

New B-type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation

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SUMMARY

Progression through meiosis requires two waves of maturation promoting factor (MPF) activity corresponding to meiosis I and meiosis II. Frog oocytes contain a pool of inactive 'pre-MPF' consisting of cyclin-dependent kinase 1 bound to B-type cyclins, of which we now find three previously unsuspected members, cyclins B3, B4 and B5. Protein synthesis is required to activate pre-MPF, and we show here that this does not require new B-type cyclin synthesis, probably because of a large maternal stockpile of cyclins B2 and B5. This stockpile is degraded after meiosis I and consequently, the activation of MPF for meiosis II requires new cyclin synthesis, principally of cyclins B1 and B4, whose translation is strongly activated after meiosis I. If this wave of new cyclin synthesis is ablated by antisense oligonucleotides, the oocytes

degenerate and fail to form a second meiotic spindle. The effects on meiotic progression are even more severe when all new protein synthesis is blocked by cycloheximide added after meiosis I, but can be rescued by injection of indestructible B-type cyclins. B-type cyclins and MPF activity are required to maintain *c-mos* and MAP kinase activity during meiosis II, and to establish the metaphase arrest at the end of meiotic maturation. We discuss the interdependence of *c-mos* and MPF, and reveal an important role for translational control of cyclin synthesis between the two meiotic divisions.

Key words: Cell cycle, Meiosis, Maturation promoting factor, Eg-2, *c-mos*, Antisense oligonucleotides, Cycloheximide, Cyclin-dependent kinase, *Xenopus laevis*, *Xenopus tropicalis*

INTRODUCTION

Fully grown *Xenopus* oocytes are arrested at the G₂/M boundary of the first meiotic division (MI) and contain a pool of pre-MPF (M-phase promoting factor; Cyert and Kirschner, 1988), a protein kinase composed of p34^{cdc2} and a B-type cyclin (Draetta et al., 1989; Gautier et al., 1990; Labbé et al., 1989). Progesterone induces meiotic maturation by triggering the conversion of pre-MPF to MPF, and a number of other protein kinases are activated including *c-mos*, MAP kinase, Polo kinase, p90^{rsk} and Eg-2/Aurora (for reviews see Sagata, 1997; Ferrell, 1999; Nebreda and Ferby, 2000; Yoshida et al., 2000). After germinal vesicle breakdown (GVBD) and entry into meiosis I (MI), MPF activity declines transiently, and rises again at the onset of meiosis II (MII; Furuno et al., 1994; Gerhart et al., 1984; Kobayashi et al., 1991; Ohsumi et al., 1994; Thibier et al., 1997). A second cell cycle arrest is subsequently established at metaphase of MII. This arrest is maintained by an unknown, MAP kinase and p90^{rsk}-dependent activity named cytostatic factor (CSF) (Bhatt and Ferrell, 1999;

Gross et al., 1999; Masui and Markert, 1971; Sagata et al., 1989b). Frog eggs remain arrested at this stage with high levels of MPF until fertilisation triggers the activation of the anaphase promoting complex (APC/C), permitting sister chromatid separation and destruction of B-type cyclins (King et al., 1995; Murray et al., 1989; Sudakin et al., 1995; Zachariae and Nasmyth, 1999).

Protein synthesis is necessary for the activation of pre-MPF (Barkoff et al., 1998; Gerhart et al., 1984; Roy et al., 1996; Sagata et al., 1988; Wasserman and Masui, 1975). Newly synthesised proteins are required to activate Cdc25, which removes inhibitory Thr-14 and Tyr-15 phosphates on p34^{cdc2}, and to inhibit Myt1 protein kinase (Gautier et al., 1991; Karaiskou et al., 1998; Kumagai and Dunphy, 1991; Mueller et al., 1995). Apart from a few exceptions, the nature of the newly synthesised proteins that are required for oocyte maturation remains elusive. Thus, it is well established that *c-mos* synthesis is necessary for MAP kinase and MPF activation (Freeman et al., 1990; Sagata et al., 1989a; Sagata et al., 1988; Sheets et al., 1995), yet it is not sufficient for this process. In the absence of

protein synthesis, *c-mos* cannot induce pre-MPF activation (Daar et al., 1993; Huang et al., 1995; Murakami and Vande Woude, 1997; Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993; Yew et al., 1992). More recently, a novel p34^{cdc2} binding protein named Ringo (Speedy) has been identified, whose translation seems to be both required and sufficient for GVBD (Ferby et al., 1999; Lenormand et al., 1999).

After GVBD, continued protein synthesis is required for successful completion of MI, the reappearance of MPF at the onset of MII (Gerhart et al., 1984), for suppression of interphase between MI and MII and for the establishment of the metaphase arrest at the end of MII (Furuno et al., 1994; Iwabuchi et al., 2000; Nakajo et al., 2000; Thibier et al., 1997). The key feature of the meiotic cell cycle, the direct succession of two M-phases, without intermediate interphase and DNA replication is thus clearly dependent on newly synthesised proteins, but the identity of these proteins remains to be established. Although there is good evidence that *c-mos* synthesis is required for suppression of DNA replication after exit from MI (Furuno et al., 1994; Kanki and Donoghue, 1991), Roy et al. (Roy et al., 1996) found that active *c-mos* could not prevent entry into interphase during meiosis in the presence of cycloheximide (CHX).

B-type cyclins are newly synthesised in response to progesterone (Frank-Vaillant et al., 1999; Kobayashi et al., 1991), and are able to induce entry into meiosis even in the absence of protein synthesis (Nebreda et al., 1995; Roy et al., 1991). They are good candidates to help account for the protein synthesis requirement during oocyte maturation besides *c-mos* and Ringo. A small pool of newly synthesised active MPF is able to bring about Cdc25 activation and inactivation of Myt1 (Hoffmann et al., 1993; Mueller et al., 1995; Solomon et al., 1990) and hence to establish a positive feedback loop of MPF activation. Furthermore, cyclins are degraded during MI by the APC/C (Peter et al., 2001; Taieb et al., 2001) and accumulate again during entry into MII (Gross et al., 2000; Kobayashi et al., 1991; Ohsumi et al., 1994; Thibier et al., 1997). Most likely, the synthesis and (or) stabilisation of B-type cyclins are involved in suppression of interphase during the transition from MI to MII (Gross et al., 2000; Iwabuchi et al., 2000; Nakajo et al., 2000). Experiments with dominant negative (kinase-dead) p34^{cdc2} support this idea. Whereas injection of kinase-dead p34^{cdc2} into stage VI oocytes blocks GVBD, probably by sequestering newly synthesised cyclins or other p34^{cdc2} binding proteins (Nebreda et al., 1995), injection of the same reagent after GVBD induces DNA replication at the MI/II transition (Furuno et al., 1994).

Conversely, inhibition of cyclin B1 and B2 synthesis using antisense oligonucleotides did not interfere with either entry or progression through meiosis (Minshull et al., 1991). This result suggested that cyclin synthesis is not required for the initial activation of pre-MPF at GVBD (Gautier and Maller, 1991; Kobayashi et al., 1991). These studies also implied that the stockpile of cyclin B bound to p34^{cdc2} in immature oocytes is sufficient to allow progression from MI to MII. If this were true, the transition from MI to MII would be more dependent on cyclin stabilisation rather than new cyclin synthesis after MI, as suggested by the results of Gross et al. (Gross et al., 2000).

These arguments are undermined, however, by the discovery of two new embryonic B-type cyclins in *Xenopus* oocytes as reported in this paper. These novel B-type cyclins are closely related to cyclins B1 and B2 but are sufficiently diverged in

mRNA sequence to avoid ablation by the oligonucleotides used in Minshull et al.'s antisense experiments (Minshull et al., 1991). Here, we reinvestigate the requirements for cyclin synthesis during *Xenopus* oocyte maturation, including the newly discovered B-type cyclins. We show that cyclin synthesis is indeed not required for the activation of MPF, but it is essential and apparently sufficient for progression from MI to MII and the establishment of CSF-arrest.

