially during the cell cycle (Arion and Meijer, 1989). Nevertheless post-translational control of p34^{cdc2} may be cell-cycle dependent and crucial for H_1K activation. In HeLa cells p34^{cdc2} is a very heavily tyrosine phosphorylated protein (Draetta *et al.*, 1988). It has been found in mouse 3T3 cells that entry into mitosis is accompanied by dephosphorylation of tyrosine and threonine residues of p34^{cdc2} in the p34^{cdc2}−P62 complex and further that inhibition of dephosphorylation of the protein kinase is associated with failure to enter mitosis (Morla *et al.*, 1989). p34^{cdc2} dephosphorylation may thus be a necessary step for activation of H_1K. Preliminary results have shown this to be the case in sea urchin eggs (Meijer *et al.*, in preparation).

The striking correlation between the presence of the phosphorylated form of cyclin and H_1K activation suggests that the phosphorylation of cyclin might be a crucial step

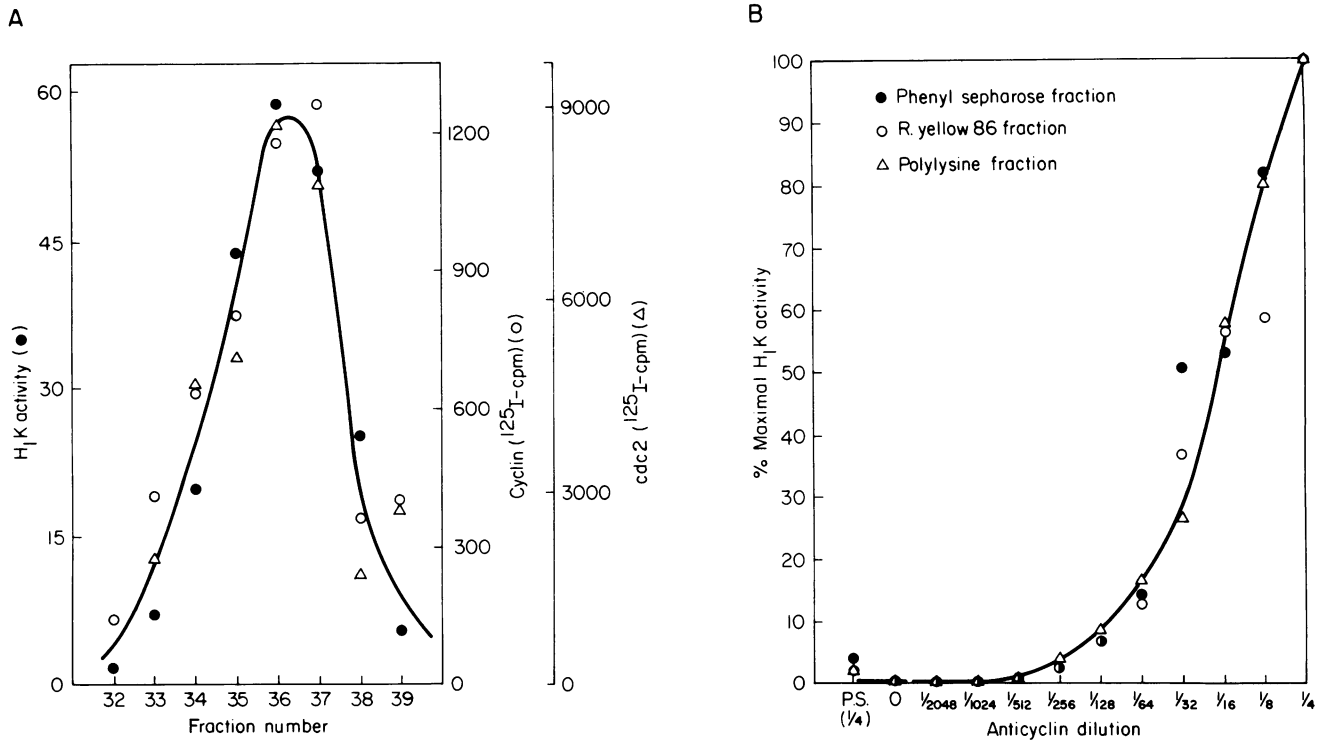

Fig. 8. Cyclin cdc2 and H_1K co-purification. (**A**) Elution of H_1K (●), cyclin (○) and p34 (△) in DEAE−Sepharose chromatography. The fractions from of the DEAE−Sepharose elution were assayed for H_1K activity or run on SDS−PAGE gels before immunoblotting with anti-p34 and anti-cyclin sera. The ^{125}I-labelled bands were excised from the immunoblots and counted. (**B**) Anti-cyclin antibodies immunoprecipitate H_1K throughout the purification procedure. H_1K prepared from mitotic eggs was purified through various chromatographic steps. Immunoprecipitation with various dilutions of anticyclin antibodies was performed on the peak fraction of the phenyl−Sepharose (●), Reactive yellow 86−agarose (○) and polylysine−agarose (△) columns. The immunoprecipitate was then assayed for H_1K activity. No detectable H_1K was observed in the pre-immune serum precipitate (P.S.). Results are expressed as the % of maximal H_1K precipitated by the antibody (i.e. ~30−50% of the initial H_1K in the peak fraction).

in H_1K activation. Thus full activation of the M-phase-specific H_1K could be achieved by coordinate phosphorylation of the cyclin and dephosphorylation of $p34^{cdc2}$.

