Development 102, 505–516 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

Meiosis reinitiation in the mollusc *Patella vulgata*. Regulation of MPF, CSF and chromosome condensation activity by intracellular pH, protein synthesis and phosphorylation

I. NÉANT and P. GUERRIER*

Developmental Biology, LP 4601 CNRS, Station Biologique, 29211 Roscoff, France

* Present address: Cellular and Molecular Biology, Ecole Normale Supérieure, 69007 Lyon, France

Summary

The dependency of some key events of the cell cycle upon pH, phosphorylation and protein synthesis was investigated during meiosis reinitiation of *Patella vulgata* oocytes stimulated by ammonia. In this report, we show, through heterologous microinjection experiments, that a stable maturation promoting factor (MPF) is actually produced under these conditions. This factor, which may be amplified in recipient germinal vesicle (GV)-blocked oocytes of the starfish *Asterias rubens*, but not of *Patella*, remains present in the metaphase-1-blocked oocytes for at least 2·5 h. Using effective concentrations of the protein synthesis inhibitor emetine, we further demonstrate that the appearance of this factor does not depend on newly made proteins, whereas the maintenance of metaphase-1 conditions and chromosome condensation activity (CCA), which has been related to the simultaneous presence of a cytostatic factor (CSF), requires a continuous supply of new short-lived proteins. Without this, the chromosomes decondense and the nuclear envelope reforms. Finally, we show that the entry into M-phase is accompanied by extensive protein phosphorylation, while the drug 6-dimethylaminopurine (6-DMAP), which induces protein dephosphorylation without affecting protein synthesis or phosphoprotein phosphatase activity, produces the same cytological effects as emetine. The fact that the effect of 6-DMAP, both on protein phosphorylation and chromosome structure, is perfectly reversible indicates that *Patella* CSF activity must also be controlled at the posttranslational level, possibly through the activation of relevant protein kinases.

Key words: chromosome condensation, cytostatic factor, interspecific microinjection, intracellular pH, maturation promoting factor, meiosis reinitiation, *Patella vulgata*, protein phosphorylation, protein synthesis.

Introduction

GV prophase-arrested oocytes of most animals are triggered to resume meiotic divisions in response to various stimuli (Masui & Clarke, 1979; Maller & Krebs, 1980; Guerrier *et al.* 1982; Meijer & Guerrier, 1984). In the prosobranch mollusc *Patella vulgata*, it has been shown that meiosis reinitiation proceeds in two steps and is regulated by ionic factors (Guerrier *et al.* 1986*a*). The first step, which leads to GVBD, chromosome condensation and formation of the metaphase-1 spindle, involves an increase in the intracellular pH. The second step, which releases the oocyte from the metaphase-1 block depends directly upon a rise in intracellular calcium. Although, at present, we ignore how these signals act intracellularly, more generally it has been established by cell fusion and heterologous transfers of cytoplasm that a universal nonspecies-specific factor, known as M-phase or maturation promoting factor (MPF), controls both the release of oocytes from the prophase block and the progression of somatic cells from G_2 to mitosis (Masui & Markert, 1971; Reynhout & Smith, 1974; Bałakier & Czolowska, 1977; Bałakier, 1978; Wasserman & Smith, 1978; Sunkara *et al.* 1979; Nelkin *et al.* 1980; Tarkowski & Bałakier, 1980;

Weintraub *et al.* 1982; Kishimoto *et al.* 1982, 1984; Schatt *et al.* 1983; Gerhart *et al.* 1984). It is generally agreed that this factor, which promotes GVBD, nuclear envelope disruption and chromosome condensation, may be a protein kinase or a phosphoprotein, if not a phosphoprotein phosphatase inhibitor (Pondaven & Meijer, 1986). Indeed, MPF activity appears always to be associated with a high level of endogenous protein phosphorylation, as observed during meiosis reinitiation of the amphibians (Maller *et al.* 1977), mammals (Wassarman *et al.* 1979; Crosby *et al.* 1984), starfishes (Guerrier *et al.* 1977; Dorée *et al.* 1983; Guerrier *et al.* 1988), echiuroids (Meijer *et al.* 1982), polychaetes (Peaucellier *et al.* 1981, 1982) and bivalves (Dubé *et al.* 1987; Eckberg *et al.* 1987). This correlation between phosphorylation and MPF activity has been observed also after activation of the amphibian oocyte (Capony *et al.* 1986), during early synchronous cleavage of the sea urchin (Schatt *et al.* 1983) or division of somatic mammalian cells (Westwood *et al.* 1985).

In addition to MPF, a second factor, CSF, has been described also, which arrests the cell cycle in metaphase, preserves MPF activity (Gurdon, 1968; Masui & Markert, 1971; Masui, 1974, 1985; Masui *et al.* 1980; Ryabova, 1983; Gerhart *et al.* 1984) but is sensitive to protein synthesis inhibitors (Clarke & Masui, 1983, 1985; Newport & Kirschner, 1984) or to the injection of lysolecithin-treated sperm nuclei (Shibuya & Masui, 1982).

In amphibians, the appearance of MPF prior to GVBD requires the synthesis of a protein initiator, while its autocatalytic amplification following cytoplasm microinjection into a recipient unstimulated GV-blocked oocyte does not depend on protein synthesis (Wasserman & Masui, 1975; Schuetz & Samson, 1979; Gerhart *et al.* 1984). The same situation prevails for ovine oocytes (Osborn & Moor, 1983; Moor & Crosby, 1986), whereas no new transcription or protein synthesis is required for triggering GVBD and chromosome condensation in the bivalve *Spisula* (Swenson *et al.* 1986), the mouse (Jagiello, 1969; Stern *et al.* 1972; Golbus & Stein, 1976; Bałakier & Czolowska, 1977; Crozet & Szollosi, 1980) and the starfish (Guerrier & Dorée, 1975; Dorée, 1982). This implies that oocytes of these animals contain a stockpile of MPF precursor molecules which are only activated at the posttranslational level. However, after the first meiotic cycle, as after each mitotic cycle, protein synthesis inhibitors have been shown to block subsequent cleavage (Wilt *et al.* 1967; Wagenaar & Mazia, 1978; Westwood & Wagenaar, 1983) and to preclude MPF reappearance (Gerhart *et al.* 1984; Picard *et al.* 1985). Such an absolute requirement for protein synthesis suggests a potential role for those proteins, known as cyclins, which are destroyed at the metaphase–anaphase transition and must be restored at each cell cycle (Evans *et al.* 1983; Standart *et al.* 1987). Recently, it has even been shown that a mRNA from *Spisula*, coding for cyclin A, could reinitiate meiosis of GV-arrested *Xenopus* oocytes, even when cyclin A was not present or needed to trigger GVBD in *Spisula* (Swenson *et al.* 1986). Obviously, more information is required to understand better the metabolic and ionic conditions that are likely to regulate those cytoplasmic factors responsible for driving on the cell cycle. We therefore decided to study this question further, using the oocyte of *Patella vulgata* as a model system which may contain both MPF and CSF activities.

In this paper, we show that MPF activation and amplification in *Patella* are pH-dependent but protein-synthesis-independent events that actually result in an increased phosphorylation of endogenous proteins. Instead, cytostatic factor and chromosome condensation activity seem to depend on a continuous supply of new proteins which may be required to protect MPF against dephosphorylation or to activate relevant protein kinase.