MATERIALS AND METHODS

Isolation and characterisation of new cyclin clones

Clones for cyclins B3, B4 and B5 were identified in *X. laevis* oocyte cDNA libraries and sequenced by the shotgun method. B-type cyclin clones in *X. tropicalis* were identified in a cDNA library and in oocyte RNA by PCR methods using pairs of specific oligonucleotide primers.

The accession numbers of *X. laevis* cyclins B3, B4 and B5 are AJ304990-AJ304992 respectively. *X. tropicalis* partial cDNA sequences have the accession nos. AJ303451-AJ303454 for cyclins B1, 2, 4 and 5.

Antisense oligonucleotides

The sequences of antisense oligonucleotides were: 'cyc8', **gtacatctctcatatt**; anti-B1/4, gcagctttcttccagcattc; anti B5-2, tccatctgtctgta. Both ends of 'cyc8' (bold letters) were synthesised using 2'-O-methyl ribonucleotide-CE phosphoramidites on a 2'-O-methyl ribonucleotide column. The innermost nine nucleotides were synthesised using deoxynucleotide-CE phosphoramidites. Deprotection occurred in concentrated ammonia at 55°C for 8 hours. Oligonucleotides were purified through NAP-10 columns (Amersham Pharmacia).

Production of specific antisera against *Xenopus* cyclins

N-terminal fragments comprising the first 108 amino acids of cyclins B1, B2 and B4 were subcloned into pET21b and expressed as C-terminally His-tagged proteins in *E. coli* BL21(DE3). They were purified using Ni-agarose columns (Qiagen), and used to immunise rabbits to generate polyclonal antisera by standard methods. For cyclin B5, a 16 residue synthetic peptide corresponding to the N terminus (WAAMRTGQMDNAVVKK) was coupled to KLH and used for immunisation. Antisera against cyclins B1, B2 and B4 gave similar signals on immunoblots in response to titrations of the antigens. All the antisera were affinity purified with the appropriate antigen. Their specificity was tested by immunoblotting in vitro translation reactions using each cyclin mRNA in the reticulocyte lysate. Fig. 1 shows that these reagents were highly specific for their cognate cyclin, reflecting their variable N-terminal sequences (see Fig. 2). Titrations of the antibodies against B1, B2 and B4 against their cognate bacterial antigens showed that the anti-B2 antiserum gave a signal approximately 2.5 time higher than those of anti-B1 and -B4, which gave similar signals to each other. The sensitivity of detection of the anti-B5 antiserum could not be tested in this manner, because it was raised against a peptide, and expression of the cyclin B5 N terminus in bacteria was poor.

In vitro transcription and translation

Plasmids encoding cyclin B1 wild-type or destruction box mutant (RTALGDIGN to ATAAGDIGN) were transcribed using the Ambion 'message machine' kit and translated in reticulocyte lysate using TnT translation kit (Promega) following the manufacturers' protocols.

Translation of endogenous mRNA in *Xenopus* egg extracts

Cytostatic factor (CSF)-arrested *Xenopus* egg extracts were prepared as described by Murray (Murray, 1991). Translation reactions were

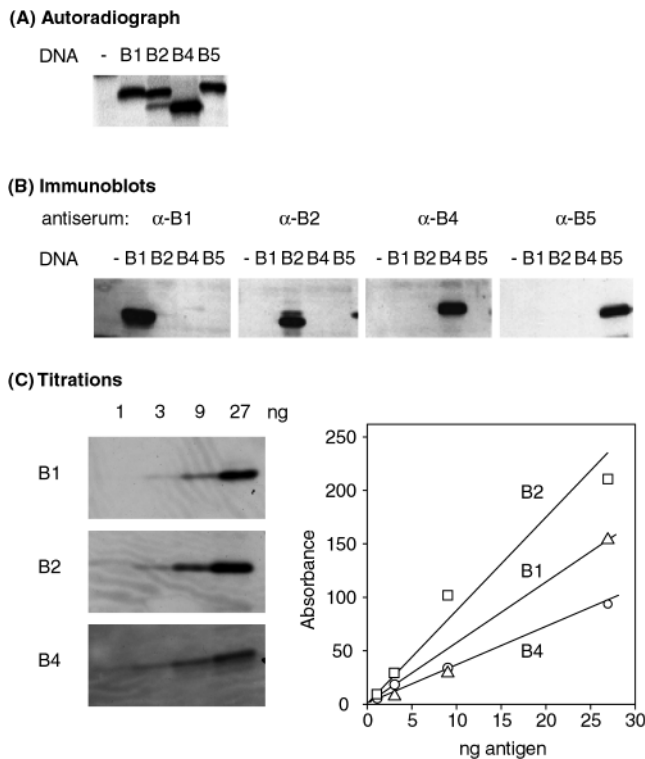


Fig. 1. Specificity of the antisera against *Xenopus* B-type cyclins. (A) Five coupled transcription-translation reactions were set up, programmed with no added DNA (–) or with the indicated cyclin plasmids. (B) Aliquots were analyzed by SDS-PAGE in sets of 5, transferred to nitrocellulose and immunoblotted separately with the indicated affinity-purified antisera. The volume of translation mix was identical in all lanes. (C) The sensitivity of the antisera against cyclins B1, B2 and B4 were tested against dilutions of the bacterial antigens, and quantitated by scanning the immunoblots.

prepared by mixing equal volumes of egg extract and nuclease-treated rabbit reticulocyte lysate (Ambion). The mixture was incubated at 22°C for 30 minutes in the presence of antisense oligonucleotides at 1 μ M. After this preincubation, 1 μ l (1.4 μ Ci) of [³⁵S]Pro-mix™ (>1000 Ci/mmol; Amersham) was added per 20 μ l reaction volume to initiate labelling, and incubated at 22°C for a further 90 minutes. Aliquots of 20 μ l were subjected to immunoprecipitation and analysed by SDS-PAGE and fluorography.

Handling of *Xenopus* oocytes

Ovaries were suspended in OR2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM K-Hepes pH 7.5; Eppig and Dumont, 1976), treated with 1 mg/ml collagenase (Boehringer Mannheim) for 3 hours, washed and resuspended in modified Barth medium (Heasman et al., 1991). Stage VI oocytes were manually sorted and left for no longer than 12 to 24 hours at 18°C. They were induced to mature with 5 μ g/ml progesterone (Sigma) at 22°C. Populations of oocytes that synchronously underwent GVBD were used for cytological and biochemical analysis of the two meiotic divisions, by collecting oocytes that had just formed the white spot (within a window of 15 minutes) from a pool of about 1000 oocytes.

Microinjection and cycloheximide treatment of oocytes

Oligonucleotides were dissolved in water and 50 nl of the solution was injected into oocytes 30 minutes after adding progesterone. 'cyc8' was used at a concentration of 1.5 mg/ml; anti-B5-2 at 0.5 mg/ml and anti-B1/B4 at 2 mg/ml; control oligonucleotides at 2 mg/ml. About 600

oocytes were injected to obtain pools of 150–200 oocytes that underwent GVBD synchronously. Cyclin B1 mRNAs (50 ng) were dissolved in water. Bacterially expressed sea urchin (*Arbacia*) cyclin B Δ 90 (from J. Gannon) was dissolved in PBS and injected at the indicated concentrations. To measure the stability of radiolabelled cyclin B1, 50 nl of TnT translation mix (Promega) programmed with cyclin B1 plasmid DNA in the presence of ³⁵S-labelled amino acids was injected into each of 20 oocytes. Samples of 3 oocytes were harvested at 20 minutes intervals. CSF-arrested oocytes were incubated in a Ca²⁺-limited medium (120 mM NaCl, 7.5 mM KCl, 22.5 mM Hepes, 400 μ M EDTA, 500 μ M MgSO₄, 150 μ M CaCl₂) before injection in order to avoid activation. Where indicated, 100 μ g/ml cycloheximide (final concentration) was added to the medium.

