J. Cell Sci. Suppl. 12, 99–116 (1989)
Printed in Great Britain © The Company of Biologists Limited 1989

Temporal regulation of cdc2 mitotic kinase activity and cyclin degradation in cell-free extracts of *Xenopus* eggs

MARIE-ANNE FELIX[1], JONATHAN PINES[2], TIM HUNT[3] AND ERIC KARSENTI[1]

[1]*European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstr. 1, 6900 Heidelberg, FRG*
[2]*The Salk Institute, P.O. Box 858800, San Diego, California 92138-9216, USA*
[3]*University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, England*

Summary

In cleaving *Xenopus* eggs, the cell division cycle is abbreviated to a rapid succession of S and M phases. During mitosis a number of proteins show increased phosphorylation due to the activation of a histone H1 kinase, the homologue of the *cdc2*$^+$ gene product of the yeast *Schizosaccharomyces pombe*. We have studied the regulation of the activity of this enzyme in cell-free extracts of *Xenopus* eggs. In extracts of activated eggs incubated at 22 °C, histone H1 kinase activity shows two peaks of activation and disappearance. Activation occurs in two stages. The first stage requires protein synthesis, whereas the second does not. The second stage of activation involves post-translational activation of the kinase. Kinase activity rises to a peak and then abruptly disappears. Added sea urchin cyclin is degraded at the time of disappearance of kinase activity. The oscillation in kinase activity is then repeated, usually with lower amplitude.

Post-translational activation of the kinase requires a membrane-containing particulate cellular component, whose role has yet to be defined. The kinase can still be activated in the presence of EDTA or in the presence of the ATP analogue, 6-dimethylaminopurine, which implies that phosphorylation of the kinase complex is not required for activation. Under these conditions, however, the kinase activity does not show its normal sudden disappearance, and added cyclin is perfectly stable. These observations are consistent with the idea that post-translational activation of the kinase involves protein phosphatase activity, whereas switching off the kinase requires an ATP–Mg^{2+}-dependent reaction, perhaps due to protein phosphorylation.

Introduction

During the first few hours following fertilization of *Xenopus laevis* eggs, the cell division cycle is abbreviated to a rapid succession of S and M phases, without any significant G_1 and G_2 phases (Newport and Kirschner, 1982; Signoret and Lefresne, 1971). The rapid alternation between two cytoplasmic states, interphase and mitosis, has been interpreted in terms of a 'master oscillator' that controls the various structural and metabolic events which characterize S and M phases (Kirschner *et al.* 1985).

The molecular nature of this oscillator is beginning to be defined. The level of overall protein phosphorylation increases in M phase in all eukaryotes (Ajiro *et al.* 1981; Bradbury *et al.* 1973; Capony *et al.* 1986; Dorée *et al.* 1983; Karsenti *et al.* 1987; Maller *et al.* 1977; Meijer *et al.* 1982; Peaucellier *et al.* 1984). This is correlated

Key words: *cdc2*$^+$, cyclin, *Xenopus* eggs, protein kinase.

with the activation of a major histone H1 kinase and a cytoplasmic activity called MPF for maturation promoting factor (Capony *et al.* 1986; Cicarelli *et al.* 1988; Guerrier *et al.* 1977; Picard *et al.* 1987; Sano, 1985; Maller, 1985). Using a cell-free assay developed from frog eggs, in which MPF activity can be followed by the induction of nuclear envelope breakdown and the formation of mitotic chromosomes (Lohka and Maller, 1985; Lohka and Masui, 1984; Miake-Lye and Kirschner, 1985), Lohka *et al.* (1988) obtained a high degree of MPF purification. Two major polypeptides of 32 and 45K ($K=10^3 M_r$) are present, and a histone H1 kinase activity is associated with MPF. The histone H1 kinase activity is apparently due to the 32K polypeptide that has been identified as the homologue of the *S. pombe* $cdc2^+$ gene product in frogs, starfish and human (Arion *et al.* 1988; Gautier *et al.* 1988; Labbé *et al.* 1988*b*; Lee and Nurse, 1987). It thus appears that the kinase activity associated with the homologue of the $cdc2^+$ gene product plays a fundamental role in the initiation of mitosis. Since the level of $cdc2^+$ polypeptide does not appear to fluctuate during the cell cycle (Draetta and Beach, 1988; Labbé *et al.* 1988*b*; Simanis and Nurse, 1986), whereas its activity does, the focus of attention turns to the matter of how the activity of this enzyme is controlled to give the metastable mitotic state.

It is well established that synthesis of new proteins is required for turning mitotic kinase on at each cell cycle (Karsenti *et al.* 1987; Miake-Lye *et al.* 1983; Picard *et al.* 1985; Wagenaar, 1983), and a family of proteins, the cyclins, are prime candidates to account for the protein synthesis that is required to drive the cell cycle. They are synthesized continuously, accumulate during interphase and are destroyed abruptly during mitosis (Evans *et al.* 1983; Pines and Hunt, 1987; Standart *et al.* 1987; Swenson *et al.* 1986). Antisense ablation of cyclin mRNA from *Xenopus* extracts prevents them entering mitosis (Minshull *et al.* 1989), and conversely, sea urchin cyclin mRNA is able to restore mitotic cycles to such extracts after their mRNA has been destroyed by RNase (Murray and Kirschner, 1989). This shows that cyclin synthesis is required for activation of the kinase. Genetic analysis in yeast has revealed that regulation of $cdc2^+$ kinase activity involves at least two other kinases (Russell and Nurse, 1987). This suggests that the mechanism of kinase activation requires a series of kinases and phosphatases that comprise a cascade of reactions. It is not yet clear what role cyclin plays in this cascade. In order to study the control of mitosis, a cell-free system is needed that mimics as closely as possible the activation and inactivation kinetics of the $cdc2^+$ kinase observed *in vivo*.

In this paper, we report the characterization of cell-free extracts from *Xenopus* eggs, in which the temporal regulation of the mitotic histone H1 kinase activity can be studied under physiological conditions. We show that histone H1 kinase activity oscillates at least twice in low speed extracts of activated eggs incubated at 22°C. The oscillation can be divided into two phases. The first requires protein synthesis, while in the second one, the kinase activates spontaneously. This is similar to what is observed *in vivo*. Kinase activity is lost abruptly, coincident with destruction of added sea urchin cyclin at the end of metaphase.

When these extracts are fractionated by successively higher speed centrifugation, it emerges that two regulatory components involved in the activation of the histone

kinase are associated with particulate material. One component sediments above 100 000 ***g*** and seems to inhibit kinase activation; it appears to be inactivated by phosphorylation. Another component sediments only at very high speed (more than 2 h at 250 000 ***g***). It is part of the activation system of the kinase. Our data suggest, however, that the final activation of the kinase involves protein dephosphorylation.

These results show that the normal physiological mechanism of activation of the mitotic kinase is very complex. It involves several steps, not all of which are controlled by soluble molecules. Further studies of this system should allow us to identify the various elements involved in the control of the mitotic protein kinase.

Materials and methods

Preparation of eggs

Female *Xenopus laevis* were purchased from the Service d'élevage d'Amphibiens CNRS (France). Eggs were obtained from females injected 3 to 8 days before use with 100 units of pregnant mare serum gonadotropin (PMSG) (Intervet, France) and the day before with 1500 units of human chorionic gonadotropin (HCG) (Sigma). The eggs were collected in 0.1 M-NaCl to prevent activation. The jelly coat was removed with 2% cysteine–HCl, pH 7.8 and the eggs were then washed extensively with modified Ringer's solution MMR/4 (25 mM-NaCl, 0.4 mM-KCl, 0.25 mM-$MgSO_4$, 0.5 mM-$CaCl_2$, 1.25 mM-Hepes, 25 μM-EDTA, pH 7.2).