Materials and methods

Handling of oocytes

Adult limpets, collected from September to March in the vicinity of Roscoff, were maintained at 10°C under a shallow flow of running sea water. Prophase-blocked oocytes were obtained by cutting and agitating the gonad either in filtered (FSW) or in artificial sea water (ASW) prepared according to MBL formula (Shapiro, 1941; Cavanaugh, 1975) and adjusted to pH 8·2–8·5 following addition of 6 mM-Tris. They were washed repeatedly, filtered through cheese cloth and resuspended in FSW. Under these conditions, only a few batches of oocytes (2–5 %) had to be discarded for undergoing spontaneous maturation. All the experiments reported here were performed on follicle-enclosed oocyte populations which did not present a percentage of spontaneous maturation higher than 5 %. Meiosis reinitiation was triggered by adding to the oocyte suspensions 5–20 mM-NH_4Cl from a 2 M-stock solution containing 10 mM-Tris, pH 8·5. Maturation also occurred up to metaphase 1 in some batches of oocytes which were shaken for 30 s in a 10 ml glass tube covered with Parafilm (Guerrier *et al.* 1986*a*).

Intracellular microinjections of cytoplasm were carried out according to the method Hiramoto (1974), as precisely described by Meijer *et al.* (1984) and Kishimoto (1986). The tip of the micropipette was broken to a diameter wider than 5 μm in order to allow free motion of the huge cytoplasmic inclusions from *Patella*. Recipient oocytes were suspended in 80 % sea water.

For incorporation studies, the cultures were continuously stirred at 60 revs min^{-1} with a glass paddle.

Protein synthesis measurements

For continuous or preloading experiments [^{14}C]arginine (140 mCi mM^{-1}) or [^{3}H]leucine (50 Ci mM^{-1}) from CEA (France) were added to an oocyte suspension containing about 10^4 oocytes ml^{-1}. Pulse experiments were performed using 1 ml samples. Sampling was performed at the desired times by centrifuging 1 ml of the oocyte suspension through a preocooled isotonic 1·1 M-sucrose/ASW cushion (30–70 %). The eggs were then washed four times with cold ASW and the final pellet resuspended into 1 ml NaOH, 0·5 N. The same washing procedure was used for preloading experiments to remove the remaining amino acids. In this case, manipulations were done at room temperature. Total uptake was determined by counting 50 µl of the NaOH solution diluted with distilled water up to 0·5 ml in 8 ml ACS liquid scintillant (Amersham). For measuring incorporation into proteins, remaining samples were reprecipitated overnight with t.c.a., washed again twice with t.c.a. and redissolved in 0·5 ml NaOH. Protein concentration was determined using the method of Lowry *et al.* (1951). All determinations were made in duplicates. Final results are expressed in cts min^{-1} mg^{-1} protein.

Incorporation of phosphate into proteins

Oocytes were preloaded with 10 µCi ml^{-1} carrier-free $^{32}PO_4$ (aqueous solution, PBS13, Amersham) for 4 h, washed free of external label and incubated in various conditions. 1 ml samples were injected in 4 ml ice-cold 10 % t.c.a., washed twice in the same medium, dissolved in 1 ml NaOH 0·5 N and reprecipitated overnight. The final pellet, washed again with 5 ml t.c.a., was dissolved in NaOH 0·5 N. Protein concentration was determined and cts min^{-1} recorded from 0·5 ml samples suspended in 8 ml of ACS. All experiments were performed at the constant temperature of 20°C.

SDS–PAGE

For electrophoresis and autoradiography, oocytes (10^4 cells ml^{-1}) were incubated with 50 µCi ml^{-1} [^{35}S]methionine (SJ 1515, Amersham). 0·2 ml samples were withdrawn from the culture, spun down in a microfuge, washed and dissolved into 0·4 ml of the SDS gel sample buffer, which contained 60 mM-Tris–HCl, pH 6·8, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol and a drop of bromophenol blue. Samples were vortexed and heated for 5 min in boiling water. They were deposited on top of 15 % or 10–15 % linear gradient SDS–PAGE according to Laemmli (1970). Gels were stained with Coomassie blue R 250 (0·25 % in 50 % (v/v) methanol and 10 % (v/v) acetic acid or t.c.a., destained in 40 % (v/v) methanol and 7 % (v/v) acetic acid. After drying in a Hoefer Scientific Instruments slab gel dryer, gels were processed for autoradiography, using Kodak X-OM AR film or Amersham hyperfilm β-max, in the presence of an intensifying screen.

Cytological observations

GVBD, formation of the metaphase spindle or resting nuclei were easily observed *in vivo* after the eggs were mounted and flattened by removing excess fluid at the edge of the coverslip. These observations were routinely confirmed after the oocytes had been fixed for 10 min in Carnoy's fluid, stained in some drops of acetic carmine and glycerol and mounted between slide and coverslip. For ultrastructural observations, specimens were fixed in 4 % glutaraldehyde in 0·1 M-cacodylate pH 7·4 for 1 h at 0°C, washed overnight in 0·2 M-cacodylate, 2 % NaCl and post-fixed 1 h at 0°C in 1 % osmium tetroxide prepared in 0·1 M-cacodylate, 2·5 % NaCl. Dehydrated samples were embedded in Spurr's resin. Ultrathin sections were stained with 0·5 % uranyl acetate and 2 % lead citrate. Observations were performed on a JEOL JEM 100 CX transmission electron microscope at 80 kV.

Chemicals

All chemicals were obtained from Sigma, except 2,4-dinitrophenol (DNP) which was purchased from Prolabo and prepared in 10 mM-Tris, pH 8·2.

Results

MPF production following ammonia stimulation

When exposed to 5–10 mM-NH_4Cl in SW, pH 8·2, GV-arrested oocytes of *Patella vulgata* undergo GVBD within 7 min. They further condense their chromosomes and form a stable metaphase-1 spindle by 30–45 min (Fig. 1A), while the follicle cells detach from the plasma membrane and gather in a small clump at the animal pole (Guerrier *et al.* 1986*a*). Since all these features are usually related to MPF appearance, at least in the starfish (Kishimoto *et al.* 1984), we decided to check for the presence of this factor by injecting cytoplasm from such maturing oocytes into recipient GV-arrested oocytes. Unfortunately, these experiments, which involved the injection of up to 180 pl of cytoplasm (6 % of the oocyte volume) and were performed on more than 10 different batches of oocytes, always failed to produce GVBD in the recipient oocytes. This could signify either that a too small amount of MPF was injected or, at least, that MPF amplification could not occur at the original low intracellular pH that characterized the unstimulated *Patella* oocyte. To answer this question, we performed heterologous microinjection experiments, taking as recipients GV-arrested starfish oocytes, which are known to exhibit a higher intracellular pH, close to neutrality (Moody & Hagiwara, 1982; Picard & Dorée, 1983). Even when only a few experiments of this type could be performed, due to a rather bad overlap of the breeding seasons for both *Patella vulgata* and *Asterias rubens*, these clearly confirmed that *Patella* maturing oocytes did actually produce amplifiable active MPF. Indeed, in three independent assays, which involved both donor and recipient oocytes issued from different animals, we found that the microinjection of about 100 pl of *Patella* maturing cytoplasm was sufficient to produce GVBD in the recipient starfish oocytes. Similar results were also obtained after microinjection of cytoplasm taken from a batch of GV-disrupted