RNA extraction, northern blotting and RNase protection assays

RNA was extracted from samples of 20 oocytes following the proteinase K/phenol method described in section 7.16 of Sambrook et al. (Sambrook et al., 1989). Total RNA (10 μ g) was separated on a 1% agarose gel in 20 mM Mops; 5 mM sodium acetate; 1 mM EDTA and 2% formaldehyde, transferred to Hybond-N+, and UV cross linked. We used PCR-fragments of the last 400 nucleotides of the B-type cyclin N-termini as probes. RNase protection assays were performed using 5 μ g of total RNA. Antisense RNA probes of N-terminal fragments of different cyclins were transcribed from pGEM vectors using T7 RNA polymerase and [α -³²P]CTP (Amersham). The RNase protection assays used Ambion RPAII Kit following the manufacturer's protocol. The protected fragments were analysed by electrophoresis on a 5% acrylamide/8 M urea gel and autoradiography.

Immunoblotting

Cell-free extracts of *Xenopus* oocytes and embryos were prepared by crushing 10 oocytes in 100 μ l EB buffer (Gerhart et al., 1984), followed by centrifugation for 10 minutes in an Eppendorf centrifuge. The clear portion of the supernatant was precipitated in 3 vol. acetone and dissolved in SDS sample buffer. An aliquot corresponding to one oocyte was analysed by SDS-PAGE. The lanes were electrophoretically transferred to nitrocellulose and immunoblotted with the indicated antisera. Equal loading of different lanes was confirmed by staining the blots with 0.3% Ponceau in 3% TCA. The bound antibodies were detected with the appropriate HRP-coupled secondary antibody and the Amersham-Pharmacia ECL kit.

Histone kinase assays

Five oocytes were homogenised in EB buffer (10 μ l/oocyte), centrifuged briefly, and 5 μ l of the clear supernatant was assayed for histone H1 kinase activity as described previously (Furuno et al., 1994).

Confocal immunofluorescence microscopy of meiotic spindles in *Xenopus* oocytes

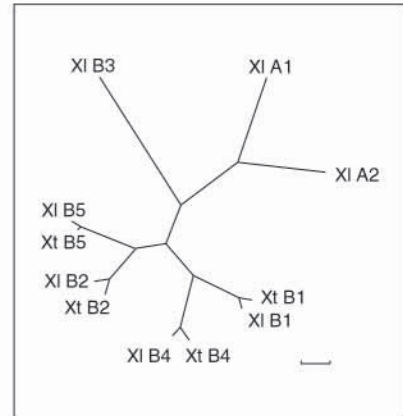
Xenopus oocytes were fixed and stained following the protocol described by Gard (1993). Primary antibodies were 10 μ g/ml anti-phospho-histone H3 rabbit polyclonal (Upstate Biotechnology) and 10 μ g/ml Tat-1 anti-tubulin mouse monoclonal (Woods et al., 1989). Secondary antibodies were used at a dilution of 1000-fold (Alexa 488 and Alexa 546, Molecular Probes). Images were obtained with a Zeiss laser scanning microscope.

RESULTS

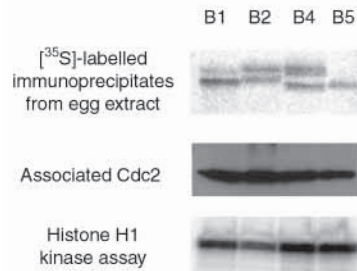
Expression patterns of B-type cyclins during oocyte maturation and early embryonic cell cycles

Xenopus oocytes and embryos contain mRNAs for two new B-type cyclins, B4 and B5; cyclin B4 is very similar to cyclin B1, and cyclin B5 strongly resembles cyclin B2 (see Fig. 2). These

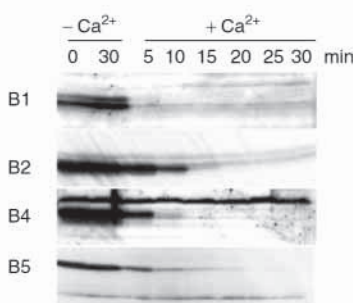
(A) Alignment of cyclins B1, B2, B4 and B5

(B) *Xenopus laevis* & *X. tropicalis* mitotic cyclins

(C) Abundance and kinase activity



(D) Destruction assay



(E) Early development

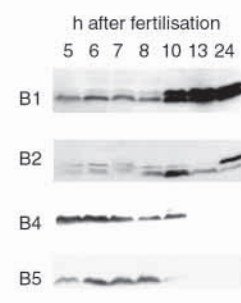


Fig. 2. Characterisation of *X. laevis* B-type cyclins. (A) Sequence alignment of cyclins B1, B2, B4 and B5; bars show the conservation, the destruction boxes are marked by a rectangle, and the nuclear export sequences indicated in red. (B) The dendrogram shows the family of mitotic cyclins in *X. laevis* and *X. tropicalis*. The scale bar represents 10 amino acid substitutions in the compared length of 180 residues. (C) Egg extracts were labelled with ^{35}S -labelled amino acids and immunoprecipitated with the indicated antisera. These immunoprecipitates were analysed by autoradiography and assayed for binding of Cdc2 and histone H1 kinase activity. (D) Immunoblot showing the destruction of each B-type cyclin after addition of CaCl_2 to an egg extract. (E) Cyclin levels during early embryogenesis determined by immunoblotting with the indicated antibodies. MBT occurred at about 10 hours in this experiment.

additional cDNAs were originally discovered by chance, but are well represented in EST libraries. Fig. 2A shows an alignment of these four B-type cyclins, which have only 10 identities in the N-terminal 100 residues compared to 128 identities in the last 300 residues. We looked for these genes in *X. tropicalis* to check whether they had arisen by the genome duplication that is thought to have occurred in *X. laevis* (*X. tropicalis* has 3.6 pg DNA/cell and 20 chromosomes, compared to *X. laevis*'s 6.4 pg DNA/cell and 36 chromosomes; Tinsley and Kobel, 1996). We searched for cyclin B4 and B5 mRNAs directly by RT-PCR and also screened a cDNA library from *X. tropicalis*. The same complement of B-type cyclin genes were present in *X. tropicalis* as in *X. laevis*, as displayed in the dendrogram in Fig. 2B, which is based on a highly conserved protein sequence of 180 residues starting at the MRAIL motif. The N-terminal 100 residues of *X. laevis* cyclins B1, 2, and 4 were expressed in bacteria and used to raise polyclonal antibodies which were specific for each of the full-length polypeptides, and which had comparable sensitivities as judged by their ability to detect the bacterial antigens (Fig. 1). The antibody against cyclin B5 was raised against an N-terminal peptide, and although its sensitivity could not be assessed in the same way, it recognised cyclin B5

produced by cell-free translation with a similar intensity on immunoblots as the other three antisera recognised their cognate antigens. Fig. 2C shows that all four cyclins were present in *Xenopus* egg extracts, and were bound to CDK1, as judged by co-immunoprecipitation. No binding to CDK2 could be detected (data not shown). The immunoprecipitates from egg extracts displayed similar levels of histone H1 kinase activity (Fig. 2C). Oocyte maturation was triggered by injection of any one of the synthetic B-type cyclin mRNAs (data not shown). All these B-type cyclins have a readily identifiable destruction box (RXXLXXIXN), and although the B5 destruction box is somewhat unusual (RPPLEEISN), cyclins B1, B2, B4 and B5 were all rapidly degraded after triggering anaphase by addition of CaCl_2 to a frog egg extract (Fig. 2D). The immunoblot in figure 2E shows that cyclins B1, B2, B4 and B5 are expressed during the cleavage cell cycles, but cyclins B4 and B5 disappeared after the mid blastula transition, which occurred at about 10 hours in this experiment. Neither protein nor mRNAs of these two cyclins were detectable in embryonic fibroblasts or tissues of adult frogs, except for ovaries and testis (data not shown). Cyclins B4 and B5 thus appear to represent purely embryonic B-type cyclins.