It is highly probable that inactivation of the histone H_1K at the onset of anaphase is the consequence of cyclin degradation by proteolysis (Draetta *et al.*, 1989). Strong support for this hypothesis has recently been obtained by Murray *et al.* (1989) who have shown that a truncated form of cyclin resists degradation and maintains cell-free *Xenopus* extracts in M phase. The mechanism of cyclin destruction and its temporal precision are of considerable interest, as is the question of exactly why loss of cyclin causes loss of histone H_1K activity.

Materials and methods

Material—preparation of gametes—fertilization

All experiments presented in this paper were performed on the sea urchin *Sphaerechinus granularis* which were collected by diving or at low tide in Brittany (Brest area) and kept in running seawater until use. Shedding of gametes was induced by injection of 0.2 ml of 0.2 M acetylcholine through the peribuccal membrane. Sperm was collected 'dry' and kept undiluted at +4°C. Eggs were collected in Millipore-filtered natural seawater (NSW). They were washed once with NSW and the egg jelly was removed in most cases by washing the eggs twice with buffer A. Eggs were rinsed three times with NSW and resuspended as a 10% (v/v) suspension. Just before fertilization, glycine (final concentration 0.1%, w/v) was added to the egg suspension; this procedure facilitates the elevation of the fertilization membrane in this species. Sperm was diluted just before insemination (1 drop 'dry' sperm/5 ml NSW; 1 drop of this dilution/10 ml egg suspension). At 2−3 min after sperm addition the eggs were checked for successful fertilization (100% in all experiments) and the excess sperm was removed by washing the eggs once with NSW. All experiments were performed at +20°C.

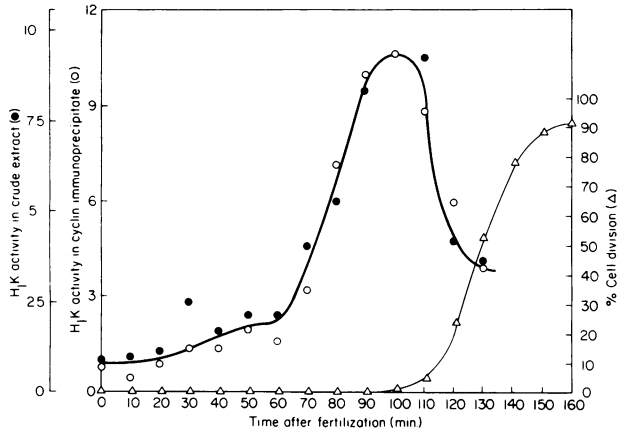

Fig. 9. Immunoprecipitation of cell-cycle dependent H_1K by anti-cyclin. At intervals after fertilization extracts were prepared for H_1K activity determination either in crude extract (●) or after immunoprecipitation with anti-cyclin antibodies (○). The data shown is representative of two independent experiments.