Preparation of extracts

Eggs were activated by an electric shock (Karsenti *et al.* 1984), incubated for 55–60 min at 22 °C and then transferred to cold acetate buffer (100 mM-K-acetate, 2.5 mM-Mg-acetate, 60 mM-EGTA, 5 μg ml^{-1} cytochalasin D (Sigma), 1 mM-DTT, 250 mM-sucrose, pH 7.2). Excess acetate buffer was removed prior to centrifugation. The 2000 ***g*** supernatants were obtained by crushing the eggs by gentle pipetting with a 1 ml Gilson pipette and centrifuging the homogenate at 2000 ***g*** for 10 min at 4 °C. This removed the yolk, but left most of the cytoplasmic components in the supernatant. The 10 000 ***g*** and high-speed supernatants were obtained from eggs crushed by centrifugation at 10 000 ***g*** for 10 min at 4 °C in an L5-65 Beckman centrifuge with maximum acceleration rate. In both cases, the cytoplasmic material between the upper lipid layer and the yolk pellet was collected and an ATP regenerating system was added. We used a final concentration of 10 mM creatine phosphate (Boehringer, from a 0.5 M stock in H_2O), 80 μg ml^{-1} creatine kinase (Boehringer, 4 mg ml^{-1} stock in 50 % v/v glycerol/H_2O), 1 mM-ATP (disodium salt, Boehringer, 50 mM stock in H_2O, pH 7). The 2000 and 10 000 ***g*** supernatants were kept on ice and used the same day. High-speed extracts were obtained by further centrifugation at 100 000 ***g*** for 1 h, or 250 000 ***g*** for 2 h at 4 °C in the SW50.1 or SW60 rotors with adaptors for 0.6 ml tubes. These supernatants were frozen in 50 μl aliquots in liquid nitrogen. The protein concentration (determined by the Bradford procedure using bovine serum albumin as a standard) was 35–45 mg ml^{-1} in the 2000, 10 000 ***g*** and 100 000 ***g*** supernatants and approximately 20 mg ml^{-1} in the 250 000 ***g*** supernatants.

Histone kinase assay

To assay histone H1 kinase, 2 μl of extract to be assayed was transferred into 50 μl of extraction buffer (EB; 80 mM-β-glycerophosphate, 20 mM-EGTA, 15 mM-$MgCl_2$, 1 mM-DTT, 1 mM-PMSF, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin, 10 μg ml^{-1} aprotinin, pH 7.3) to stabilize kinase activity. The samples were frozen in liquid nitrogen and stored at −20 °C. The histone kinase activity was assayed by adding 10 μl of this sample to 6 μl of a mix containing 3.3 mg ml^{-1} histone H1 (H III-S from calf thymus, Sigma), 1 mM-ATP, 0.25 μCi μl^{-1} [γ-^{32}P]ATP (Amersham PB 218). The reaction was performed for 15 min at 20 °C and stopped by pipetting 12 μl of the reaction mixture on P81 phosphocellulose paper (Whatman) precut in 1.5 cm^2 squares. The filters were washed three times for 15 min in 150 mM-H_3PO_4 (Merck) (at least 20 ml/filter), rinsed in ethanol, allowed to dry and Cerenkov-counted. The background obtained in the absence of extract was

subtracted. In some cases, histone phosphorylation was analysed by SDS–PAGE. The reaction was then stopped by addition of an equal volume of SDS sample buffer and the samples were run on a 15% minigel according to Laemmli (1970). The gels were fixed in three changes of 12.5% isopropanol, 10% acetic acid, dried and exposed using Kodak X-AR or X-AS paper.

Assay for cyclin degradation in the extracts

Arbacia punctulata cyclin mRNA was transcribed with T7 RNA polymerase from a full length clone (cyc4) in pGEM1 as described by Pines and Hunt (1987). The mRNA was translated in the presence of [^{35}S]methionine in a reticulocyte lysate. The total translation mix containing [^{35}S]cyclin was added to the *Xenopus* extract in a proportion of 1 volume per 5 vol. of extract. The extract was incubated at room temperature and 4 μl samples were transferred into 16 μl of SDS gel sample buffer. The samples were run on a 10% gel. The gel was fixed in 45% methanol, 7% acetic acid and processed using the 'Intensify' (DuPont) procedure.

Preparation of the particulate 'activator' material

The particulate material of an extract of *Xenopus* eggs prepared 60 min after egg activation was obtained as follows. The 10 000 ***g*** supernatant was further centrifuged at 100 000 ***g*** in a completely full 5 ml SW 50.1 tube. The supernatant, including the fluffy part of the pellet, was recovered and centrifuged at 150 000 ***g*** for 30 min at 4°C in 280 μl aliquots in a fixed angle rotor (TLA 100) in the TL 100 Beckman centrifuge. The pellets were resuspended in 3 vol. of acetate buffer containing 10 mM-EGTA and the ATP regenerating system. Aliquots of 100 μl were frozen and stored in liquid nitrogen.

Sucrose gradient centrifugation

A discontinuous sucrose gradient was prepared by layering successively 100 μl of 70%, 50%, 40%, 30%, 20% w/v sucrose in acetate buffer (W/W) in 0.6 ml SW 50.1 tubes. One hundred μl of the TL 100 pellet was layered on top of the gradient and centrifuged at 100 000 ***g*** for 1 h. Fractions of 50 μl were collected from the top of the gradient. The 'kinase activator' activity in the fractions was assayed by adding one volume of the fractions (13 μl) to one volume of a TL 100, 60 min supernatant in which the kinase could not get activated. Histone H1 kinase activation–inactivation was followed as usual in the reconstituted system.