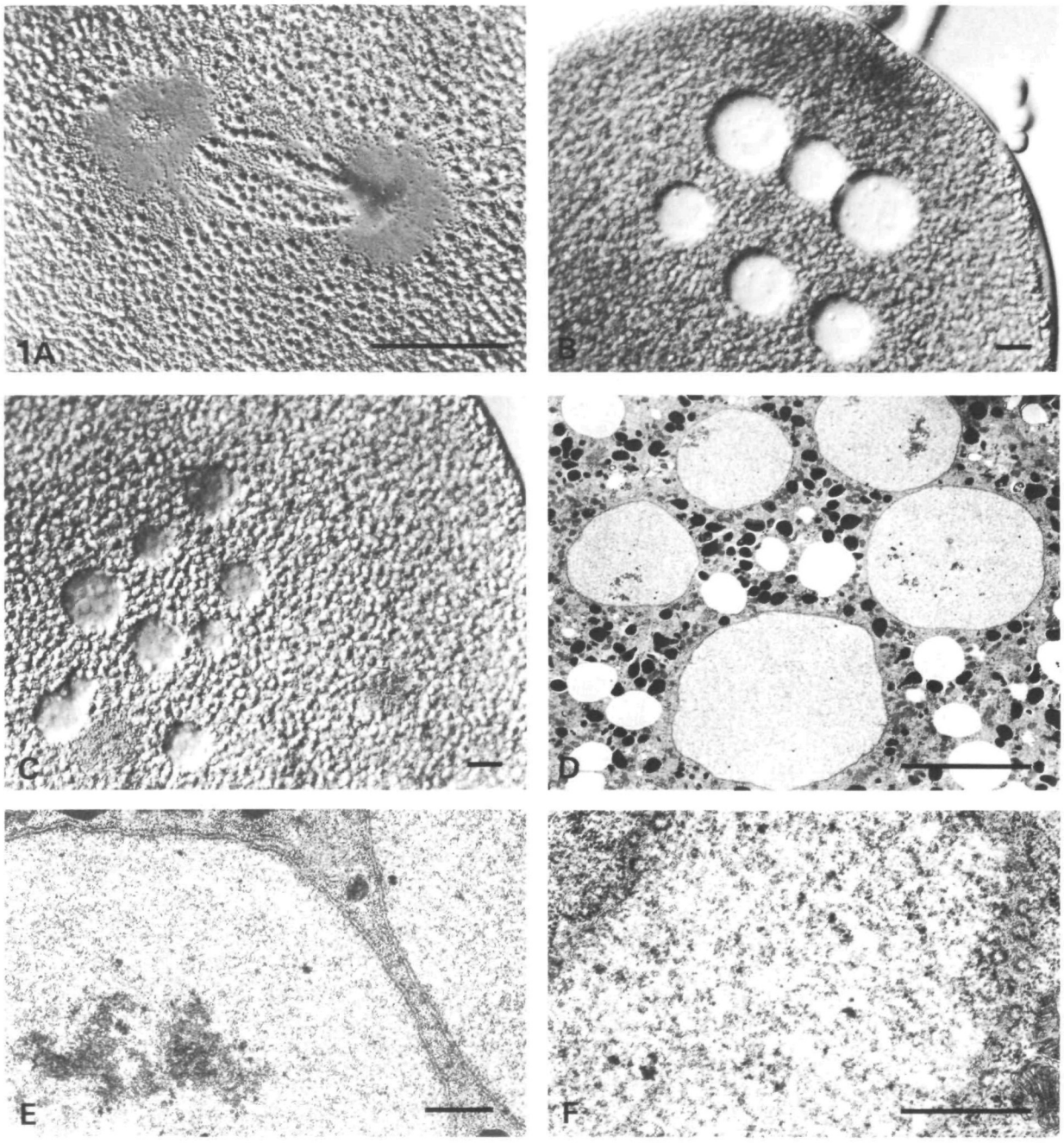

Fig. 1. Effect of inhibitors on metaphase-1-arrested oocytes of *Patella vulgata*. (A) A living metaphase spindle as observed in a compressed oocyte 5 h after ammonia stimulation. No inhibitor added. (B) Resting nuclei observed 90 min after the addition of 90 μM-emetine to metaphase-1 oocytes obtained following ammonia stimulation. (C) Resting nuclei observed 90 min after adding 600 μM-6-DMAP to ammonia-induced metaphase-1 oocytes. (D) Thin section through emetine-induced resting nuclei, 90 min after emetine addition. (E) Higher magnification of the same preparation showing the nuclear envelope. (F) A more peripheral section demonstrating nuclear pores. Bar, 10 μm in A–D, 1 μm in E and F.

shaken oocytes that proved able to reach the metaphase-1 stage in the absence of previous ammonia stimulation (Guerrier *et al.* 1986). In any case, it was found (1) that GVBD always occurred between 17 and 21 min after microinjection of the *Patella* donor cytoplasm, i.e. according to the same kinetics as observed following microinjection of starfish MPF (Guerrier & Néant, 1986*a*); (2) that the metaphase-1-blocked oocyte preserved its MPF activity for at least 2·5 h following GVBD; (3) that cytoplasm from GV-blocked oocytes of *Patella* never produced maturation of the recipient starfish oocytes.

Effects of metabolic inhibitors upon NH_4Cl-induced maturation

NH_4Cl-induced meiosis reinitiation was not inhibited when the oocytes had been previously treated for more than 2 h with 200 μM-forskolin, a drug that has been shown to stimulate adenylate cyclase and to delay or inhibit maturation in amphibian (Schorderet-Slatkin & Baulieu, 1982), mammalian (Eppig *et al.* 1983; Schultz *et al.* 1983) and starfish oocytes (Meijer & Zarutskie, 1987). The same absence of effect was observed following 30 min incubation in the presence of either 1 mM-dibutyryl-cyclic AMP (dbc-AMP), cyclic GMP (c-GMP) or the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) used separately or in association. GVBD and spindle formation also occurred normally, following incubation of the oocytes with 20 $\mu g\,ml^{-1}$ actinomycin D or with 90 μM-emetine.

In contrast, ammonia-induced maturation was inhibited by the uncoupler DNP and the puromycin analogue 6-dimethylaminopurine (6-DMAP) which blocked GVBD at 200–500 μM and 1·5–2 mM, respectively. At lower concentrations, these drugs only delayed GVBD which, for example, took some 30 min to occur in the presence of 120 μM-DNP or 1 mM-6-DMAP. Under these conditions, the metaphase-1 spindles formed normally and the chromosomes condensed, giving rise to nine typical tetrads which, however, did not persist as long as observed in control oocytes. Indeed, while cyclic nucleotides had no specific cytological effect upon oocyte maturation, except that IBMX alone could block follicle cell detachment, all the other treatments listed were found to severely affect the mitotic apparatus and chromosome structure. Thus, both 6-DMAP (600 μM) and emetine (75 μM) rapidly induce disappearance of the metaphase-1 spindle. Within 40 min, the chromosomes disperse and decondense to produce a set of resting nuclei which exhibits well-formed nucleoli and a typical nuclear envelope with nuclear pores (Fig. 1A–F). This process is only delayed when lower concentrations of emetine (18 μM) or 6-DMAP (150 μM) are used. In any case, it does not result from activation and true mitosis since no spindle cycle is observed under these conditions. Moreover, the number of nuclei that appear simultaneously in the treated oocytes is never in great excess over the number of tetrads originally present in the metaphase plate. It is worth noting that this evolution is also triggered when emetine or 6-DMAP are only applied 1 h after ammonia stimulation when the metaphase-1 spindle is already attached to the animal pole cortex. The effects of emetine and 6-DMAP only differ in one respect, their reversibility. Indeed, whereas emetine-induced nuclei persist after washing out the inhibitor, in 6-DMAP-depleted oocytes new asters are rapidly produced, nuclei break down and the chromosomes recondense (Fig. 2A–D). This rescue process also occurs in the presence of 1 mM-cyclic nucleotides and IBMX, in contrast to the situation found in puromycin-treated mouse oocytes (Clarke & Masui, 1985).

Finally, it was found that the application of DNP to the metaphase-1-blocked oocyte also induced chromosome decondensation which, however, did not usually result in the production of multiple resting nuclei. These figures were only present in some instances, after the asters have completely disappeared and the oocytes had been washed out from the inhibitor.