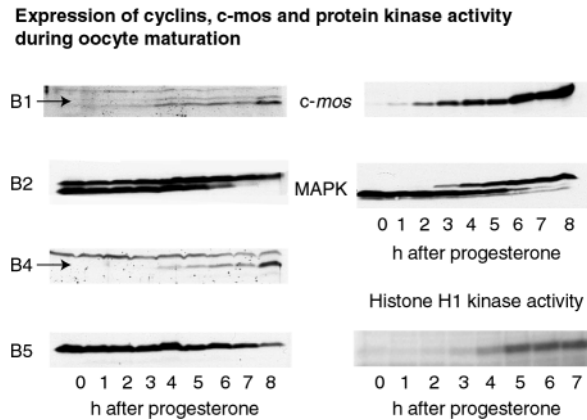


Fig. 3. Oocyte maturation time course. White spot formation started 3.25 hours after addition of progesterone, and half the oocytes had undergone GVBD by 4.5 hours. All the panels are immunoblots using the indicated antisera, except for autoradiograph of the histone H1 kinase assay.

Frog oocytes also contain a mRNA that encodes cyclin B3, a more distant relative of the other B-type cyclins with closer similarity to the cyclin A family (see Fig. 2B). Cyclin B3 was first identified in chickens, nematodes and flies (Gallant and Nigg, 1994; Jacobs et al., 1998; Kreutzer et al., 1995; Sigrist et al., 1995), and there is a human version of the gene close to the centromere of chromosome 5 as well as several human ESTs. We have been unable to detect significant levels of cyclin B3 protein during frog oocyte maturation, however, or during early embryonic cell cycles (data not shown). Reconstruction experiments with translated synthetic mRNA showed that the limits of detection were below 1 ng/oocyte. For this reason, we consider it highly unlikely that cyclin B3 is involved in *Xenopus* oocyte maturation.

Stage VI oocytes contain cyclins B2 and B5, but very low levels of B1 and B4 (Fig. 3). Cyclin B2, but not cyclin B5 (which has the sequence EPPPIP in place of VPSPVP), undergoes a phosphorylation-dependent shift in electrophoretic mobility after progesterone treatment (Gautier and Maller, 1991; Kobayashi et al., 1991), and the levels of both these cyclins drop at later stages of oocyte maturation. Cyclins B1 and B4 started to accumulate around the time of MPF activation, 4 hours after progesterone addition (at which time 30% of the oocytes had a white spot in this experiment). The accumulation of *c-mos* and activation of MAP kinase (correlated with an electrophoretic mobility shift; Haccard et al., 1993) preceded these events by approximately 1 hour. A second more dramatic increase in levels of cyclins B1 and B4 is reproducibly seen late in meiosis, between 7 and 8 hours after progesterone treatment.

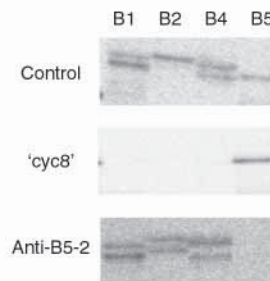
Antisense ablation of B-type cyclin mRNAs

Minshull et al. (Minshull et al., 1991) studied the cyclin synthesis requirements during oocyte maturation using separate antisense oligonucleotides directed against cyclins B1, B2 and A1. In another study, Weeks et al. (Weeks et al., 1991) reported the successful ablation of all B-type cyclin mRNA in fertilized *Xenopus* eggs, using an antisense oligonucleotide ('cyc8') that targeted a conserved region in the cyclin-box. They found, very surprisingly, that cell division continued

(A) Antisense alignment

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'cyc8'  AATATGAAGAGATGTAC
B1      AATATGAAGAGATGTAC
B2      AATATGAAGAGATGTAC
B4      AATATGAAGAAATCTAT
B5      AGTATGAAGAAATGTAT
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(B) Immunoprecipitates



(C) Northern blots

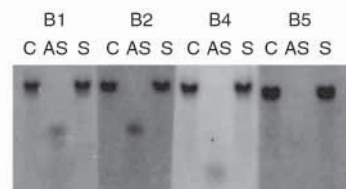


Fig. 4. Characterisation of anti-B-type cyclin antisense oligonucleotides. (A) Sequence alignment of oligonucleotide 'cyc8' with different B-type cyclins. Mismatches are shown in red. (B) Effects of oligonucleotides 'cyc8' and anti B5-2 on cyclin synthesis monitored by the indicated immunoprecipitates of [³⁵S]methionine-labelled egg extracts. (C) Northern blot analysis of mRNA isolated from oocytes which were, uninjected (C), injected with a mixture of 75 ng 'cyc8' and 25 ng of anti B5-2 (AS) or injected with the same oligonucleotides in the sense orientation (S).

more or less normally despite the supposed loss of all CDK1 activators. Obviously, the interpretation of both these studies is compromised by the discovery of unsuspected new B-type cyclins. Neither B4 nor B5 mRNAs are targeted by the oligonucleotides used by Minshull et al. (Minshull et al., 1991) (data not shown). The mRNAs for cyclins B4 and B5 have 3 mismatches in a sequence alignment with the oligonucleotide used by Weeks et al. (Fig. 4A). We tested the ability of the 'cyc8' oligonucleotide to target the newly discovered B-type cyclins by measuring cyclin synthesis in frog egg extracts after 30 minutes preincubation with 'cyc8'. Fig. 4B shows that even in the presence of 1 μM 'cyc8', an [³⁵S]methionine-labelled band corresponding to cyclin B5 could be detected in the immunoprecipitates. This probably accounts for Weeks et al.'s failure to inhibit cell cycle progression in embryos, although we have not checked this point.

In order to inhibit all B-type cyclin synthesis, we selected a potent anti-B5 oligonucleotide using scanning arrays (Milner et al., 1997; Sohail et al., 2001; Southern et al., 1994). Oligonucleotide anti-B5-2 specifically and efficiently inhibited cyclin B5 synthesis in egg extracts (Fig. 4B). A combination of 'cyc8' and anti-B5-2 could therefore be used to ablate the mRNAs encoding all four B-type cyclins in *Xenopus* oocytes. We used a nuclease-resistant form of 'cyc8', but the other oligonucleotides were unmodified (see Methods). Fig. 4C

shows that injection of a mixture of 75 ng 'cyc8' and 25 ng anti-B5-2 efficiently cleaved more than 90% of the B-type cyclin mRNA as judged by northern blotting. Using this strategy we were thus able to inhibit the synthesis of all known B-type cyclins in *Xenopus* oocytes, which we confirmed by immunoblotting extracts from progesterone-treated oocytes (see Fig. 6 below). When tested in cell-free egg extracts, these oligonucleotides always caused some degree of non-specific inhibition of protein synthesis, which inevitably affected some (unknown) mRNAs more than others (Sohail et al., 2001). This should be borne in mind in interpreting the following results.

Oocytes can initiate maturation without new cyclin synthesis

We investigated the maturation of *Xenopus* oocytes in which the mRNAs of cyclins B1, B2, B4 and B5 had been ablated with the mixture of 'cyc8' and anti-B5-2 oligonucleotides. These oocytes underwent GVBD in response to progesterone without any delay compared to uninjected oocytes or oocytes injected with control oligonucleotides. Approximately 1.5 hours after the appearance of the white spot, however, the antisense-injected oocytes started to look different from the control oocytes. They took on a mottled appearance, and time lapse videos revealed white ring-shaped pigment-free areas that migrated progressively across the surface of the oocytes (Fig. 5A, 90-180 minute time points). Four to five hours after GVBD, the oocytes injected with antisense oligonucleotides had lost all pigmentation and looked as though they were dying (data not shown). Oocytes injected with control oligonucleotides matured without such ill effects. Above a total concentration of 150 ng/oocyte, however, any oligonucleotide or combination of oligonucleotides delayed GVBD. Moreover, oocytes treated with high concentrations of control oligonucleotides appeared to undergo activation 3-4 hours after white spot formation and eventually decayed (data not shown).

We analysed the meiotic spindles of the antisense-treated oocytes following the methods introduced by Dave Gard (Gard, 1992; Gard et al., 1995). Glutaraldehyde-fixed and laterally bisected oocytes were stained with Tat1 anti-tubulin (green) and anti-phospho-histone H3 (red) antibodies (see Methods). Shortly (5-10 minutes) after nuclear envelope breakdown the condensed chromosomes are embedded in a transient microtubule organising centre (data not shown). This microtubule array moves towards the animal pole of the oocytes and forms a small bipolar spindle (Fig. 5B; 30 and 60 minutes). This spindle is orientated parallel to the oocyte surface and subsequently rotates and elongates to form the MI metaphase spindle (Fig. 5B; 90 minutes). No difference in MI spindle morphology could be detected in oocytes injected with the combined anti-B-type cyclin oligonucleotides. Thus, as previously concluded (Minshull et al., 1991), new B-type cyclin synthesis is not required for the initiation of oocyte maturation or for assembly of the first meiotic spindle.