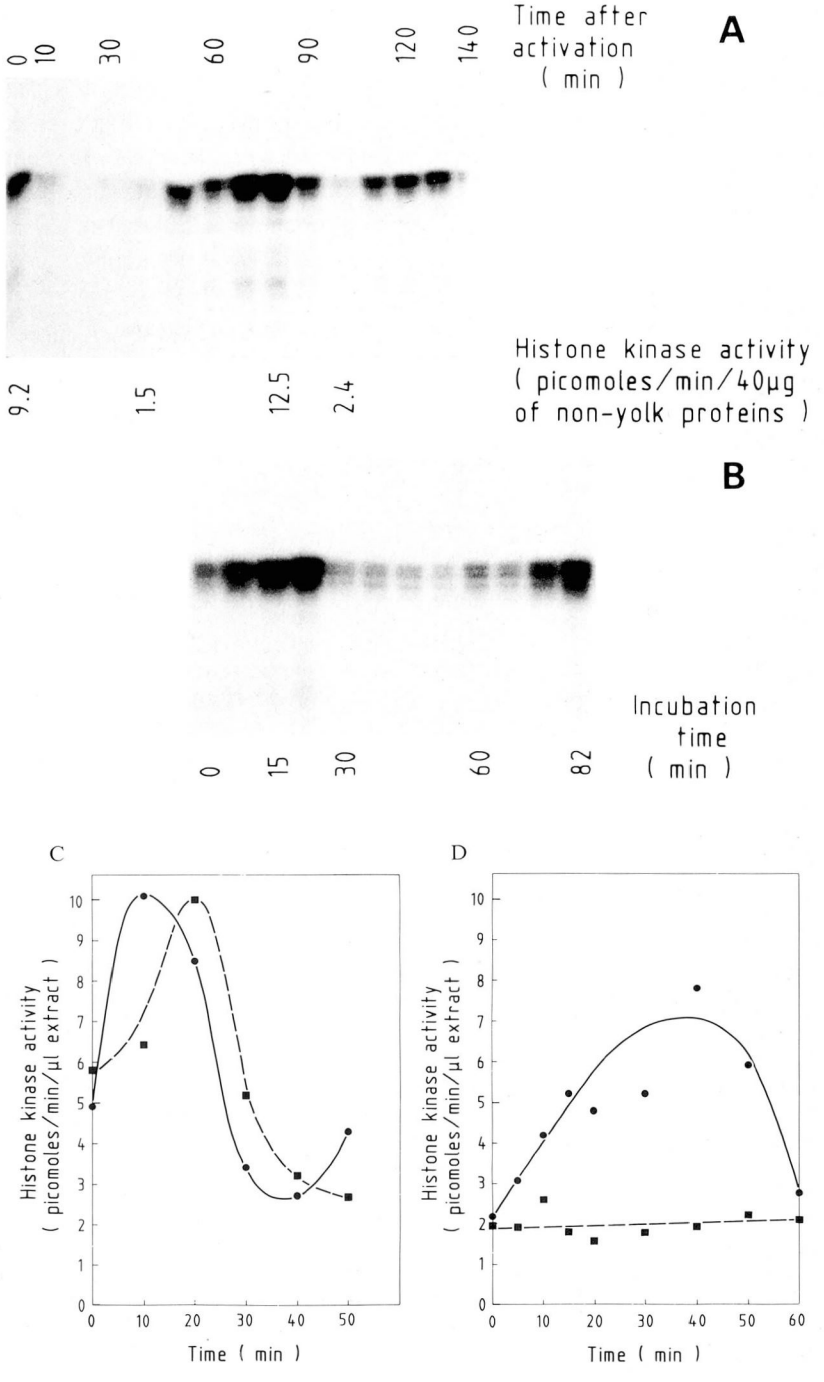
Results

Histone kinase activity oscillates in extracts of activated eggs

Xenopus eggs are stably arrested in second metaphase of meiosis and contain high histone H1 kinase activity, as shown in the first lane of Fig. 1A. Fertilization or artificial activation releases the metaphase block, leading to a drop in histone H1

Fig. 1. Histone H1 kinase activity oscillates in activated *Xenopus* eggs and in low speed supernatants of activated eggs. Panel A: a batch of eggs was activated, and at the indicated times two eggs were crushed directly in 50 μl of extraction buffer. The yolk was removed by centrifugation, and the supernatant assayed for histone H1 kinase activity and analyzed on 15% SDS–PAGE. The phosphorylated histone bands were revealed by autoradiography. The quantitative data indicated below the autoradiogram were obtained by adsorption of the histones on phosphocellulose filters. Panel B: eggs were activated and homogenized 60 min later. The 2000 ***g*** supernatant was incubated at 22°C. Aliquots sampled at the indicated times were diluted in EB every 7.5 min and used to phosphorylate histones as described previously. Panel C: cycloheximide (100 μg ml^{-1}) was added to a 2000 ***g*** supernatant of eggs homogenized 60 min after activation and, panel D, to a 2000 ***g*** supernatant of eggs homogenized 80 min after activation. In panels C and D, histone kinase activity was measured by adsorption of the histones on phosphocellulose filters. (■---■, with cycloheximide; ●——●, control.)

kinase activity. This is followed by the reappearance of H1 kinase activity, which peaks at about 80 min after fertilization. This corresponds to the time of first metaphase in the intact eggs. Next, the kinase activity drops rapidly, followed by a



second round of increase and disappearance peaking at 120 min. Thereafter the kinase activity in intact embryos oscillates with a period of about 30 min.

Fig. 1B shows the results of histone H1 kinase assays performed on low-speed extracts prepared from eggs sampled 60 min after activation ('60 minute' extract). Initially these extracts contained a low level of histone kinase activity that was similar to the *in vivo* value (compare the first lane of Fig. 1B with the 60 min time point of Fig. 1A). Upon incubation of the '60 minute' extract at room temperature, the histone kinase activity increased, reached a peak at about 20 min and then suddenly dropped to a very low value at 30 min. This occurred very close to the time of kinase inactivation in intact eggs (see Fig. 1A, 100 min time point). Later, the histone H1 kinase activity increased again in the extract, but much more slowly than in the intact eggs. The first rise and fall of kinase activity observed in the extracts did not require protein synthesis, since it was not inhibited by cycloheximide (Fig. 1C). The second *in vitro* cycle absolutely required protein synthesis since activation of the kinase did not occur in the presence of cycloheximide (Fig. 1D). Thus the slow appearance of kinase activity during the second *in vitro* cycle was probably due to a low rate of protein synthesis. It was indeed only 20 % of the rate measured in intact embryos (data not shown).

Histone kinase activity oscillates in concert with endogenous protein phosphorylation and exogenous cyclin degradation

As shown in the top panel of Fig. 2, extracts prepared 60 min after egg activation could be centrifuged at 250 000 ***g*** and still retain their ability to activate and turn off histone H1 kinase. The peak of activity in these supernatants varied from extract to extract, often being lower than in the corresponding 10 000 ***g*** supernatant. The appearance of kinase activity was accompanied by a large increase in ^{32}P labelling of endogenous proteins in the extract when assayed with added [γ-^{32}P]ATP (Fig. 2, middle panel). These proteins corresponded closely to the proteins we previously found to be labelled *in vivo* during metaphase (Karsenti *et al.* 1987; Felix *et al.* 1989).

To test if the disappearance of the kinase activity in the extract was accompanied by destruction of cyclin, we added labelled sea urchin cyclin (produced by translation of synthetic cyclin mRNA in a reticulocyte lysate with [^{35}S]methionine). The added cyclin was indeed degraded between 20 and 30 min, at the very moment of kinase inactivation (Fig. 2, bottom panel). These experiments show that basic biochemical events of the cell cycle previously characterized *in vivo* can be reproduced in high-speed frozen supernatants of activated *Xenopus* egg extracts. This should permit study of the activation and inactivation mechanism of the mitotic histone H1 kinase *in vitro* under almost physiological conditions. In addition, the mechanism of cyclin degradation seems equally amenable to biochemical investigation. We have begun to use this system for both purposes, and the initial results are submitted for publication elsewhere (Felix *et al.* 1989). We describe below some recent results obtained on the study of the activation mechanism of the mitotic kinase.

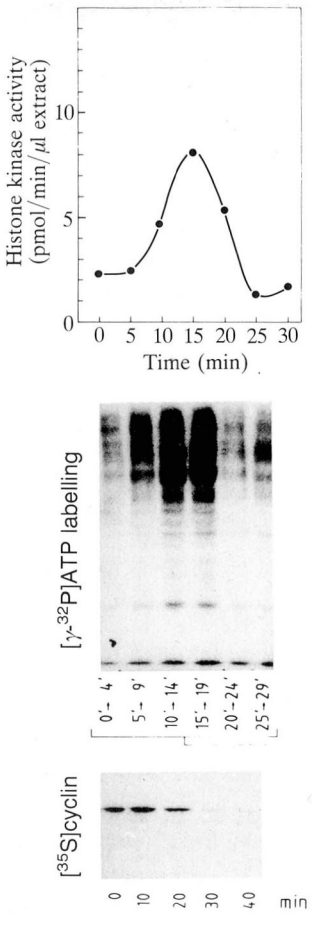


Fig. 2. Histone H1 kinase activity and endogenous protein phosphorylation oscillate and cyclin is degraded when kinase activity falls. A 10 000 ***g*** supernatant of eggs sampled 60 min after activation was further centrifuged at 250 000 ***g*** for 2 h at 4 °C. Upon incubation at room temperature, the histone H1 kinase oscillates in this supernatant (upper panel), endogenous protein phosphorylations increase and drop in synchrony (middle panel), and added exogenous cyclin is degraded at the very moment of histone H1 kinase inactivation. Exogenous cyclin added after the drop of kinase activity was stable for at least one hour in this extract (not shown). Endogenous protein phosphorylation was measured by incubating aliquots of the extracts for 4 min with 20 μCi of [γ-^{32}P]ATP at the indicated times. The samples were analysed by SDS–PAGE on a 10 % SDS gel, followed by autoradiography. *Arbacia punctulata* cyclin was synthesized in the presence of [^{35}S]methionine in a reticulocyte lysate and added to the extract at the beginning of the incubation at room temperature. The 10 % SDS gel was analyzed by autoradiography.