Changes in protein synthesis following ammonia stimulation

In preliminary experiments, we found that ammonia stimulated by 100 % the uptake and incorporation of amino acids into proteins to reach a steady-state level within 1 h. Pulse experiments also indicated that the rate of protein synthesis might increase during the course of maturation and that emetine (90 μM) efficiently blocked amino acid incorporation (data not shown). Preloading experiments were also performed which showed that ammonia did actually stimulate this incorporation independently of the uptake. They also confirmed that emetine was fully effective within 5 min of its application, in great contrast to the effect of 6-DMAP (Fig. 3), a drug that has been already shown to block the sea urchin cell cycle without inhibiting protein synthesis (Rebhun *et al.* 1973). These important conclusions are corroborated by the data produced in Fig. 4, where the rates of incorporation, as expressed in percentage of the uptake, have been determined in pulse experiments performed within 1 h after ammonia stimulation.

Qualitative differences were also recorded in the patterns of protein synthesis observed after [^{35}S]methionine incorporation, SDS–PAGE and autoradiography. In particular, a new protein of about 48×10^3 M_r was found to appear within 20 min after ammonia stimulation, which was not synthesized by the unstimulated GV-blocked oocyte and remained stable following an 8 min treatment with emetine (Fig. 5). Since this protein is also produced in the presence of inhibiting concentrations of 6-DMAP (150 μM–1 mM), it is thus clear that the emetine-like cytological effects produced by 6-DMAP cannot be explained by a failure of the protein synthesis machinery.

Effects of ammonia and inhibitors upon endogenous protein phosphorylation

Within 2 min following ammonia addition, the rate of endogenous protein phosphorylation increases by

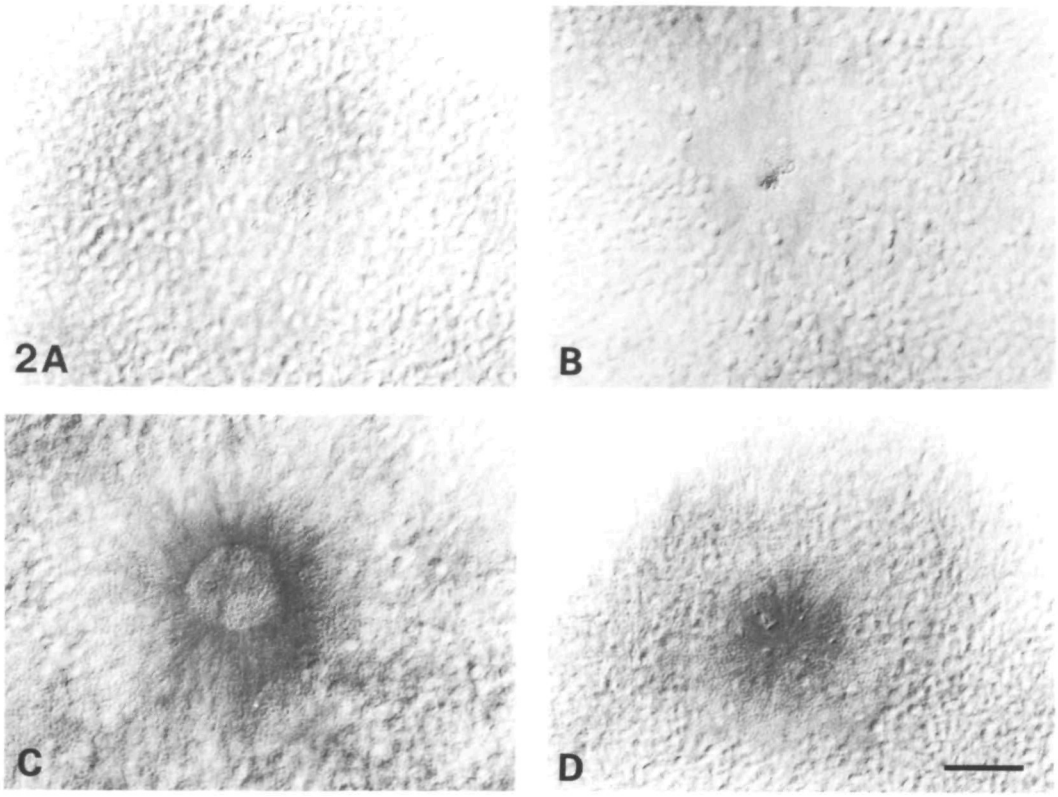

Fig. 2. Various aspects of chromosome recondensation and aster reconstitution in *Patella vulgata* oocytes that formed resting nuclei upon treatment with 600 μM-6-DMAP and were returned to fresh seawater. (A) Outline of the nucleus is still visible (5 min recovery). (B,C) Recondensing chromosomes are visible in the centre of a radiating astral zone (10 min). (D) Final aspect observed 20 min after washing out the inhibitor. Acetocarmine. Bar, 20 μm.

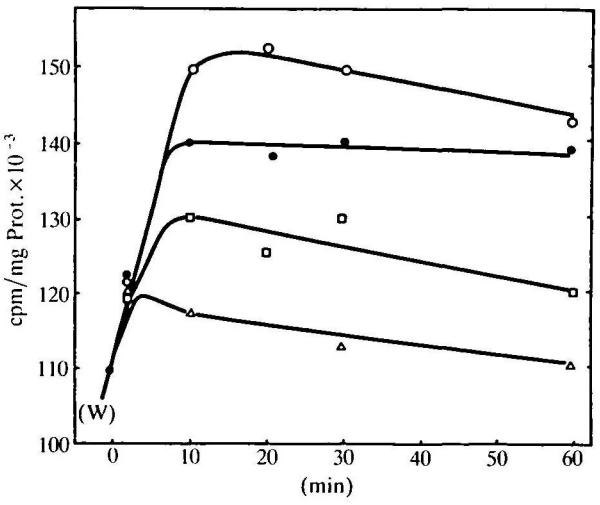

Fig. 3. Incorporation of [^{3}H]leucine (500 μCi ml^{-1}) into *Patella* oocytes preloaded for 20 min and stimulated by 10 mM-NH_4Cl alone (●) or in the presence of 600 μM-6-DMAP (○), 100 μM-2,4-DNP (□) or 90 μM-emetine (△). Mean of duplicates. Drugs were added at 0 min.

about 20-fold, showing a small inflexion around GVBD (Fig. 6). As shown on Fig. 7, plateau level is reached within 1 h, at which time the metaphase spindle has already attached to the animal pole. This figure also illustrates the fact that a 30 min incubation with 90 μM-emetine neither blocks meiosis reinitiation nor the phosphorylation burst induced by ammonia, which persists at least for 30 min and allows the formation of a typical metaphase spindle bearing condensed chromosomes. At this stage, however, a rapid dephosphorylation process occurs, while resting nuclei begin to appear in the cytoplasm after destruction of the spindle. The same features are also triggered when emetine is only added to the metaphase-1 oocytes, 1 h after ammonia stimulation.

Phosphorylation was also reduced following a 30 min incubation with 100 μM-DNP, a concentration that allowed GVBD to occur but blocked spindle formation, and definitively suppressed with 400 μM, a concentration that precluded GVBD. Addition of the same concentrations of DNP to metaphase-1 oocytes also produced a 50 % reduction in the extent of

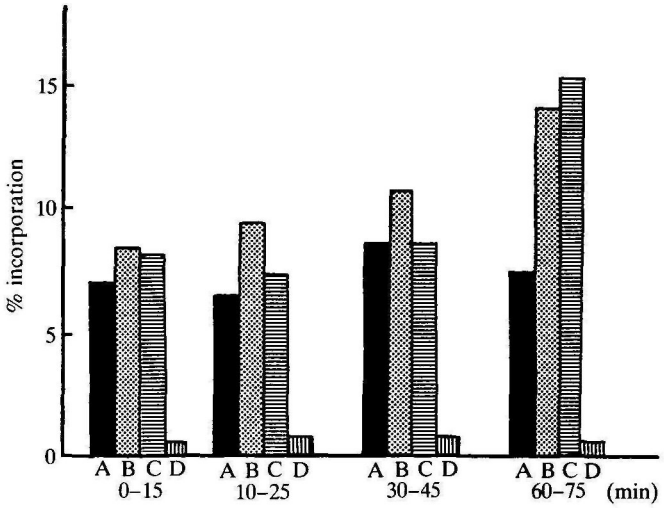

Fig. 4. Effects of emetine (90 μM) and 6-DMAP (600 μM) on the rate of incorporation of [^{3}H]leucine (5 μCi ml^{-1}) as expressed in percentage of the uptake. (A) Control unstimulated oocytes; (B) 10 mM-ammonia-stimulated oocytes. C and D received 600 μM-6-DMAP or 90 μM-emetine, respectively, before NH_4Cl addition. Pulses lasting for 15 min were performed starting at 0, 10, 30 and 60 min after ammonia addition. Mean of duplicate determinations.