Buffers

Buffer A: 50 mM Tris, 50 mM maleic acid, 520 mM NaCl, 1 mM EDTA, pH to 8.0.

Homogenization buffer: 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl-phosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 0.1 mM sodium fluoride,

10 μg leupeptine/ml, 10 μg aprotinin/ml, 10 μg/ml soybean trypsin inhibitor, 100 μM benzamidine. This buffer had previously been shown to stabilize the starfish meiotic oocyte and sea urchin mitotic egg M-phase-specific histone H_1K (Meijer *et al.*, 1987; Pelech *et al.*, 1987; Meijer and Pondaven, 1988).

Buffer C: Homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors.

KI: 25 mM β-glycerophosphate, 15 mM nitrophenyl phosphate, 25 mM MOPS pH 7.2, 1 mM EGTA, 15 mM $MgCl_2$, 2 mM DTT, 0.1 mM NaF.

KII: 25 mM β-glycerophosphate, 25 mM MOPS pH 7.2, 1 mM EGTA, 15 mM $MgCl_2$, 2 mM DTT, 0.1 mM NaF.

KIII: 220 mM phosphate buffers at pH 7.2 in KII.

KIV: KII diluted 1:1 with distilled water.

KV: 0.1% Brij 35 in KIV.

KVI: 200 mM NaCl in KV.

Bead Buffer: 50 mM Tris pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 10 μg leupeptine/ml, 10 μg aprotinin/ml, 10 μg soybean trypsin inhibitor/ml, 100 μM benzamidine.

Transfer Buffer: 39 mM glycine, 48 mM Tris, 0.37% SDS, 20% methanol.

'Net Gel': 150 mM NaCl, 5 mM EDTA, 2.5 g gelatine/l, 0.01% NP40, 50 mM Tris (pH 7.4).

Preparation of egg extracts

At various times after addition of sperm, 1 or 2 ml aliquots of the egg suspension were rapidly centrifuged (5 s full speed in an Eppendorf centrifuge). The supernatant was removed by aspiration and 400 μl of homogenization buffer was added to the 100 or 200 μl egg pellet. The tube was immediately frozen in liquid nitrogen and kept at −20°C until further processing (within 24 h). Eggs were homogenized by a 10-s sonication. After centrifugation for 10 min at 14 000 *g* at 4°C, the supernatant was recovered and either frozen in liquid nitrogen and stored at −20°C until the kinase assay or immediately loaded on p13 beads or exposed to anti-cyclin antibodies.

Purification of H_1K

Eggs were pelleted by a brief centrifugation (1 min at 1000 *g*) about 80−90 min after insemination and the sea water was removed. The cells were homogenized with 3 or 4 vol of homogenization buffer in a glass homogenizer. After centrifugation at 100 000 *g* for 30 min at 4°C, the supernatant was immediately loaded on an 80 ml hydroxylapatite column (IBF, France) equilibrated with KI. The resin was washed with KI and elution of the kinase performed by buffer KIII. The active fractions were pooled, and loaded onto a 60 ml phenyl−Sepharose column (Pharmacia) equilibrated with KII. The column was then extensively washed with KII before elution with 2% Brij 35 in KII. The pooled active fractions were then diluted five-fold and loaded onto a 60 ml Reactive Yellow 86−agarose column (Sigma) equilibrated with KV. After washing with KV, the active H_1K was eluted with KVI. The active fractions were pooled, diluted and loaded on a 50 ml DEAE−Sepharose column (Pharmacia) equilibrated with KV. The resin was washed with KV and H_1K was eluted with a 0−0.4 M NaCl linear gradient (in KV). The active fractions were diluted and loaded on a 30 ml polylysine−agarose column (Sigma) equilibrated with KV. Active H_1K was eluted with 0.5 M NaCl in KV.