Light particulate material is required for histone H1 kinase activation

As reported in the preceding section, it is possible to obtain one cycle of kinase activation and inactivation in 250 000 ***g*** supernatants of extracts prepared 60 min after egg activation. These centrifugation conditions would usually be considered

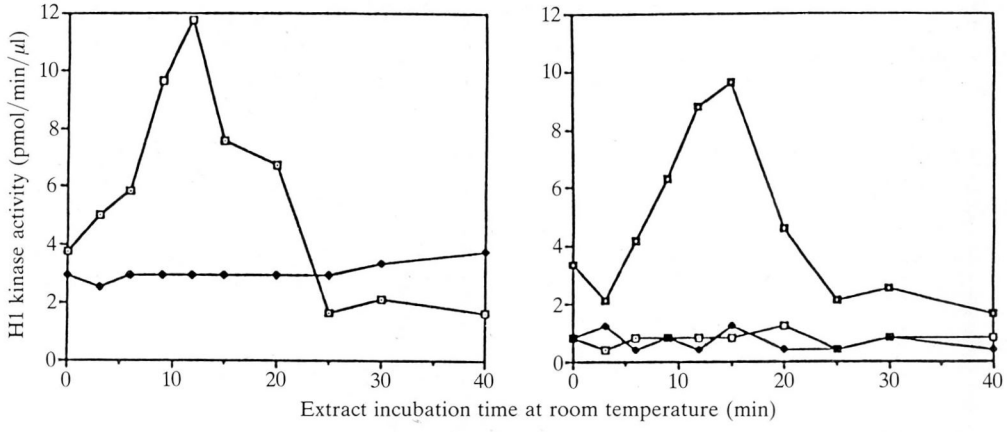


Fig. 3. Particulate material is required for histone H1 kinase activation in extracts prepared 60 min after egg activation. A 10 000 ***g*** supernatant of eggs homogenized 60 min after activation was submitted to a further centrifugation at 100 000 ***g***. In this supernatant the histone kinase showed a normal oscillation (left panel, ⊡——⊡). After a further centrifugation for 30 min at 150 000 ***g*** in the TL 100 ultracentrifuge, no oscillation occurred in the supernatant (left panel, ◆——◆). No oscillation occurred in the resuspended pellet diluted 1:1 in buffer (right panel, ◆——◆), nor in the supernatant diluted 1:1 in buffer (right panel, ⊡——⊡), but a normal oscillation was restored when the resuspended pellet was recombined with the supernatant in a 1:1 (v/v) proportion (right panel, □——□).

sufficient to completely eliminate all particulate material. However, the extracts used in this study were highly concentrated, and we were concerned that some low-density particulate material might not be sedimented. We therefore did several experiments to investigate this possibility. The left panel of Fig. 3 shows what happened when 200 μl of a 100 000 ***g*** supernatant was recentrifuged for 30 min at 150 000 ***g*** in the table top BeckmanTL 100 centrifuge. The kinase did not activate in the supernatant of the extract centrifuged in this way, probably because the small distance between the top and bottom of the tubes in this centrifuge allows slowly sedimenting material to be pelleted. The right panel of Fig. 3 shows that this was not a centrifugation artefact, since addition of the resuspended pellet (which itself had no histone kinase activity) to the supernatant (also devoid of kinase activity) restored a normal round of histone H1 kinase activation and inactivation. It thus appeared that particulate material that is difficult to pellet by normal centrifugation of concentrated extracts was involved in the activation process of the mitotic histone H1 kinase.

In the preceding experiment, the pellet and the inactive supernatant were both obtained from eggs homogenized 60 min after activation, a time when further protein synthesis is not needed to support activation of the mitotic histone H1 kinase. It was of interest to test whether the particulate material had to be prepared at a specific time of the cell cycle in order to activate the kinase. We therefore prepared a 100 000 ***g*** supernatant from eggs sampled only 20 min after activation. At this time in the cell cycle, the component that has to be synthesized in order to trigger kinase activation (which we strongly suspect is cyclin, according to the recent results of Minshull *et al.*

1989, and Murray and Kirschner, 1989) should be either missing or in low concentration. Indeed, incubation of such an extract at room temperature for 1 h did not result in any activation of the histone kinase (data not shown). This extract was fractionated as described above into a TL100 pellet and supernatant. Homologous reconstitution between the 20 min pellet and supernatant did not result in kinase activation (Fig. 4D). In Fig. 4A, we show an homologous reconstitution between a 60 min TL 100 supernatant and its pellet. As expected, the slight activation of the histone kinase that occurred in the supernatant (open symbols) was strongly increased by addition of the pellet. Addition of the '20 minute' pellet to the '60

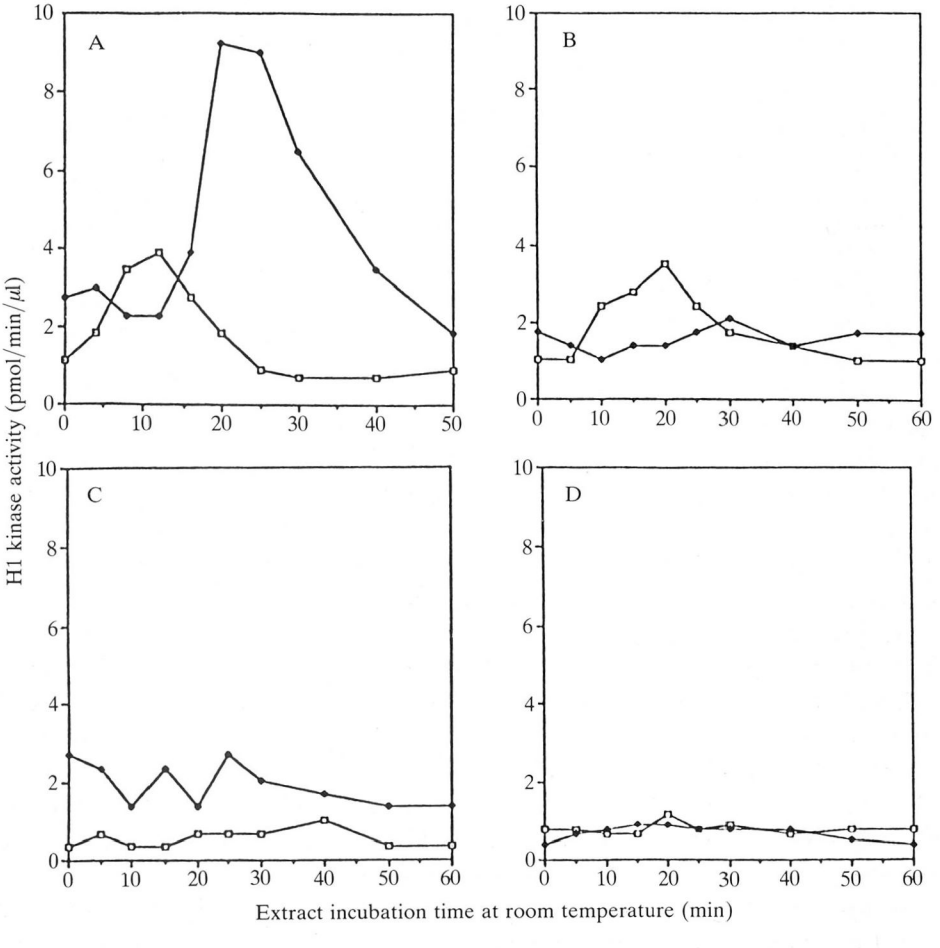


Fig. 4. Only homologous complementation between particulate material and supernatant of eggs sampled 60 min after activation results in cycling of the histone H1 kinase activity. Panel A: TL 100 supernatant and pellet of eggs sampled 60 min after egg activation. Panel B: TL 100 supernatant of eggs sampled 60 min after activation and TL 100 pellet of eggs sampled 20 min after activation. Panel C: TL 100 supernatant of eggs sampled 20 min after activation and pellet of eggs sampled 60 min after activation. Panel D: TL 100 supernatant and pellet of eggs sampled 20 min after egg activation. (□——□, supernatant+buffer; ◆——◆, supernatant+resuspended pellet.