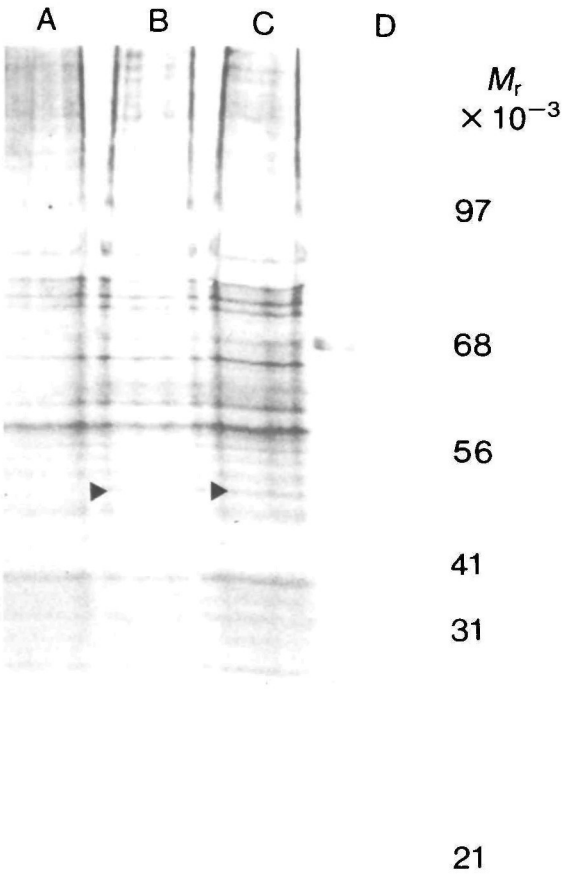

Fig. 5. Changes in the pattern of protein synthesis as revealed following [^{35}S]methionine incorporation. 30 min after beginning of the incorporation, GV-blocked oocytes were stimulated with 10 mM-NH_4Cl for 15 min (lane A) or 30 min (lane B). In lane C, emetine (90 μm) was added for 8 min, starting 25 min after ammonia stimulation. Labelled standard proteins in lane D include soybean trypsin inhibitor (21K), carbonic anhydrase (31K), creatine kinase (41K), glutamate dehydrogenase (56K), bovine serum albumin (68K), phosphorylase (97K).

phosphorylation (data not shown). The same progressive reduction in phosphorylation capacity has been recorded following the application of various concentrations of 6-DMAP, which also correlated perfectly with the cytological effects of this compound. Data presented in Fig. 8 demonstrate, moreover, that this drug, which does not affect protein synthesis, is quite effective in reducing endogenous protein phosphorylation, whenever it is applied. However, as illustrated in Fig. 9, this effect is perfectly reversible, an observation supported by the fact that resting nuclei rapidly disappear and that chromosomes recondense under these conditions. In contrast, phosphorylation did not start again after washing out such inhibitors as emetine or DNP, which permanently affected the oocyte (data not shown).

Possible targets for 6-DMAP action

The effect of 6-DMAP upon protein phosphorylation can be explained in different ways, i.e. by a stimulation of the protease or phosphoprotein phosphatase activities, by an inhibition of the protein kinase activity or by a reduction in the availability of the ATP nucleotide pool. To investigate these possibilities, we first tried to characterize the components that may affect dephosphorylation *in vitro*. Results from such experiments demonstrate that 6-DMAP (750 μM) as well as emetine (90 μM) have no influence on the rate of dephosphorylation observed under these conditions (Fig. 10). Moreover, when used in association with phosphoprotein phosphatase or protease inhibitors, these drugs did not influence their protective effect. This indicates that they do not directly activate proteolysis or the phosphoprotein phosphatases that were present in our assay and that we could inhibit using α-naphtyl phosphate (Pondaven & Meijer, 1986). At present, we cannot eliminate the possibility that 6-DMAP might have affected the ATP pool level. However, the rapidity with which oocytes recover from this inhibitor and the fact that its cytological effects are closer to those produced by emetine than by DNP makes this proposition rather unlikely. We are thus inclined to think that 6-DMAP

may directly affect protein kinase activity as we have recently shown to be the case in starfish ooctyes, where 6-DMAP and emetine exert the same biochemical and cytological effects as observed in *Patella* (Guerrier & Néant, 1986*b*).

Discussion

We have shown previously that GV-blocked oocytes of *Patella vulgata* reinitiate meiosis up to metaphase 1 when their intracellular pH is increased by about 0·6 units (Guerrier *et al.* 1986). Shaking experiments also demonstrated that the threshold pH required for chromosome condensation and metaphase-1 spindle formation was slightly lower than that needed to obtain GVBD. In the present study, we confirm that these treatments induce the appearance of a stable MPF activity which can be amplified following heterologous cytoplasm transfers using GV-arrested starfish oocytes as recipients. In contrast, homologous

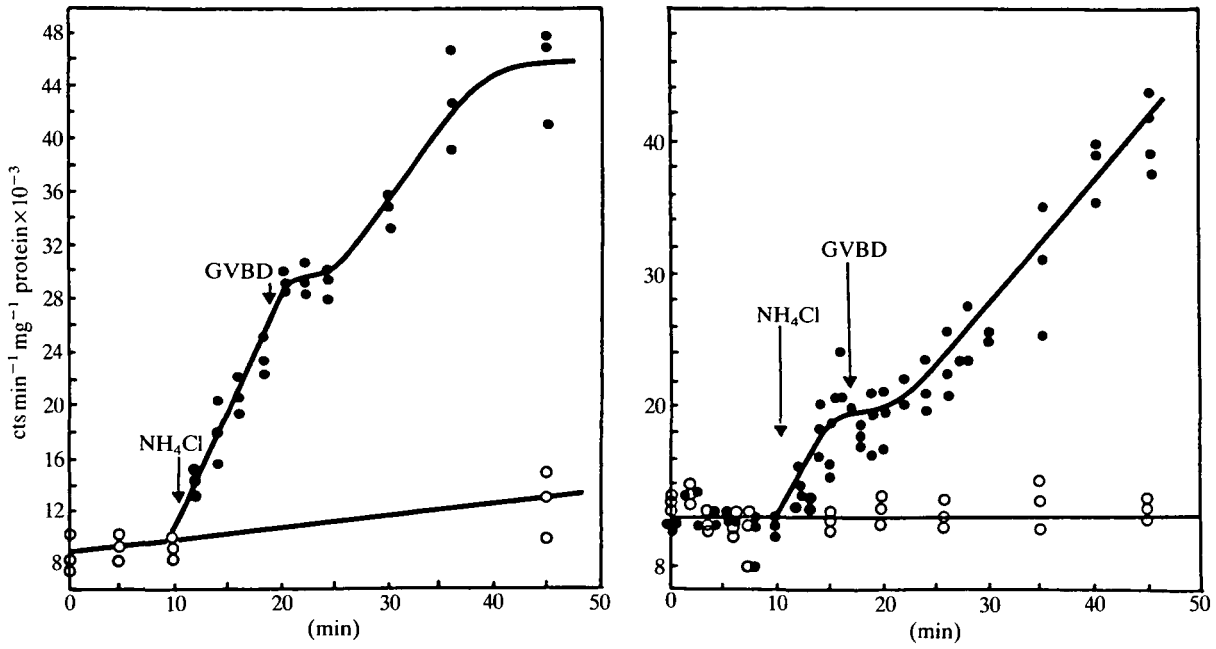

Fig. 6. Stimulation of the incorporation of ^{32}P into proteins following stimulation of *Patella* GV-blocked oocytes by 10 mM-ammonia. Two independent experiments with triplicate determinations.