Progression from MI to MII requires new cyclin synthesis

After formation of the first polar body, a new microtubule array forms underneath the surface of the oocyte. This array quickly transforms into the second meiotic spindle that rotates again into the final axial position, where it arrests in metaphase II (Fig. 5C). In oocytes injected with anti-B-type cyclin

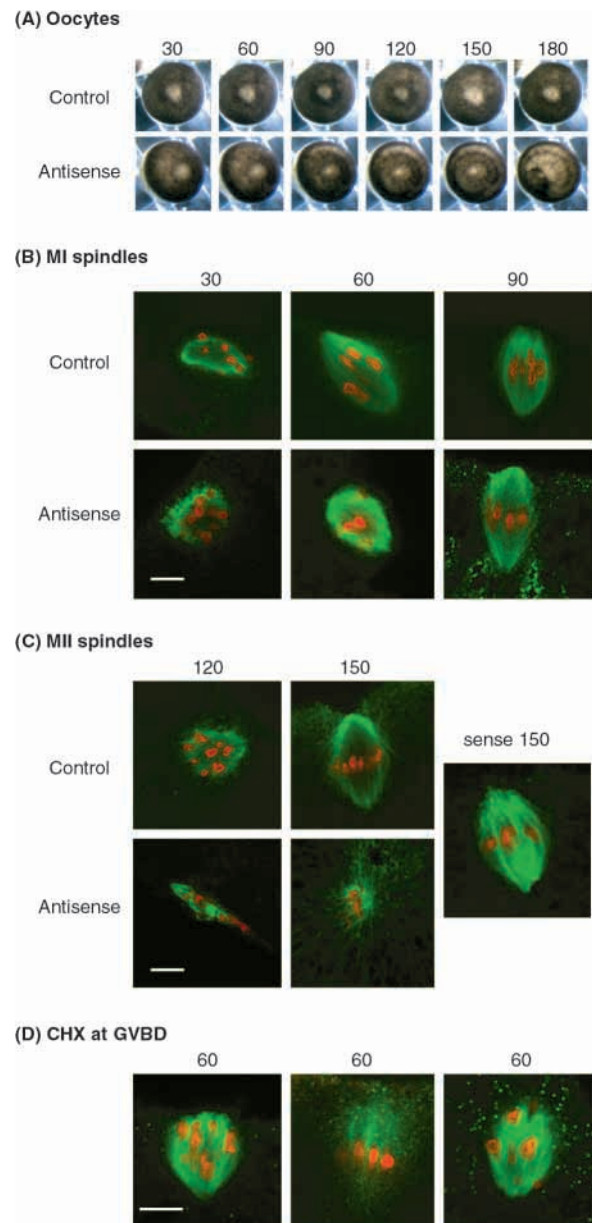


Fig. 5. Cytological characterisation of oocyte maturation (minutes after white spot formation are shown above each panel). (A) Images from a time lapse video of maturing oocytes. An uninjected 'Control' oocyte is compared with an 'Antisense' injected oocyte containing 75 ng 'cyc8' and 25 ng of anti B5-2 antisense oligonucleotides. (B) Spindles and chromosomes during MI. Microtubules are green, phosphorylated histone H3 red. (C) MII meiotic spindles of control, antisense- and sense-injected oocytes. (D) Meiotic spindles in CHX-treated oocytes. Scale bars, 10 μm.

oligonucleotides, the second meiotic spindle failed to assemble. The chromosomes eventually moved towards the centre of the cell and became embedded in a dense microtubule network (Fig. 5C; antisense), where they remained partly condensed and continued to give a detectable signal with the anti-phospho-histone H3 antibody until approximately 2.5 hours after GVBD. Oocytes injected with the same concentration of random or sense oligonucleotides arrested in MII with a healthy looking metaphase II spindle (Fig. 5C; sense).

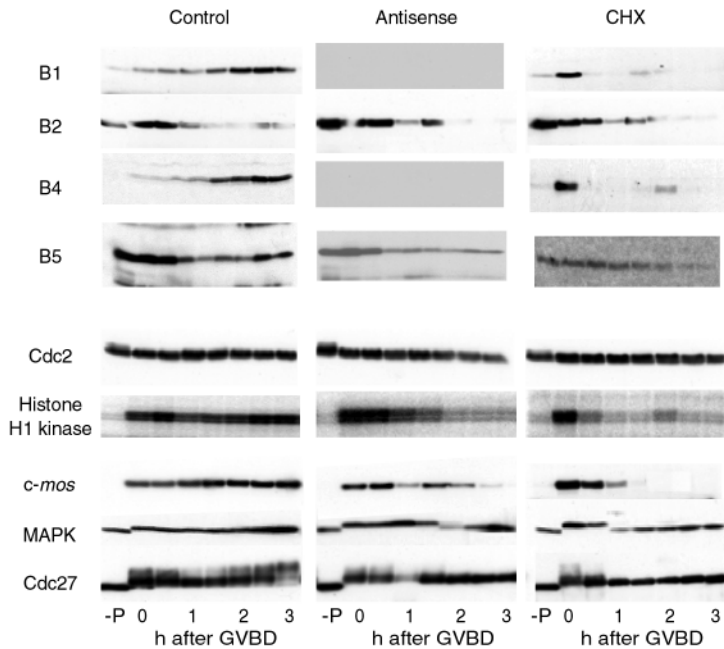
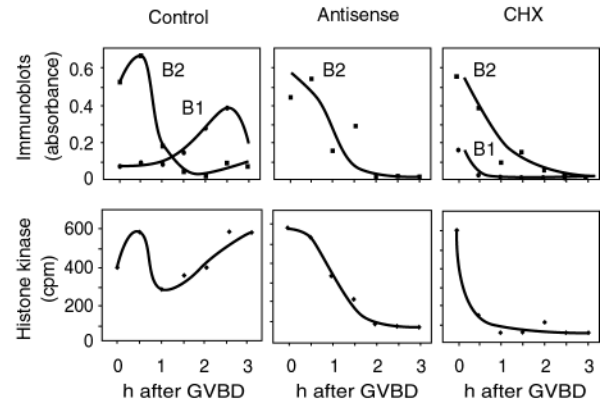
(A) Immunoblots and kinase assays**(B) Quantitation**

Fig. 6. Analysis of the MI/MII transition in progesterone-treated oocytes (Control), oocytes that were injected with 75 ng 'cyc8' and 25 ng of anti B5-2 oligonucleotides (Antisense) and oocytes treated with 100 µg/ml CHX 10 minutes after GVBD (CHX). (A) Immunoblots with indicated antibodies and histone H1 kinase assay of oocytes at the indicated times after GVBD; -P denotes stage VI oocytes. (B) Quantitation of the data for cyclins B1 and B2 and histone H1 kinase activity shown in A.

Inhibition of protein synthesis with CHX after GVBD interferes with completion of MI

In contrast to oocytes injected with antisense oligonucleotides, oocytes exposed to CHX shortly after GVBD did not complete MI. As previously reported by several groups, CHX applied at the time of GVBD caused rapid entry into interphase and initiation of DNA replication at the end of MI (Furuno et al., 1994; Huchon et al., 1993; Wasserman and Masui, 1975). Cytological analysis revealed that under these conditions, a compact spindle formed without properly aligned chromosomes. This structure persisted for approximately 1 hour after GVBD (Fig. 5D and data not shown). After this, the signal from the anti-phospho-histone H3 antibody rapidly disappeared and by 90 minutes the microtubule arrays in these oocytes were undetectable (data not shown). These data show that protein synthesis is required for proper completion of MI and entry into MII, whereas the antisense data imply that new cyclin synthesis is only necessary to enter and maintain MII.