minute' supernatant did not activate the kinase. Instead, it inhibited the residual activation that occurred in the supernatant alone (Fig. 4B). Adding the '60 minute' pellet to the '20 minute' supernatant did not result in kinase activation either (Fig. 4C).

Therefore, the slowly sedimenting particulate material involved in the mechanism of kinase activation is active only when prepared from eggs that have begun to enter mitosis. Moreover, active particulate material is not sufficient to activate the kinase in a supernatant prepared from eggs sampled during interphase.

The results of a preliminary attempt to fractionate the particulate material involved in activation of the kinase on a discontinuous sucrose gradient are shown in Fig. 5. A TL 100 pellet was centrifuged for 1 h at 100 000 ***g*** on a 20–70 % discontinuous sucrose gradient, and the fractions added to different aliquots of a TL 100 supernatant. The kinetics of kinase activation was followed for each reconstitution. In this experiment, the kinase showed a low amplitude cycle in the TL 100 supernatant alone (the level of activation of the kinase in the TL 100 supernatants varied somewhat from extract to extract). We attribute the residual cycle of kinase activity observed in some supernatants to incomplete removal of the particulate material. In any case, Fig. 5 clearly shows that a normal level of kinase activation could be restored in the TL 100 supernatants by adding back fractions 3 to 5 of the sucrose gradient (the 20–30 % sucrose interface). No other fractions had detectable activity. This confirms that the kinase activator is associated with a slowly sedimenting particle (as a reference, centrosomes would sediment in 50–60 % sucrose under the same conditions), and is in agreement with the sedimentation properties of this activity in the concentrated extracts. We have not yet determined precisely the sedimentation coefficient or the density of the activator.

Effect of kinase and phosphatase inhibitors on histone H1 kinase activation in egg extracts

As already mentioned in the introduction, activation of the mitotic kinase requires the presence of cyclin (Minshull *et al.* 1989; Murray *et al.* 1989). It also involves a cascade of phosphorylation and dephosphorylation events, as suggested by genetic analysis in yeast (Russell and Nurse, 1987). In fact, the genetic analysis predicts that activation of $p34^{cdc2}$ kinase occurs when another kinase (the product of the *wee1*$^+$ gene) is inhibited by phosphorylation. This suggests that final activation of $p34^{cdc2}$ may be achieved by dephosphorylation, a hypothesis that is strongly supported by biochemical results from several laboratories working on both *Xenopus* and starfish (Labbé *et al.* 1988*a*,*b*; Gautier *et al.* 1989; Dunphy and Newport, 1989; Draetta *et al.* 1989).

As a first approach to test the involvement of protein phosphorylation in kinase activation we added EDTA to the extracts to block all ATP–Mg^{2+}-dependent reactions, or used 6-dimethylaminopurine (6-DMAP). This adenine analog blocks mitosis in fertilized sea urchin eggs at a concentration of 10^{-4} M, and seems to be mainly a protein kinase inhibitor (Néant *et al.* 1988). Both these reagents strongly inhibited ^{32}P incorporation into the proteins of the treated extracts. As Fig. 6 shows,

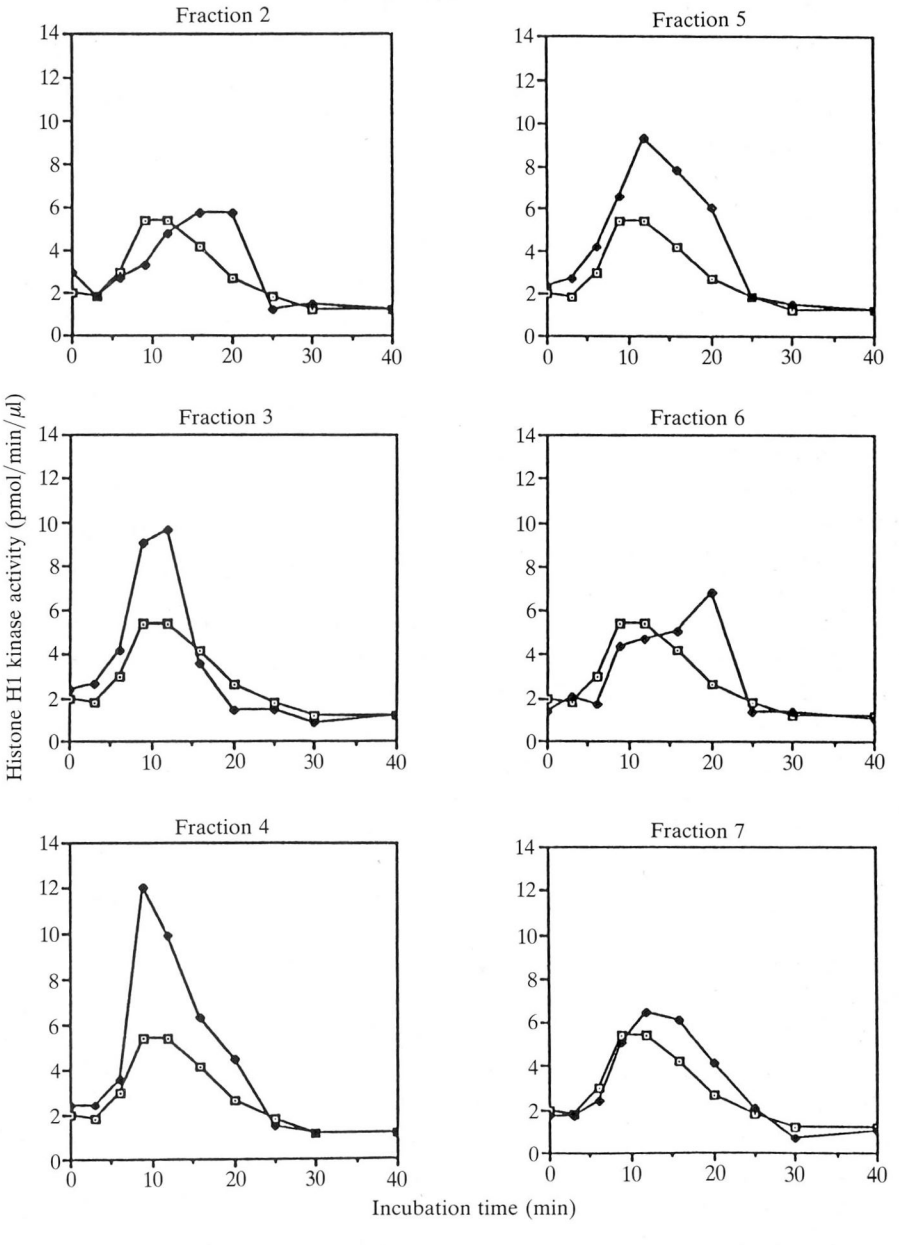


Fig. 5. Sucrose gradient fractionation of the particulate activator of the H1 kinase. 100 μl of particulate material of eggs sampled 60 min after activation was centrifuged for 1 h at 100 000 ***g*** on a discontinuous 20–70 % sucrose gradient as described in Materials and methods. The fractions were collected from top to bottom. The fractions showed correspond to the 20–45 % region of the gradient. Each panel represents an assay in which the fraction was added to a TL 100 supernatant, and the mixture incubated at room temperature for 40 min. Histone H1 kinase activity was assayed at various times during the incubation. (⊡——⊡, TL 100 supernatant+buffer. ◆——◆, TL 100 supernatant+ gradient fraction.)