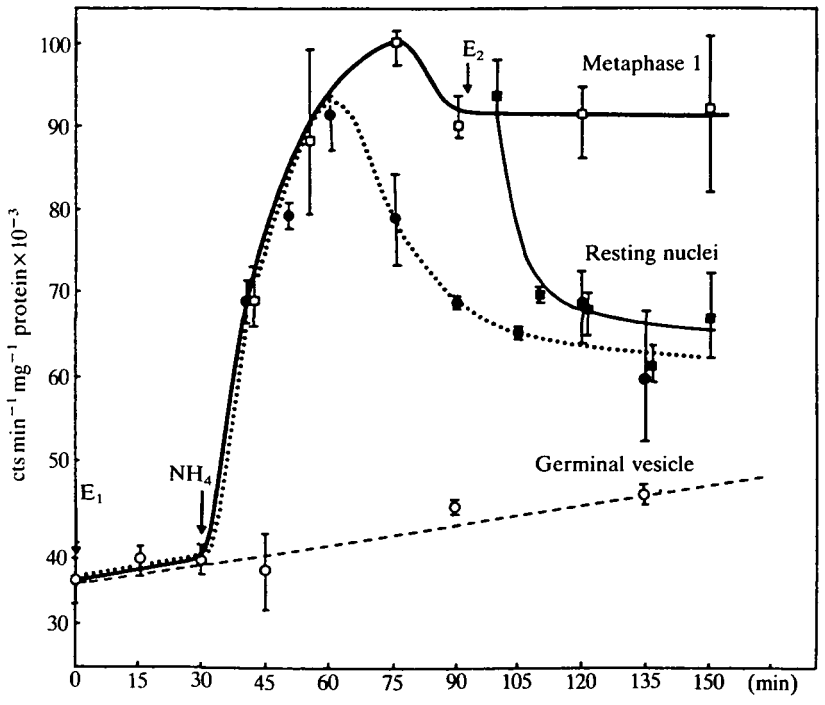

Fig. 7. Effect of emetine on ammonia-induced meiosis reinitiation and protein phosphorylation. Emetine (90 μM) was added (arrow E_1) 30 min before the addition of 10 mM-NH_4Cl or 30 min later, at the metaphase-1 stage (arrow E_2). ○, control unstimulated oocytes; □, stimulated oocytes; ●, oocytes stimulated 30 min after emetine addition; ■, metaphase-1 oocytes treated with emetine.

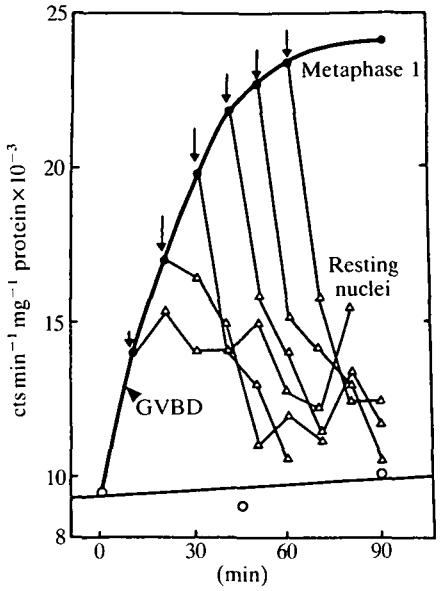

Fig. 8. *In vivo* induction of dephosphorylation by 6-DMAP. The evolution of protein phosphorylation was followed in various samples taken from batches which received 600 μM-6-DMAP (arrows) at various times after stimulation by 10 mM-NH_4Cl. ○, control unstimulated oocytes; ●, stimulated oocytes; △, stimulated oocytes treated with the inhibitor.

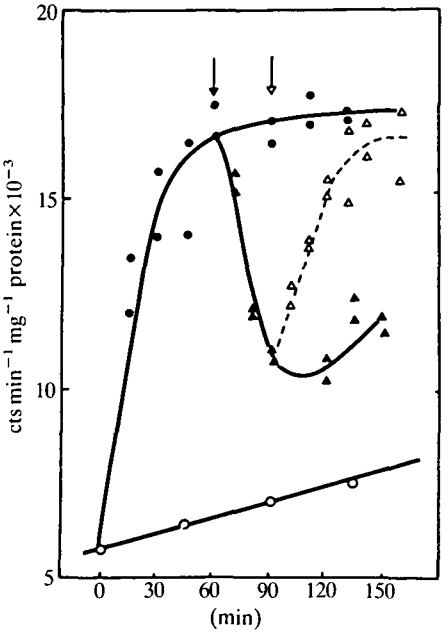

Fig. 9. Reversibility of the dephosphorylating effect of 6-DMAP. ○, control unstimulated oocytes; ●, 10 mM-NH_4Cl-stimulated oocytes; ▲, stimulated oocytes + 600 μM-6-DMAP; △, 6-DMAP washed stimulated oocytes. The inhibitor was added at 60 min (closed arrow) and removed at 90 min (open arrow).

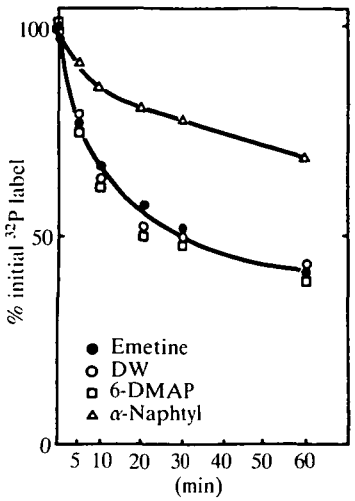

Fig. 10. Protease and phosphoprotein phosphatase activities measured in a homogenate of *Patella* oocytes (5×10^4 oocytes ml^{-1}) preloaded for 4 h, with [^{32}P]orthophosphate (90 μCi ml^{-1}). During the last hour of incubation, the oocytes were induced to reach metaphase 1 following stimulation with 10 mM-ammonia. After washing out the external radioactivity, the homogenate was prepared in a buffer containing 50 mM-Tris pH 7, 250 mM-sucrose, 1 mM-EDTA and 0·1 % β-mercaptoethanol. The incubation medium contained 300 μl buffer, 200 μl homogenate and 100 μl of the drugs tested. 100 % incorporation level corresponded to 2×10^6 cts min^{-1}. ●, emetine (90 mM); ○, distilled water; □, 6-DMAP (750 μM); △, α-naphtyl phosphate (26 mM).

transfers performed from metaphase-1 to GV-arrested *Patella* oocytes failed to trigger GVBD. This indicates that, in *Patella*, MPF amplification as well as MPF formation depend on an elevated intracellular pH that may approach the values reported to occur in GV-arrested oocytes of the starfish, i.e. 7·09 ± 0·8, according to Moody & Hagiwara (1982) or from 6·8 to 7·04, according to Picard & Dorée (1983).