Comparisons between CHX and antisense-treated oocytes

To further investigate this point, the levels of B-type cyclins and histone H1 kinase activity were measured in a set of progesterone-treated oocytes that were either not injected (control), or injected with 'cyc8' and anti-B5-2 oligonucleotides, or exposed to CHX just after GVBD (Fig. 6). In this experiment, a synchronously maturing cohort of oocytes was selected by hand. We also analysed the expression of *c-mos* and the phosphorylation status of MAP kinase and the APC/C subunit Cdc27, as deduced from their electrophoretic mobility.

During MI, the pool of B-type cyclins consisted mainly of cyclins B2 and B5. Histone kinase activity dropped partially and transiently after entry into meiosis, reflecting the overall cyclin levels: in this experiment, cyclin B2/B5 levels declined somewhat before cyclins B1/B4 accumulated. In the antisense-

injected oocytes, histone H1 kinase activity declined roughly in parallel with the loss of B2/B5 after MI, and never recovered. By contrast, addition of CHX led to a much more rapid decay of histone kinase activity, even though cyclins B2 and B5 disappeared with similar kinetics in the CHX and antisense-treated oocytes. The reason for this difference is not clear. The expression patterns of *c-mos* and the activity of MAP kinase followed the pattern of histone kinase activity. In the absence of cyclin synthesis, MAP kinase was inactivated during MII and *c-mos* was degraded 120-150 minutes after GVBD. In CHX-treated oocytes, loss of MAP kinase and the disappearance of *c-mos* occurred much earlier, 30-60 minutes after GVBD, reflecting the earlier fall in histone kinase activity. The levels of cyclins B1 and B2 and the total histone H1 kinase activity are plotted in Fig. 6B.

Fig. 6 also shows the phosphorylation-dependent mobility shift of Cdc27 during oocyte maturation as described previously (Thibier et al., 1997; Gross et al., 2000), which appears to correlate with the activity of the APC/C (Vorlaufer and Peters, 1998). The phosphorylation status of Cdc27 partially reflected the changes in histone kinase activity during oocyte maturation. Cdc27 became phosphorylated early in MI and remained mainly in an intermediate state of electrophoretic mobility until the end of MII, regardless of the histone H1 kinase activity. A hyperphosphorylated band was present only when histone kinase activity was at a maximum. About 3 hours after GVBD in the control oocytes, all the Cdc27 was converted into this hyperphosphorylated species, which seems to be correlated with cyclin stabilisation and CSF activity (Vorlaufer and Peters, 1998). In oocytes injected with antisense oligonucleotides or treated with CHX, the hyperphosphorylated form of Cdc27 was never as obvious, and the upper smear had completely disappeared by 90 minutes in both cases.

Fig. 6 suggests that cyclins B2 and B5 do not accumulate to their previous levels during MII, but this turned out to be a variable feature of different batches of oocytes. Cyclin B2 and

B5 were synthesised strongly during MII in some experiments, while in other cases (as in Fig. 6) they were not. We found that specific ablation of cyclins B1 and B4 was sufficient to inhibit MII in those batches of oocytes in which cyclins B2 and B5 did not accumulate. In the oocytes in which high levels of the B2 and B5 cyclins were synthesized after MI, antisense ablation of cyclin B1 and B4 had no effect on progression through meiosis (data not shown).

Cyclin B $\Delta 90$ reverts the effects of cyclin ablation

The simplest interpretation of these results is that B-type cyclin synthesis is essential for progression through the meiotic cell cycle after MI metaphase. This conclusion relies on treatment of oocytes with antisense oligonucleotides, which can cause a variety of non-specific side effects (Minshull and Hunt, 1992). We therefore tested if the ill effects of the anti-cyclin oligonucleotides could be rescued by introduction of a B-type cyclin protein at the appropriate time. One and a half hours after GVBD (at the end of MI) recombinant sea urchin cyclin B $\Delta 90$ was injected into oocytes whose mRNA for cyclins B1 and B4 had been ablated by antisense oligonucleotides. Fig. 7A shows that these antisense oligonucleotides caused the usual disturbance of the pigmented oocyte surface in this batch of oocytes, and that the mRNA for cyclins B1 and B4 was efficiently ablated. Histone kinase activity fell to low levels in these antisense-treated oocytes (Fig. 7C). The pigment disturbances, the loss of histone kinase and of *c-mos* were all prevented by injection of 25 ng cyclin B $\Delta 90$. Fig. 7D shows that oocytes injected with cyclin B $\Delta 90$ contained somewhat disorganised spindles with condensed but scattered chromosomes. In the antisense-injected oocytes that did not receive cyclin B $\Delta 90$, microtubule arrays like those shown in Fig. 5C were observed at the equivalent time (3 hours after GVBD). The peculiar morphology of the 'rescued' spindles occurred even without any DNA injection when the *Arbacia* cyclin B $\Delta 90$ was introduced (Fig. 7D), as has previously been reported in egg extracts (Glotzer et al., 1991; Luca et al., 1991; Minshull et al., 1994; Murray et al., 1989).

Cyclin synthesis is sufficient to allow progression through MII

B-type cyclin synthesis is clearly essential during MII, and we wanted to test if it was the only new protein required to be synthesised at this stage of oocyte maturation. Other newly synthesized proteins might be required for successful completion of meiosis, and such proteins could explain why CSF is established only at metaphase II, and not at metaphase I. These unknown proteins could be responsible for chromosome condensation and histone H3 phosphorylation, which persist for some time in the absence of cyclin synthesis, but are only briefly maintained when all protein synthesis is inhibited by CHX. Fig. 8A shows the experimental approach designed to address these questions. We injected mRNA encoding an indestructible form of *Xenopus* cyclin B1 into oocytes that had just completed MI. Translation was allowed to continue for about 30 minutes, and then CHX was added to inhibit further protein synthesis.

In the uninjected control oocytes, it took about 30 minutes for CHX to cause similar changes in the pigment pattern on the oocyte surface as were observed in oocytes injected with antisense oligonucleotides. Large pigment-free rings appeared

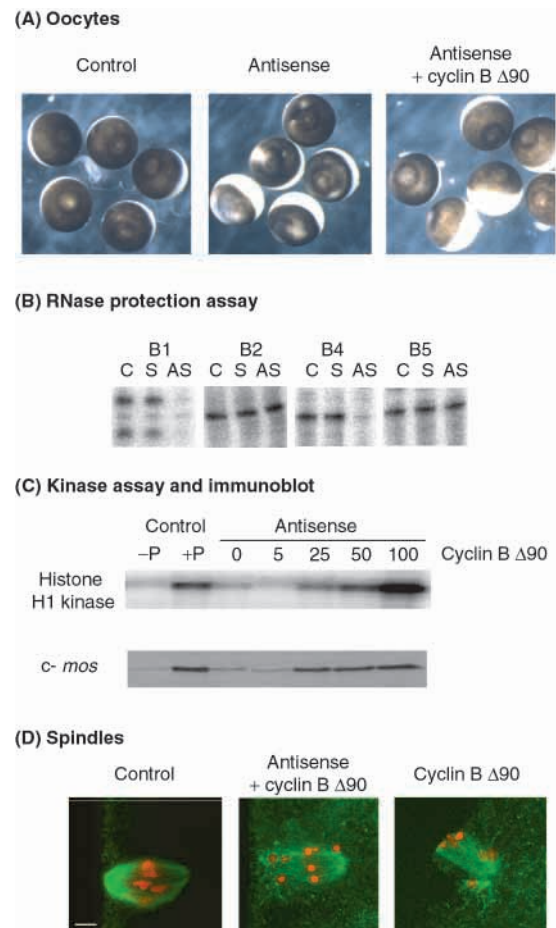


Fig. 7. Effects of *Arbacia* cyclin B $\Delta 90$ on oocytes injected with 100 ng of anti B1/B4 antisense oligonucleotide. (A) Images of progesterone-treated oocytes 3 hours after GVBD (6 hours after addition of progesterone). Control, uninjected oocytes; Antisense, 100 ng anti B1/B4 antisense oligonucleotide injected 15 minutes after addition of progesterone; Antisense + cyclin B $\Delta 90$, as before with 100 ng cyclin B $\Delta 90$ injected 1.5 hours after GVBD. (B) RNase protection assay of mRNA cleavage in these oocytes; C, uninjected controls; S, sense; AS, antisense injected. (C) Histone H1 kinase assays and *c-mos* immunoblots of the oocytes. Cyclin B $\Delta 90$ levels were as indicated above each lane. (D) Meiotic spindles; Control, 6 hours after progesterone addition in an uninjected oocyte; Antisense + cyclin B $\Delta 90$, oocytes injected with 100 ng anti B1/B4 antisense oligonucleotide 15 minutes after progesterone addition and 100 ng cyclin B $\Delta 90$ 1.5 hours after GVBD, sampled 1.5 hours later; Cyclin B $\Delta 90$, as before without antisense. Scale bar, 10 μ m.