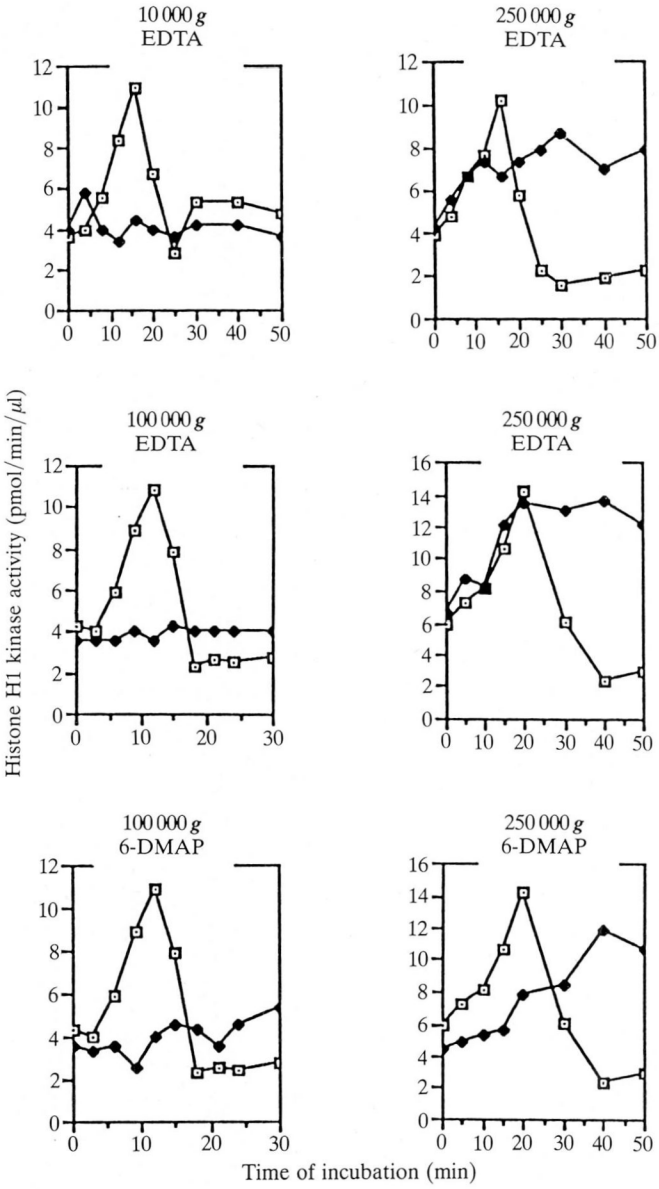


Fig. 6. Differential effect of both EDTA (5 mM) and 6-DMAP (0·5 mM) on H1 kinase oscillation in 100 000 ***g*** and 250 000 ***g*** supernatants of '60 minute' extracts. Histone H1 kinase activity was followed in supernatants obtained by centrifugation at various speeds and incubated at room temperature in the presence (◆——◆) and absence (□——□) of the drugs indicated in each panel. The 250 000 ***g*** supernatant used for the experiment shown in top right panel was derived, without intermediate freezing, from the 10 000 ***g*** supernatant used in the top left panel.

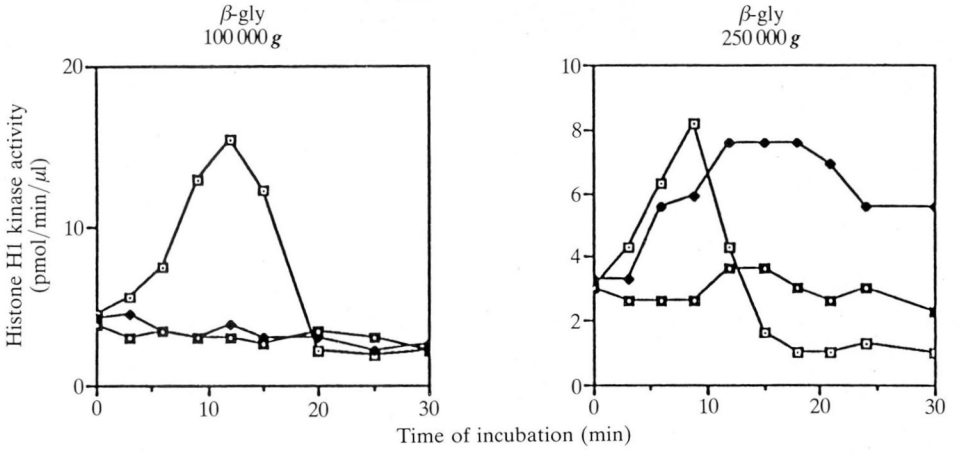


Fig. 7. Differential effect of β-glycerophosphate on H1 kinase oscillation in 100 000 ***g*** and 250 000 ***g*** supernatants of '60 minute' extracts. Histone H1 kinase activity was followed in supernatants obtained by centrifugation at indicated speeds and incubated at room temperature in the presence of 20 mM (◆——◆) or 50 mM β-glycerophosphate (■——■) and in the absence of the drug (□——□).

EDTA and 6-DMAP completely inhibited activation of the histone kinase in 10 000 ***g*** and 100 000 ***g*** supernatants. The activity of the histone kinase could be measured in the extracts containing phosphorylation inhibitors because the EDTA or 6-DMAP were diluted below their active concentration when the extract was diluted in the histone H1 kinase assay buffer. Neither EDTA and 6-DMAP had any effect on the measured kinase activity when added to extracts in which the histone kinase was already activated.

To our surprise, the 250 000 ***g*** supernatants gave a completely different result with these inhibitors. Neither EDTA nor 6-DMAP prevented the increase in histone kinase activity, which reached a level equivalent to *in vivo* values. However, once activated, the activity never dropped. This is described in more detail elsewhere (Felix *et al.* 1989).

These results draw attention to two important points. First, a particulate material present in 100 000 ***g*** supernatants is not needed for activation of the histone H1 kinase when protein phosphorylation is inhibited. Second, the particulate material can be removed by centrifugation at 250 000 ***g*** for 2 h. In the absence of the particulate material, the kinase activates normally when all phosphorylations are inhibited in the extract. This is consistent with the idea that the final activation step of the histone H1 kinase represents dephosphorylation and further suggests the existence of a particulate inhibitor of the kinase, which is inactivated by phosphorylation (either directly or indirectly).