Our data also reveal that ammonia-induced MPF formation is accompanied by a dramatic increase in protein phosphorylation up to a plateau level characteristic of the metaphase-1 stage. The inhibition of protein synthesis by emetine allows development of the phosphorylation burst, GVBD and formation of the metaphase spindle but soon results in protein dephosphorylation. Simultaneously, the metaphase-1 spindle disappears and the chromosomes disperse and decondense, giving rise to typical resting nuclei. This suggests that MPF formation in *Patella* does not require the synthesis of an initiator but that CSF, which maintains phosphorylation, chromosome condensation and metaphase-1 spindle structure depends on a continuous supply of short-lived proteins. These may be new MPF molecules differing in some way to the previously activated MPF precursor molecules

and/or regulatory factors which may activate protein kinases or counteract dephosphorylation. Indeed, one of the most important findings made during this study was the observation that chromosome condensation activity was sensitive to the dephosphorylations induced by emetine, DNP or the drug 6-DMAP which had no direct effect upon protein synthesis or phosphoprotein phosphatase activity.

Thanks are due to Mrs C. Guerrier for excellent technical assistance throughout this work and for preparing the illustrations, to Mrs N. Guyard for typing the text, to G. Sinquin, who is responsible for the electron microscope in Brest and to the fishermen of the Biological station at Roscoff who provided us with animals throughout the breeding season. This work was partly supported by ATP 84C0939 from the ministry of Research and Industry and by the ARC (fellowship to I.N.).

References

Bałakier, H. (1978). Induction of maturation in small oocytes from sexually immature mice by fusion with meiotic or mitotic cells. *Expl Cell Res.* **112**, 137–141.

Bałakier, H. & Czołowska, R. (1977). Cytoplasmic control of nuclear maturation in mouse oocytes. *Expl Cell Res.* **110**, 466–469.

Capony, J. P., Picard, A., Peaucellier, G., Labbé, J. C. & Dorée, M. (1986). Changes in the activity of the maturation-promoting factor during maturation and following activation of amphibian and starfish oocytes: their correlations with protein phosphorylation. *Devl Biol.* **117**, 1–12.

Clarke, H. J. & Masui, Y. (1983). The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. *Devl Biol.* **97**, 291–301.

Clarke, J. J. & Masui, Y. (1985). Inhibition by dibutyryl cyclic AMP of the transition to metaphase of mouse oocyte nuclei and its reversal by cell fusion to metaphase oocytes. *Devl Biol.* **108**, 32–37.

Crosby, I. M., Osborn, J. C. & Moor, R. M. (1984). Changes in protein phosphorylation during the maturation of mammalian oocytes *in vitro*. *J. exp. Zool.* **229**, 459–466.

Crozet, N. & Szollozi, D. (1980). Effects of actinomycin D and α-amanitin on the nuclear ultrastructure of mouse oocytes. *Biol. Cellulaire* **38**, 163–170.

Dorée, M. (1982). Protein synthesis is not involved in initiation or amplification of the maturation-promoting factor (MPF) in starfish oocytes. *Expl Cell Res.* **139**, 127–133.

Dorée, M., Peaucellier, G. & Picard, A. (1983). Activity of the maturation-promoting factor and the extent of the protein phosphorylation oscillate simultaneously during meiotic maturation of starfish oocytes. *Devl Biol.* **99**, 489–501.

Dubé, F., Golsteyn, R. & Dufresne, L. (1987). Protein kinase C and meiotic maturation oocytes. *Biochem. biophys. Res. Commun.* **142**, 1072–1076.

Eckberg, W. R., Szuts, E. Z. & Carroll, A. G. (1987). Protein kinase C activity, protein phosphorylation and germinal vesicle breakdown in *Spisula* oocytes. *Devl Biol.* **124**, 57–64.

Eppig, J. J., Freter, R. R., Ward-Bailey, P. F. & Schultz, R. M. (1983). Inhibition of oocyte maturation in the mouse: participation of c-AMP, steroids, and a putative maturation inhibitory factor. *Devl Biol.* **100**, 39–49.

Evans, T., Rosenthal, E. T., Yongblom, J., Distel, D. & 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.

Gerhart, J., Wu, M. & Kirschner, M. (1984). Cell cycles dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **98**, 1247–1255.

Golbus, M. S. & Stein, M. P. (1976). Qualitative patterns of protein synthesis in the mouse oocyte. *J. exp. Zool.* **198**, 337–342.

Guerrier, P., Brassart, M., David, C. & Moreau, M. (1986*a*). Sequential control of meiosis reinitiation by pH and Ca^{2+} in oocytes in the prosobranch mollusk *Patella vulgata*. *Devl Biol.* **114**, 315–324.

Guerrier, P. & Dorée, M. (1975). Hormonal control of reinitiation of meiosis in starfish. The requirement of 1-methyladenine during nuclear maturation. *Devl Biol.* **47**, 341–348.

Guerrier, P., Guerrier, C., Néant, I. & Moreau, M. (1986*b*). Germinal vesicle nucleoplasm and intracellular pH requirements for cytoplasmic maturity in oocytes of the prosobranch mollusk *Patella vulgata*. *Devl Biol.* **116**, 92–99.

Guerrier, P., Moreau, M. & Dorée, M. (1977). Hormonal control of meiosis in starfish: stimulation of protein phosphorylation induced by 1-methyladenine. *Molec. cell. Endocrinol.* **7**, 137–150.

Guerrier, P., Moreau, M., Meijer, L., Mazzei, G., Vilain, J. P. & Dubé, F. (1982). The role of calcium in meiosis reinitiation. In *Membranes in Growth and Development, Progress in Clinical and Biological Research*, vol. 91 (ed. J. J. Hoffman, G. H. Giebisch & L. Bolis), pp. 139–155. New York: Alan R. Liss.

Guerrier, P. & Néant, I. (1986*a*). Metabolic cooperation following fusion of starfish ootid and primary oocyte restores meiotic-phase-promoting activity. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4814–4818.

Guerrier, P. & Néant, I. (1986*b*). Factors regulating MPF activity during starfish oocyte maturation. In *Advances in Invertebrate Reproduction*, vol. 4 (ed. M. Porchet, J. C. Andries & A. Dhainant), pp. 265–273. Amsterdam: Elsevier Science Publishers.

Guerrier, P., Néant, I. & Clédon, P. (1988). Urea-induced meiosis reinitiation in oocytes of the starfish *Marthasterias glacialis*. *Dev. Growth Differ.* (in press).

Gurdon, J. C. (1968). Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. exp. Morph.* **20**, 401–414.

HIRAMOTO, Y. (1974). A method of microinjection. *Expl Cell Res.* **87**, 403–406.

JAGIELLO, G. M. (1969). Meiosis and inhibition of ovulation in mouse eggs treated with actinomycin D. *J. Cell Biol.* **42**, 571–574.

KISHIMOTO, T. (1986). Microinjection and cytoplasmic transfer in starfish oocytes. In *Methods in Cell Biology* **27** (ed. T. H. Schroeder), pp. 379–394. New York: Academic Press.

KISHIMOTO, T., KURIYAMA, R., KONDO, H. & KANATANI, H. (1982). Generality of the action of various maturation-promoting factors. *Expl Cell Res.* **137**, 121–126.

KISHIMOTO, T., USUI, N. & KANATANI, H. (1984). Breakdown of starfish ovarian follicle cells induced by maturation-promoting factor. *Devl Biol.* **101**, 28–34.

KISHIMOTO, T., YAMAZAKI, K., KATO, Y., KOIDE, S. & KANATANI, H. (1984). Induction of starfish oocyte maturation by maturation-promoting factor of mouse and surf clam oocytes. *J. exp. Zool.* **231**, 293–295.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **302**, 76–79.

LOWRY, O. M., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. G. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MALLER, J. L. & KREBS, E. G. (1980). Regulation of oocyte maturation. *Curr. Top. Cell. Regul.* **16**, 271–311.

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

MASUI, Y. (1974). A cytostatic factor in amphibian oocytes: its extraction and partial characterization. *J. exp. Zool.* **187**, 141–147.

MASUI, Y. (1985). Meiotic arrest in animal oocytes. In *Biology of Fertilization*, vol. 1 (ed. C. B. Metz & A. Monroy), pp. 189–219. New York: Academic Press.