Histone H_1K assays

All kinase assays contained 50 nM TTYADFIASGRTGRRNAIHD (a synthetic peptide with a structure corresponding to the inhibitory site of cAMP-dependent protein kinase inhibitor, Scott *et al.*, 1986), 1 mg histone H_1/ml (Sigma, type III-S), 15 μM [γ-^{32}P]ATP at ~800 c.p.m./pmol, 5 μl of diluted extract or column fraction and buffer C, in a final volume of 25 μl. Kinase reactions were started by the addition of radioactive ATP and were of 10 min duration at 30°C. Assays were terminated by spotting 20 μl aliquots onto 2.5 × 3 cm pieces of Whatman P81 phosphocellulose paper, and, after 20 s, the filters were washed five times (for at least 5 min each time) in a solution of 10 ml phosphoric acid/liter of water. The wet filters were transferred into 6 ml plastic scintillation vials, 5 ml of ACS (Amersham) scintillation fluid was added and the samples were counted in a Packard Counter. The kinase activity was expressed in pmol phosphate incorporated in 5 μg histone H_1/fraction or extract volume/10 min incubation.

To assay H_1K activity on material bound to p13 beads, the bead pellet was resuspended in 30 μl of a mixture containing 1 mg histone H_1/ml, 500 nM of the PKI-synthetic peptide and 15 μM [γ-^{32}P]ATP. After 10 min incubation at 30°C, the p13 beads were pelleted and 20 μl of supernatant was removed and spotted onto Whatman P81 phosphocellulose papers which were further processed as described above. H_1 Kinase assay on material bound to protein A beads was essentially the same with the exception of H_1 concentration (1.7 mg/ml) and ATP concentration (25 μM).

Cyclin labelling in vivo

To observe cyclin appearance, conversion and disappearance, eggs were labeled with [^{35}S]methionine (Amersham, final concentration: 50 μCi/ml) added shortly (8−15 min) after fertilization. In some experiments, 50 μl samples of the egg suspension were placed at various intervals into 100 μl of cold 25% TCA; these samples were centrifuged for 2 min at 10 000 *g* and the supernatant was removed. The pellet was washed once in 50 μl of water and twice in 1 ml of ice-cold acetone. The pellet was dried with a stream of compressed air and solubilized in 100 μl of SDS sample buffer. Samples were analysed by SDS−PAGE and autoradiography as described further in the text. In experiments involving p13 beads, 2 ml samples of the egg suspension were withdrawn at various times after fertilization and the eggs were rapidly pelleted, resuspended in 400 μl of homogenization buffer and frozen in liquid nitrogen. After homogenization as described under 'preparation of egg extracts', the [^{35}S]methionine labelled samples were loaded on p13 beads as described further. The proteins bound to the p13 beads were then analysed by SDS−PAGE and autoradiography.

Preparation and use of p13−Sepharose beads

p13 was purified from an overproducing strain of *Escherichia coli* by gel filtration on Sepharose CL-6B (Pharmacia) as described previously (Brizuela *et al.*, 1987) and a further step of Mono-Q (Pharmacia) ion-exchange chromatography. It was conjugated to CnBr-activated Sepharose 4B (Sigma) according to the instructions of the manufacturer. Unreacted groups on the resin were quenched with 1 M ethanolamine (pH 8.0). The concentration of coupled p13 was 5 mg/ml of gel. The p13 beads used in this study are essentially the same as those described by Dunphy *et al.* (1988). The p13 beads were kept at 4°C as a 20% (v/v) suspension in bead buffer. Just before use, 10 μl of packed p13 beads was washed with 1 ml of bead buffer and resuspended in 400 μl bead buffer. The egg extract supernatant (390 μl) was added to the beads and the tubes were kept under constant rotation at 4°C for 30 min. After a brief centrifugation at 10 000 *g*, removal of the supernatant, the beads were washed three times with 1 ml of bead buffer. The beads were then either resuspended in 60 μl of PAGE sample buffer and boiled for 3 min or immediately used for H_1K activity determination.