In order to test whether the final kinase activation step was due to protein dephosphorylation, we added different concentrations of β-glycerophosphate to 100 000 ***g*** and 250 000 ***g*** supernatants prepared 60 min after egg activation. As shown in Fig. 7, left panel, activation of the histone H1 kinase in 100 000 ***g*** supernatants was

completely inhibited by 20 mM-β-glycerophosphate. This is the concentration routinely used to stabilize MPF and histone H1 kinase activity in extracts of non-activated eggs. This same concentration of phosphatase inhibitor had no effect on the activation of the kinase in 250 000 ***g*** supernatants, but prevented the normal disappearance of kinase activity (Fig. 7, right panel). Higher β-glycerophosphate concentrations inhibited activation of the histone kinase in the 250 000 ***g*** supernatants. The effect of β-glycerophosphate was not due to chelation of Mg^{2+} ions, as was shown by adding a molar equivalent of Mg^{2+} acetate to the extract together with β-glycerophosphate (data not shown). Other phosphatase inhibitors (NaF, α-naphthyl phosphate) gave similar results (data not shown).

These experiments suggest that both activation and inactivation of the histone H1 kinase involve dephosphorylation events, but in 250 000 ***g*** supernatants, the relative absence of particulate material means that the kinase can be activated even when phosphorylation is blocked and phosphatase inhibitors are present. Higher concentrations of phosphatase inhibitors presumably act by directly inhibiting the dephosphorylation of $p34^{cdc2}$ that is thought to be the final step of kinase activation. These confusing results probably owe their explanation to the existence of a complex chain of protein modifications.

Discussion

In this paper, we describe the preparation of extracts from activated *Xenopus* eggs that are capable of more than one cycle of histone H1 kinase activation and inactivation. (Cicarelli *et al.* 1988; Dabauvalle *et al.* 1988; Labbé *et al.* 1988*a*). We have not formally identified this activity with that of the frog homologue of the $cdc2^+$ gene product of *S. pombe*. The $cdc2^+$ gene product has strong histone H1 kinase activity from yeast to humans, and this activity is activated only during mitosis (for review, see Lokha, 1989). The histone H1 kinase activity is abolished in crude extracts of a temperature-sensitive mutant of $cdc2^+$ in yeast (Moreno *et al.* 1989). The mitotic histone kinase activity of *Xenopus* eggs has been partially purified (Labbé *et al.* 1988*a*) and fractionates as a single peak through four fractionation steps, and the activity coincides with $p34^{cdc2}$. Finally, most of the frog egg histone H1 kinase activity eluted as a single peak from DEAE cellulose and binds to Sepharose beads carrying the yeast $p13^{suc1}$ protein (Félix *et al.* 1989). This protein has been shown to interact directly with $p34^{cdc2}$ (Dunphy *et al.* 1988; Arion *et al.* 1988). Therefore, it is extremely probable that the histone kinase activity we observed in this work is due to $p34^{cdc2}$.

There have been several previous reports of *Xenopus* egg extracts in which the cell cycle could be reconstituted (Cyert and Kirschner, 1988; Hutchison *et al.* 1987, 1988). In these studies, either the activity of MPF, morphological markers or the occurrence of DNA synthesis were used to follow the cell cycle. Our goal was to define a cell-free system that reconstituted the cell cycle *in vitro* with a minimum of alteration to the *in vivo* situation, in order to have a secure starting point to further simplify the system and study the physiological cascade of reactions involved in the

control of mitosis. We therefore needed to define precise molecular markers that could be used to compare the *in vivo* and *in vitro* conditions. We believe that protein phosphorylation provides such a marker, since the pattern of ^{32}P-labelled proteins observed in our extracts is virtually identical to that seen in intact fertilized eggs (Felix *et al.* 1989). Moreover, the mitotic kinase is activated and inactivated with essentially the same kinetics in our extracts as in the first cell cycle of fertilized eggs, and the regulation of cyclin degradation is correctly preserved. Moreover, it is possible to freeze high-speed extracts which preserve the activation–inactivation mechanism of the histone kinase after thawing.

We find that a particulate subcellular fraction, which is even present at a significant level in the 250 000 ***g*** supernatants, is involved in the mechanism of kinase activation. This material can be eliminated by longer centrifugation, or by dilution and recentrifugation of the extract. We find that the p34^{cdc2} kinase itself is not sedimented under any condition (Felix *et al.* 1989). Therefore, some component necessary for its activation must be particulate. Moreover, the cycle of kinase activation and inactivation can be reconstituted by recombining the pellet and the inactive supernatant. We call the particulate material the 'Light Particulate Activator' (LPA). 'Light Particulate', because it sediments only at very high speed in the crude extracts and does not enter very far into a sucrose gradient, and 'Activator' because LPA seems to be required to activate the kinase in a high-speed supernatant prepared from pre-mitotic eggs. LPA prepared from eggs homogenized just before metaphase cannot activate the histone H1 kinase in interphase supernatants. This indicates that other components – perhaps cyclins – must be present in the cytoplasm to support its activity. It is perhaps more surprising that LPA prepared from interphase eggs cannot activate the kinase in pre-mitotic supernatants. This is more difficult to understand, and seems to indicate that LPA is in a different state in interphase and in pre-mitotic eggs.

We also found that histone H1 kinase activated in 250 000 ***g*** supernatants, which contain at least some LPA, when protein phosphorylation was completely inhibited by EDTA or 6-DMAP. This strongly suggests that phosphorylation of the histone H1 kinase is not necessary for its activation. It should be recalled that phosphatase inhibitors block activation of the kinase in these extracts, suggesting that activation of the kinase is brought about by dephosphorylation of some component of the system. Accordingly, LPA could provide either this hypothetical phosphatase or an activator of the phosphatase. Much more biochemical analysis will be required, however, before all this confusing phenomenology can be clearly and rationally explained. We think that the system described here will be useful for such studies.

References

AJIRO, K., BORUN, T. W. AND COHEN, L. H. (1981). Phosphorylation states of different histone 1 subtypes and their relationship to chromatin function during the Hela S-3 cell cycle. *Biochemistry* **20**, 1445–1454.

ARION, D., MEIJER, L., BRIZUELA, L. AND BEACH, D. (1988). *cdc2* is a component of the M-phase specific histone H1 kinase: evidence for its identity with MPF. *Cell* **55**, 371–378.

BRADBURY, E. M., INGLIS, R. J., MATTHEWS, H. R. AND SARNER, N. (1973). Phosphorylation of very lysin rich histone in *Physarum polycephalum*. *Eur. J. Biochem.* **33**, 131–139.

CAPONY, T. P., PICARD, A., PEAUCELLIER, G., LABBÉ, J. C. AND DORÉE, M. (1986). Changes in the activity of the maturation-promoting factor during meiotic maturation and following activation of amphibian and starfish oocytes: their correlations with protein phosphorylation. *Devl Biol.* **117**, 1–12.

CICIRELLI, M. F., PELECH, S. L. AND KREBS, E. G. (1988). Activation of multiple protein kinases during thew burst in protein phosphorylation that precedes the first meiotic cell division in *Xenopus* oocytes. *J. biol. Chem.* **263**, 2009–2019.

CYERT, M. S. AND KIRSCHNER, M. (1988). Regulation of MPF activity *in vitro*. *Cell* **53**, 185–195.

DABAUVALLE, M. C., DORÉE, M., BRAVO, R. AND KARSENTI, E. (1988). Role of nuclear proteins in the early cell cycle of *Xenopus* embryos. *Cell* **52**, 525–533.

DORÉE, M., PEAUCELLIER, G. AND PICARD, A. (1983). Activity of the maturation-promoting factor and the extent of protein phosphorylation oscillate simultaneously during maturation of starfish oocytes. *Devl Biol.* **99**, 489–501.

DRAETTA, G. AND BEACH, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells: cell cycle dependent phosphorylation and subunit rearrangement. *Cell* **54**, 17–26.

DRAETTA, G., LUCA, F., WESENDORF, J., BRIZUELA, L., RUDERMAN, J. AND BEACH, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell* **56**, 829–838.