at the animal pole that progressed towards the oocyte equator (Fig. 8B). Biochemical analysis of these oocytes showed that B-type cyclins and *c-mos* disappeared when protein synthesis was inhibited. Histone H1 kinase and MAP kinase were inactivated, and Cdc27 was not hyperphosphorylated. Prior injection of the indestructible cyclin B1 mRNA prevented all these effects, except for the destruction of the endogenous B-type cyclins. Fig. 8C shows that the mutant B1 protein accumulated to a very similar level to the endogenous cyclin B1 when its mRNA was injected, whereas endogenous cyclin B4 was almost completely degraded. In controls using the mRNA for wild type cyclin B1, the effects of CHX were not

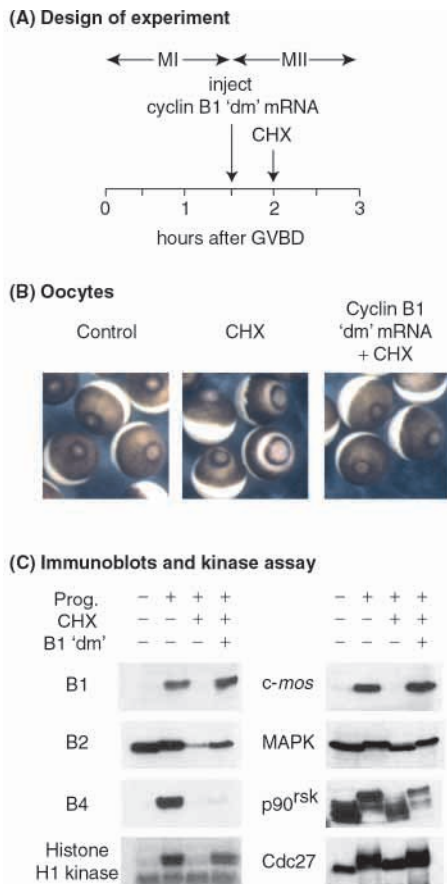


Fig. 8. Effects of indestructible cyclin B1 mRNA and CHX. (A) Experimental design; dm indicates mRNA encoding indestructible cyclin B1 (RXXL to AXXA mutant). (B) Images of MII-arrested oocytes, CHX-treated oocytes and oocytes that were injected with mRNA encoding indestructible cyclin B1 30 minutes before addition of CHX. (C) Immunoblots and histone H1 kinase assays of oocytes subjected to the indicated treatments.

reversed, and no cyclin B1 protein was detectable 1 hour after addition of CHX (data not shown). These observations indicate that the increased accumulation of cyclins B1 and B4 after MI is a consequence of increased synthesis, rather than a reduction in the rate of proteolysis. Clearly, the B-type cyclins are subject to rapid turnover during MII, until the activity of the APC/C has been inhibited by CSF about 3 hours after GVBD (data not shown).

We next tested whether the presence of indestructible cyclin B was sufficient to allow activation of CSF, in the absence of other protein synthesis. We injected [35 S]methionine-labelled cyclin B1 that had been synthesised in reticulocyte lysate into oocytes and followed its degradation during a period of 1 hour (Fig. 9A). As expected from the previous experiments of Thibier et al. (Thibier et al., 1997) and Peter et al. (Peter et al., 2001), the labelled protein was unstable during M I and M II (data not shown). Fig. 9A shows that cyclin B1 was stable in normal MII arrested oocytes. In oocytes treated with CHX, however, the labelled cyclin B1 was unstable at the equivalent time after GVBD, confirming that inhibition of protein synthesis prevented the establishment of CSF arrest. Injection of mRNA encoding stable cyclin B1 30 minutes before adding

(A) Cyclin stability assays

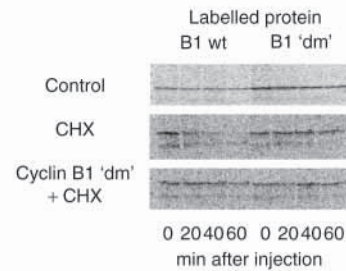


Fig. 9. Cyclin stability and spindle appearance in oocytes treated with CHX 30 minutes after injection of indestructible cyclin B1 mRNA. (A) Destruction assays in oocytes using 35 S-labelled cyclin B1, either wild type (WT) or destruction-box mutant (dm) injected into the indicated oocytes 3 hours after GVBD. Control, oocytes with no other treatment; CHX, cycloheximide added 2 hours after GVBD; Cyclin B1 dm + CHX as before, with indestructible cyclin B1 mRNA injected 1.5 hours after GVBD. (B) Control MII spindles in a progesterone-treated oocyte and images of spindles from 3 oocytes treated with CHX after translation of indestructible cyclin B1. Scale bar, 10 μ m.

CHX allowed the establishment of the CSF arrest, as indicated by the stability of the labelled cyclin B1 (Fig. 9A). We interpret these results to suggest that the presence of indestructible cyclin B1 was sufficient to support the formation of metaphase-arrested meiotic spindles, even in the absence of other protein synthesis (Fig. 9B). The spindles in these oocytes looked very similar to those in control oocytes, but were consistently slightly enlarged (with a length of approximately 60 μ m compared to 45–50 μ m in control oocytes; Fig. 9B). No spindle-like structures or condensed chromosomes were observed in oocytes after CHX treatment (data not shown), and Huchon et al. (Huchon et al., 1993) previously showed that such oocytes rapidly form nuclei.

Thus, translation of the mRNA for a cyclin B1 destruction box mutant or injection of cyclin B Δ 90 protein reverted all the observed effects of cycloheximide. We conclude that by 30 minutes after exit from MI, no other proteins apart from cyclins need to be newly synthesised in order to assemble the MII spindle and to establish CSF-mediated inhibition of the APC.

DISCUSSION

The B-type cyclin family in *Xenopus*

We report the existence of two new B-type cyclins, B4 and B5, in *Xenopus* oocytes, eggs and embryos, which bind to CDK1 to form an active protein kinase, and are degraded in an APC/C-dependent manner. These new B-type cyclins are

expressed from a stockpile of maternal mRNA until the mid blastula transition (MBT) and are not found in adult cells apart from the germline. The extra cyclins do not simply arise as a consequence of the pseudo-tetraploidy in *Xenopus laevis*, because they are also found in its diploid relative *Xenopus tropicalis*. As far as is known, amphibians are the only organisms to show such a large number of B-type cyclins, and we suspect that their existence is an adaptation to the large size of the frog egg. Sea urchin and clam eggs, for example, have a volume 1000 times smaller, and express only a single cyclin B, and similarly only one B-type cyclin is found in fish (Hirai et al., 1992). We believe that we have now identified all the B-type cyclins in *Xenopus*, because the EST libraries clearly contain cyclins B1-B5, but no others. Yet we should point out that the N-terminal antibodies against cyclins B1 and B4 recognised two sizes of polypeptide, which do not appear to be alternative phosphorylated forms (data not shown). Although the origin of these extra bands is unknown, they are ablated by the antisense oligonucleotides.