MASUI, Y. & CLARKE, H. J. (1979). Oocyte maturation. *Int. Rev. Cytol.* **57**, 185–282.

MASUI, Y. & MARKERT, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. exp. Zool.* **177**, 129–146.

MASUI, Y., MEYERHOF, P. G. & MILLER, M. A. (1980). Cytostatic factor and chromosome behaviour in early development. In *The Cell Surface, Mediator of Developmental Processes* (ed. S. Subtelny & N. K. Wessels), pp. 235–256. New York: Academic Press.

MEIJER, L. & GUERRIER, P. (1984). Maturation and fertilization in starfish oocytes. *Int. Rev. Cytol.* **86**, 129–196.

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

MEIJER, L., PONDAVEN, P., GUERRIER, P. & MOREAU, M. (1984). A starfish oocyte user's guide. *Cah. Biol. Mar.* **25**, 457–480.

MEIJER, L. & ZARUTSKIE, P. (1987). Starfish oocyte maturation: 1-methyladenine triggers a drop of c-AMP concentration related to the hormone-dependent period. *Devl Biol.* **121**, 306–315.

MOODY, W. H. & HAGIWARA, S. (1982). Block of inward rectification by intracellular H^+ in immature oocytes of the starfish *Mediaster aequalis*. *J. gen. Physiol.* **79**, 115–130.

MOOR, R. M. & CROSBY, I. M. (1986). Protein requirements for germinal vesicle breakdown in ovine oocytes. *J. Embrol. exp. Morph.* **94**, 207–220.

NELKIN, B., NICHOLS, C. & VOGELSTEIN, B. (1980). Protein factor(s) from mitotic CHO cells induce meiotic maturation in *Xenopus laevis* oocytes. *FEBS Letters* **109**, 233–238.

NEWPORT, J. W. & KIRSCHNER, M. W. (1984). Regulation of the cell cycle during early *Xenopus* development. *Cell* **37**, 731–742.

OSBORN, J. C. & MOOR, R. M. (1983). Time-dependent effects of α-amanitin on nuclear maturation and protein synthesis in mammalian oocytes. *J. Embryol. exp. Morph.* **73**, 317–338.

PEAUCELLIER, G., DORÉE, M. & DEMAILLE, J. G. (1982). Stimulation of endogenous protein phosphorylation in oocytes of *Sabellaria alveolata* (Polychaete annelid) at meiosis reinitiation induced by protease, fertilization, or ionophore A 23187. *Gamete Res.* **1**, 115–123.

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

PICARD, A. & DORÉE, M. (1983). Intracellular microinjection of alkaline buffers reversibly inhibits the initial phase of hormone action in meiosis reinitiation of starfish oocytes. *Devl Biol.* **97**, 184–190.

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

PONDAVEN, P. & MEIJER, L. (1986). Protein phosphorylation and oocyte maturation. I. Induction of starfish oocyte maturation by intracellular microinjection of a phosphatase inhibitor, α-naphtylphosphate. *Expl Cell Res.* **163**, 477–488.

REBHUN, L. L., WHITE, D., SANDER, G. & IVY, N. (1973). Cleavage inhibition in marine eggs by puromycin and 6-dimethylaminopurine. *Expl Cell Res.* **77**, 312–318.

REYNHOUT, J. K. & SMITH, L. D. (1974). Studies on the appearance and nature of a maturation-inducing factor in the cytoplasm of amphibian oocytes exposed to progesterone. *Devl Biol.* **38**, 394–400.

RYABOVA, L. V. (1983). The cytostatic effect of the cytoplasm of mature, non-activated and cleaving eggs of *Rana temporaria*, *Acipenser stellatus* and *Xenopus laevis*. *Cell Differ.* **13**, 171–175.

SCHATT, P., MOREAU, M. & GUERRIER, P. (1983). Variations cycliques de la phosphorylation des protéines et de l'activité MPF pendant la segmentation précoce de l'oeuf d'oursin. *C. r. hebd. Séanc. Acad. Sci. Paris* **296**, 551–554.

SCHORDERET-SLATKIN, S. & BAULIEU, E. E. (1982). Forskolin increases c-AMP and inhibits progesterone

induced meiosis in *Xenopus laevis* oocytes. *Endocrinol.* **111**, 1385–1387.

SCHUETZ, A. W. & SAMSON, D. (1979). Nuclear requirement for post-maturational cortical differentiation in amphibian oocytes: effects of cycloheximide. *J. exp. Zool.* **210**, 307–320.

SCHULTZ, R. M., MONTGOMERY, R. R. & BELANOFF, J. R. (1983). Regulation of mouse oocyte maturation: Implication of a decrease in oocyte c-AMP and protein dephosphorylation in commitment to resume meiosis. *Devl Biol.* **97**, 264–273.

SHAPIRO, H. (1941). Centrifugal elongation of cells, and some conditions governing the return to sphericity and cleavage time. *J. cell comp. Physiol.* **18**, 61–78.

SHIBUYA, E. K. & MASUI, Y. (1982). Sperm-induced cell cycle activities in blastomeres arrested by the cytostatic factor of unfertilized eggs in *Rana pipiens*. *J. exp. Zool.* **220**, 381–385.

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

STERN, S., RAYYIS, A. & KENNEDY, J. F. (1972). Incorporation of amino acids during maturation *in vitro* by the mouse oocyte: Effect of puromycin on protein synthesis. *Biol. Reprod.* **7**, 341–346.

SUNKARA, P. S., WRIGHT, D. A. & RAO, P. N. (1979). Mitotic factors from mammalian cells induce germinal vesicle breakdown and chromosome condensation in amphibian oocytes. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2799–2802.

SWENSON, K. I., FARRELL, K. M. & 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.

TARKOWSKI, A. K. & BAŁAKIER, H. (1980). Nucleo-cytoplasmic interactions in cell hydrids between mouse oocytes, blastomeres and somatic cells. *J. Embryol. exp. Morph.* **55**, 319–330.

WAGENAAR, E. B. & MAZIA, D. (1978). The effect of emetine on first cleavage division in the sea urchin *Strongylocentrotus purpuratus*. In *Cell Reproduction, ICN-UCLA Symp. on Mol. cell. Biol.* (ed. E. Dirkson & D. Prescott), pp. 539–545. New York: Academic Press.

WASSARMAN, P. M., SCHULTZ, R. M. & LETOURNEAU, G. E. (1979). Protein synthesis during meiotic maturation of mouse oocytes *in vitro*. Synthesis and phosphorylation of a protein localized in the germinal vesicle. *Devl Biol.* **69**, 94–107.

WASSERMAN, W. J. & MASUI, Y. (1975). Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. *Expl Cell Res.* **91**, 381–388.

WASSERMAN, W. J. & SMITH, L. D. (1978). The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. *J. Cell Biol.* **78**, 15–22.

WEINTRAUB, H., BUSCAGLIA, M., FERREZ, M., WEILLER, S., BOULET, A., FABRE, F. & BAULIEU, E. E. (1982). Mise en évidence d'une activité "MPF" chez *Saccharomyces cerevisiae*. *C. r. hebd. Séanc. Acad. Sci. Paris, série III* **295**, 787–790.

WESTWOOD, J. T., CHURCH, R. B. & WAGENAAR, E. B. (1985). Changes in protein phosphorylation during the cell cycle of Chinese hamster ovary cells. *J. biol. Chem.* **260**, 10308–10313.

WESTWOOD, J. T. & WAGENAAR, E. B. (1983). Chinese hamster cells can be reversibly blocked before mitosis with the protein synthesis inhibitor emetine. *J. Cell Sci.* **59**, 257–268.

WILT, F. H., SAKAI, H. & MAZIA, D. (1967). Old and new protein in the formation of the mitotic apparatus in cleaving sea urchin eggs. *J. molec. Biol.* **27**, 1–7.

(*Accepted 18 November 1987*)