DUNPHY, W. AND NEWPORT, J. (1989). Fission yeast p13 blocks mitotic activation of tyrosine dephosphorylation of the *Xenopus* Cdc2 protein kinase. *Cell* **58**, 181–191.

DUNPHY, W., BRIZUELA, L., BEACH, D. AND NEWPORT, J. (1988). The *Xenopus* Cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54**, 423–431.

EVANS, T., ROSENTHAL, E., YOUNGBLOM, J., DISTEL, D. AND HUNT, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389–396.

FELIX, M. A., PINES, J., HUNT, T. AND KARSENTI, E. (1989). A post-ribosomal supernatant from activated *Xenopus* eggs that displays post-translationally regulated oscillation of its cdc2$^+$ mitotic kinase activity. *EMBO J.* **8**, in press.

GAUTIER, J., NORBURY, C., LOHKA, M., NURSE, P. AND MALLER, J. (1988). Purified maturation promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*. *Cell* **54**, 433–439.

GAUTIER, J., NORBURY, C., LOHKA, M., NURSE, P. AND MALLER, J. (1988). Purified maturation promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*$^+$. *Cell* **54**, 433–439.

GUERRIER, P., MOREAU, M. AND DORÉE, M. (1977). Hormonal control of meiosis in starfish: Stimulation of protein phosphorylation induced by 1-methyladenine. *Molec. cell. Endocr.* **7**, 137–150.

HUTCHISON, C. J., COX, R., DREPAUL, R. S., GOMPERTS, M. AND FORD, C. (1987). Periodic DNA synthesis in cell-free extracts of *Xenopus* eggs. *EMBO J.* **6**, 2003–2010.

HUTCHISON, C. J., COX, R. AND FORD, C. C. (1988). The control of DNA replication in a cell-free extract that recapitulates a basic cell cycle *in vitro*. *Development* **103**, 553–566.

KARSENTI, E., NEWPORT, J., HUBBLE, R. AND KIRSCHNER, M. (1984). The interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into *Xenopus* eggs. *J. Cell Biol.* **98**, 1730–1745.

KARSENTI, E., BRAVO, R. AND KIRSCHNER, M. (1987). Phosphorylation changes associated with the early cell cycle in *Xenopus* eggs. *Devl Biol.* **119**, 442–453.

KIRSCHNER, M., NEWPORT, J. AND GERHART, J. (1985). The timing of early developmental events in *Xenopus*. *Trends Genet.* **1**, 41–47.

LABBÉ, J.-C., LEE, M. G., NURSE, P., PICARD, A. AND DORÉE, M. (1988*b*). Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene *cdc2*$^+$. *Nature, Lond.* **335**, 251–254.

LABBÉ, J.-C., PICARD, A., KARSENTI, E. AND DORÉE, M. (1988*a*). An M-phase-specific protein kinase of *Xenopus* oocytes: partial purification and possible mechanism of its periodic activation. *Devl Biol.* **127**, 157–169.

LEE, M. G. AND NURSE, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature, Lond.* **327**, 31–35.

LOHKA, M. J. (1989). Mitotic control by metaphase-promoting factor and *cdc* proteins. *J. Cell Sci.* **92**, 131–135.

LOHKA, M. J., HAYES, M. K. AND MALLER, J. L. (1988). Purification of maturation promoting factor, an intracellular regulator of early mitotic events. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3009–3013.

LOHKA, M. J. AND MALLER, J. L. (1985). Induction of nuclear envelope breakdown, chromosome condensation and spindle formation in cell free extracts. *J. Cell Biol.* **101**, 518–523.

LOHKA, M. J. AND MASUI, Y. (1984). Effects of Ca^{2+} ions on the formation of metaphase chromosomes and sperm pronuclei in cell free preparations from unactivated *Rana pipiens* eggs. *Devl Biol.* **103**, 434–442.

MALLER, J. L. (1985). Regulation of amphibian oocyte maturation. *Cell Differ.* **16**, 211–221.

MALLER, J., WU, M. AND GERHART, J. C. (1977). Changes in protein phosphorylation accompanying maturation of *X. laevis* oocytes. *Devl Biol.* **58**, 295–312.

MEIJER, L., PAUL, M. AND EPEL, D. (1982). Stimulation of protein phosphorylation during fertilization-induced maturation of *Urechis caupo* oocytes. *Devl Biol.* **94**, 62–70.

MIAKE-LYE, R. AND KIRSCHNER, M. (1985). Induction of early mitotic events in a cell free system. *Cell* **41**, 165–175.

MIAKE-LYE, R., NEWPORT, J. W. AND KIRSCHNER, M. W. (1983). Maturation promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of *Xenopus laevis*. *J. Cell Biol.* **97**, 81–91.

MINSHULL, J., BLOW, J. J. AND HUNT, T. (1989). Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis *Cell* **56**, 949–956.

MORENO, S., HAYLES, J. AND NURSE, P. (1989). Regulation of $p34^{cdc2}$ protein kinase during mitosis. *Cell* **58**, 361–372.

MURRAY, A. AND KIRSCHNER, M. (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature, Lond.* **339**, 275–280.

MURRAY, A., SOLOMON, M. J. AND KIRSCHNER, M. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature, Lond.* **339**, 280–285.

NÉANT, I. AND GUERRIER, P. (1988). 6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase. *Expl Cell Res.* **176**, 68–79.

NEWPORT, J. AND KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos: I. Characterisation and timing of cellular changes at the midblastula stage. *Cell* **30**, 675–686.

PEAUCELLIER, G., DORÉE, M. AND PICARD, A. (1984). The rise and fall of protein phosphorylation during meiotic maturation of oocytes in *Sabellaria alveolata*. *Devl Biol.* **106**, 267–274.

PICARD, A., LABBÉ, J.-C., PEAUCELLIER, G., LEBOUFFANT, F., LEPEUCH, C. J. AND DORÉE, M. (1987). Changes in the activity of the maturation promoting factor are correlated with those of a major cAMP- and calcium-independent protein kinase during the first mitotic cell cycles in the starfish embryo. *Dev. Growth. Differ.* **29**, 93–103.

PICARD, A., PEAUCELLIER, G., LE BOUFFANT, F., LE PEUCH, L. AND DORÉE, M. (1985). Role of protein synthesis and proteases in production and inactivation of maturation promoting factor activity during meïotic maturation of starfish oocytes. *Devl Biol.* **109**, 311–320.

PINES, J. AND HUNT, T. (1987). Molecular cloning and characterization of the mRNA for cyclin from sea urchin eggs. *EMBO J.* **6**, 2987–2995.

RUSSELL, P. AND NURSE, P. (1987). The mitotic inducer $nim1^{+}$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* **49**, 469–476.

SANO, K. (1985). Calcium- and cyclic AMP-independent, labile protein kinase appearing during starfish oocyte maturation: its extraction and partial characterization. *Dev. Growth Differ.* **27**, 263–275.

SIGNORET, J. AND LEFRESNE, J. (1971). Contribution à l'étude de la segmentation de l'oeuf d'axolotl I. Definition de la transition blastuléenne. *Ann. Embryol. Morphogen.* **4**, 113–123.

SIMANIS, V. AND NURSE, P. (1986). The cell cycle control gene *cdc2* of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45**, 261–268.

STANDART, N., MINSHULL, J., PINES, J. AND HUNT, T. (1987). Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte. *Devl Biol.* **124**, 248–258.

SWENSON, K. I., FARELL, K. M. AND RUDERMAN, J. V. (1986). The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in *Xenopus* oocytes. *Cell* **47**, 861–870.

WAGENAAR, E. B. (1983). The timing of synthesis of proteins required for mitosis in the cell cycle of sea urchin embryo. *Expl Cell Res.* **144**, 393–403.