MPF activation at the G₂/M transition does not require new synthesis of B-type cyclins

Complete ablation of all mRNAs for B-type cyclins did not delay the timing or extent of GVBD, suggesting that even though B-type cyclin synthesis is able to trigger MPF activation, it is dispensable for this process. We conclude from these results that the pool of pre-MPF in stage VI oocytes is sufficient to allow entry into M-phase and that cyclin B synthesis is not required for the amplification loop that leads to MPF activation. Previously, it was shown that cyclin A1 was not required for oocyte maturation (Minshull et al., 1991), and the levels of cyclin A are about 1% of the level of cyclin B1 at the time of GVBD (Kobayashi et al., 1991). We therefore consider it unlikely that any new cyclin synthesis is required for the G₂-M transition in *Xenopus* oocytes, whereas the recently discovered Ringo/Speedy protein is an excellent candidate, alongside *c-mos*, to account for the protein synthesis requirement for progesterone-induced oocyte maturation (Ferby et al., 1999; Lenormand et al., 1999; Sagata et al., 1989a).

A similar situation is found in the somatic cell cycle, where a pool of inactive cyclin B/p34^{cdc2} accumulates until the end of G₂, and new protein synthesis is required for the final activation of MPF and entry into M-phase (Altman et al., 1970; Daga and Jimenez, 1999; Nishijima et al., 1997; Wagenaar, 1983). It is thus conceivable, perhaps even likely, that the newly synthesised proteins in somatic cells are not B-type cyclins.

MPF activity during MI depends on the synthesis of unidentified proteins

The maternal stockpile of cyclin B2 and B5 protein is sufficient to support what appears to be normal assembly of the first meiotic spindle without any new cyclin synthesis. The stockpile of these maternal cyclins is then degraded slowly and with similar kinetics after antisense treatment or CHX treatment. By contrast, histone kinase activity falls much more rapidly when CHX is added to oocytes shortly after GVBD than in antisense-treated oocytes. Furthermore, complete inhibition of protein synthesis just after formation of the white spot interferes strongly with spindle assembly. Comparison

between the specific inhibition of new cyclin synthesis as opposed to complete inhibition of translation thus reveals the existence of unidentified protein(s) that need to be synthesized in order to keep MPF active after GVBD. The same analysis reveals that histone H3 phosphorylation is not directly linked to MPF activity in *Xenopus* oocytes. We could detect phosphorylated histone H3 in condensed chromosomes long after MPF had become inactive in antisense injected oocytes (Fig. 5C). In the presence of CHX, however, the signal for histone H3 phosphorylation disappeared rapidly, before MI was completed. We conclude that histone H3 phosphorylation is maintained by another protein synthesis-dependent activity during meiosis. The Aurora kinases, Eg2 and Airk2, are known to phosphorylate serine 9 in histone H3 kinase (Hsu et al., 2000; Speliotet et al., 2000), but they appear to be stable proteins in *Xenopus* oocytes (H.H., unpublished data). There may be other candidate kinases, or the activation of the Aurora family may depend indirectly on protein synthesis.

Cyclin synthesis is essential and sufficient during MII

Suppression of interphase and progression through MII until the CSF-dependent arrest at metaphase represents the final protein synthesis requirement of oocyte maturation. Passage between MI and MII is associated with high APC/C activity, so that proteins containing destruction boxes are highly unstable at this time. Addition of CHX 1.5 hours after white spot formation (i.e. after MI has been completed) leads to the rapid loss of cyclins and MAP kinase activity and returns the cell to interphase. Specific inhibition of B-type cyclin synthesis similarly prevented entry into MII: antisense-injected oocytes did not reactivate MPF, did not form a normal MII spindle, and appeared very similar to CHX-treated oocytes (the 'white puffball' phenotype). We were previously misled (Minshull et al., 1991) into concluding that the pool of pre-MPF was sufficient for completion of meiosis, because we did not suspect the existence of cyclins B4 and B5, whose continued synthesis is sufficient to support a normal MII. This revised interpretation is supported by the observation of Iwabuchi et al. (Iwabuchi et al., 2000) and Nakajo et al. (Nakajo et al., 2000) that a certain threshold level of MPF activity is required to keep newly synthesised Wee1 in check (Murakami and Vande Woude, 1998).

The deleterious effects of general inhibition of cyclin synthesis using antisense oligonucleotides were largely prevented by injection of indestructible cyclin B (sea urchin $\Delta 90$), except that the spindles were somewhat abnormal. As discussed above, *Arbacia* $\Delta 90$ cyclin B caused spindle abnormalities even in control oocytes, but it is also possible that the antisense oligonucleotides we used may have inadvertently ablated other mRNAs whose products are important for assembly of a perfect spindle. It is likely that other proteins, in particular *c-mos*, need to be synthesised in order to promote the increase in cyclin synthesis that normally occurs at the transition between MI and MII.

None of our observations can account for the long-standing paradox that CSF activity does not appear during MI metaphase. A reasonable explanation for this could be the existence of a protein X, which starts to be made some time after exposure to progesterone, and only reaches its necessary threshold after completion of MI. Our results do imply,

however, that MPF activity is required for this hypothetical protein to inhibit the APC.

The different levels of cyclins B2 and B5 synthesis in different batches of oocytes after the completion of MI reflect the possibility of significant biochemical differences between oocytes derived from different frogs, and even between sub-populations of oocytes as previously reported by Fisher et al. (Fisher et al., 1999). Cyclins B1 and B4 are dispensable if cyclins B2 and B5 accumulate to significant levels after MI. This argues against the possibility that the two families of B-type cyclins have specific substrate targeting functions that are necessary for progression into MII. Nevertheless, cyclins B1 and B4 were required in about half the batches of oocytes that we have analysed ($n=15$). Synthesis of cyclins B1 and B4 is thus the predominant event that allows progression from MI to MII, whereas cyclins B2 and B5 are the major components of the maternal stockpile of pre-MPF whose activation is required for GVBD. We have ablated each individual B-type cyclin using specific antisense oligonucleotides, and not observed any obvious defects in oocyte maturation (Sohail et al., 2001; data not shown). We conclude that there is considerable functional overlap between these four B-type cyclins.

The interdependence of MAP kinase and MPF at the MI/II transition

In this paper, we provide evidence that *c-mos* accumulation and MAP kinase activity depend on MPF. Loss of MPF after GVBD results in inactivation of MAP kinase, followed by the disappearance of *c-mos*. The activity of MAP kinase in frog oocytes requires the activity of *c-mos* (Nebreda et al., 1993; Nebreda and Hunt, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993). Hence, MPF seems to be required to maintain *c-mos* activity, possibly by phosphorylating it on S3 (Fisher et al., 2000). The subsequent disappearance of *c-mos* is probably a consequence of its dephosphorylation/inactivation (Nishizawa et al., 1992).

Conversely, accumulation of cyclins during MII is strictly dependent on the *c-mos*/MAP kinase/p90^{rsk} pathway (Furuno et al., 1994; Gross et al., 2000). The mutual interdependence of cyclins and *c-mos* is crucial during *Xenopus* oocyte maturation, and if either fails to appear, the other is powerless to act. A recent paper (Frank-Vaillant et al., 2001) disagrees with these conclusions. These investigators injected 1 μ M (final concentration) GST-p21^{cip1} into oocytes to inhibit the activity of CDK1, and we suspect that this may not have been quite sufficient to achieve complete inhibition of the kinase. The concentration of CDK1 in oocytes is about 0.5 μ M (Kobayashi et al., 1994), and p21 is not as powerful an inhibitor of cyclin B-dependent CDKs as it is of cyclin A- and E-CDKs (Strausfeld et al., 1994).

The results presented in this paper give a clearer view of why protein synthesis is needed for progression of MI to MII. The translational activation of B-type cyclins after MI ensures that MPF activity is quickly restored, even though the cyclins turn over rapidly until CSF shuts down the APC/C. What determines the timing of this translational activation, and how it is controlled by the *c-mos*/MAP kinase/p90^{rsk} pathway remain to be investigated. The most extreme interpretation of the data in this paper would be that no other protein synthesis is required for progression from MI to MII if high enough levels of B-type cyclins are present. We cannot exclude that

other key proteins are synthesized during the 30 minute window between MI and MII that (of necessity) remained open in our experiments. It is clear, however, that the strong synthesis of B-type cyclins after the completion of MI is a key event allowing proper transition between the two meiotic divisions.

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