Preparation of anti-cyclin antibodies

Arbacia punctulata B-type cyclin was expressed at high level in *E.coli* using the T7 expression system of Tabor and Richardson (1985). An *Nco*I−*Hin*dIII fragment of the sea urchin cDNA clone containing all of the coding region except for 35 nucleotides at the 5′ end was inserted into an end-filled *Nco*I−*Hin*dIII cut modified pGEM1 vector that contained the ribosome binding site of the MS2 phage replicase gene. Oligonucleotide adaptors were used to reconstruct the 5′ end of the cyclin sequence from the end-filled *Nco*I site of the vector to the *Nco*I site in the cyclin coding region. The resulting plasmid, Cyc-RBS, gave much higher expression of the protein than when it was expressed from its normal 5′ leader. Expression was induced by heat-shock in the presence of rifampicin using the system of Tabor and Richardson (1985) with the host bacteria K38/pGP1-2. Cyclin was clearly visible as a strongly staining band after induction. The bacteria were harvested and sonicated. Cyclin formed insoluble inclusion bodies, which were washed with Triton X-100 and EDTA according to Marston (1988) and then run on an SDS−polyacrylamide gel. The cyclin band was visualized by soaking the gel in 0.25 M KCl. This band was cut out, emulsified in Freund's complete adjuvant and injected into rabbits. Booster injections of 0.5−1.0 mg/animal (from a 500 ml bacterial culture) were given at 2 week intervals in Freund's incomplete adjuvant. The antisera recognized B-type cyclins from all sea urchins tested (*A.punctulata*, *Strongylocentrotus purpuratus* and *S.granularis*), but did not cross react with *Spisula* or *Xenopus* cyclins.

Immunoprecipitation

To precipitate the H_1K with anti-cyclin antibodies 30 μl of serum (at various dilutions) were mixed with 30 μl of fraction or extract; after 90 min incubation at 0°C, 20 μl of a 50% (v/v) suspension of protein A−agarose (Sigma)

were added; this mixture was kept for 30 min at 4°C under constant rotation; after a brief centrifugation and removal of the supernatant, the beads were washed twice with 1 ml of bead buffer and immediately assayed for H_1K activity.

Electrophoresis and Western blotting

Fractions from the purification columns were concentrated by lyophilization or redissolved in Laemmli sample buffer (Laemmli, 1970). Proteins bound to p13 beads were recovered with the sample buffer. Typically, samples were run in 10% polyacrylamide gels and stained with the BioRad silver staining kit. [^{35}S]methionine or [^{32}P]phosphate labelled gels were dried prior to exposure to β-Max X-ray film (Amersham) overnight. For immunoblotting, experimental samples were run on 7.5–15% SDS–polyacrylamide gradient gels; proteins were transferred to 0.1 μm nitrocellulose paper or to immobilon sheets (Millipore) in a transblot cell (Biorad) for 30–40 min at 2.5 mM/cm^2 in transfer buffer. Subsequently the filters were blocked with phosphate-buffered saline (PBS) containing 1% ovalbumin or 3% powdered milk for 1 h at 37°C. The filters were then incubated overnight, at room temperature, with a 1:1000 dilution of the G_1 serum against p34^{cdc2} or a 1:250 dilution of the serum against cyclin. After three washes of 15 min each with 'Net gel' the filters were treated with 1 μCi of [^{125}I]protein A (80 μCi/mg, ICN or Amersham) in 1% BSA in PBS for 2 h at room temperature. After four washes of 15 min each with 'Net gel' the filters were exposed to XoMat AR or β-Max X-ray films overnight. In some experiments the radioactive bands on the nitrocellulose paper were cut out and counted in a scintillation-counter.

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