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Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes

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SUMMARY

To study the mechanisms involved in the progression of meiotic maturation in the mouse, we used oocytes from two strains of mice, CBA/Kw and KE, which differ greatly in the rate at which they undergo meiotic maturation. CBA/Kw oocytes extrude the first polar body about 7 hours after breakdown of the germinal vesicle (GVBD), whilst the oocytes from KE mice take approximately 3-4 hours longer. In both strains, the kinetics of spindle formation are comparable. While the kinetics of MAP kinase activity are very similar in both strains (although slightly faster in CBA/Kw), the rise of cdc2 kinase activity is very rapid in CBA/Kw oocytes and slow and diphasic in KE oocytes. When protein synthesis is inhibited, the activity of the cdc2 kinase starts to rise but arrests shortly after GVBD with a slightly higher level in CBA/Kw oocytes, which may correspond to the presence of a larger pool of cyclin B1 in prophase CBA/Kw oocytes. After GVBD, the rate of cyclin B1 synthesis is higher in CBA/Kw than in KE oocytes, whilst the overall level of protein synthesis and the amount of messenger RNA coding for cyclin B1 are identical in oocytes from both strains. The injection of cyclin B1 messenger RNA in KE oocytes increased the H1 kinase activity and sped up first polar body extrusion. Finally, analysis of the rate of maturation in hybrids obtained after fusion of nuclear and cytoplasmic fragments of oocytes from both strains suggests that both the germinal vesicle and the cytoplasm contain factor(s) influencing the length of the first meiotic M phase. These results demonstrate that the rate of cyclin B1 synthesis controls the length of the first meiotic M phase and that a nuclear factor able to speed up cyclin B synthesis is present in CBA/Kw oocytes.

Key words: Cyclin, H1 kinase, MAP kinase, Meiosis, Mouse oocyte

INTRODUCTION

One of the striking peculiarities of meiotic maturation in mouse oocytes concerns the first meiotic M phase (MI). In contrast to all other M phases in vertebrate cells (except the metaphase II arrest), the first meiotic M phase is long, usually lasting between 7 and 10 hours. However, the precise timing of MI seems to be genetically controlled, as shown by significant variations in the speed of meiotic maturation between different strains of mice, in particular the strains CBA/Kw and KE (Polanski, 1986, 1997). CBA/Kw oocytes extrude the first polar body about 7 hours after germinal vesicle breakdown (GVBD), whilst the KE oocytes do so approximately 3-4 hours later, that is 10-11 hours after GVBD.

The major cell cycle kinase, the cyclin/p34^{cdc2} kinase (maturation promoting factor; MPF) is activated just before GVBD and its activity rises progressively until it reaches a plateau at MI (Choi et al., 1991; Verlhac et al., 1994). By this time, the microtubules have formed into a spindle, but exit from the first meiotic M phase is delayed for a further 3-4 hours.

Mitotic cyclins are the regulatory subunits of the p34^{cdc2} kinase that are synthesized throughout the cell cycle and destroyed at each cell division (Evans et al., 1983). Cyclin degradation is a proteolytic event involving the ubiquitin pathway that is regulated throughout the cell cycle, such that the cyclins remain stable in interphase and are destroyed during a short interlude just before the metaphase-anaphase transition (for a review, see Townsley and Ruderman, 1998). It was reported that the synthesis of cyclin B1 increases gradually during meiosis I in mouse oocytes (when MPF activity rises) and the newly synthesized protein becomes complexed immediately with the p34^{cdc2} kinase (Hampl and Eppig, 1995b; Winston, 1997). Thus, these data suggest that MPF activity is regulated by a translation-dependent mechanism that determines the level of cyclin synthesis. Similar increases in cyclin B synthesis have also been observed during the transition from G_2 to metaphase I in oocytes from *Patella* and *Xenopus* (Gautier and Maller, 1991; Kobayashi et al., 1991; van Loon et al., 1991; Galas et al., 1993).

In this work, we have studied the role of cyclin B1 metabolism in the timing of meiotic maturation using oocytes from the KE and CBA/Kw mouse strains.

MATERIALS AND METHODS

Collection and culture of oocytes

To obtain immature oocytes arrested at prophase I of meiosis, the ovaries were removed from 5- to 6-week-old KE or CBA/Kw female mice (bred in the laboratory), F_1 (CBA×C57/Bl) or Swiss female mice (Animalerie Spécialisée de Villejuif, Center National de la Recherche Scientifique, France) and transferred to prewarmed (37°C) M2 medium supplemented with 4 mg/ml bovine serum albumin (BSA; Whittingham, 1971) and 50 μg/ml dibutiryl cyclic AMP (dbcAMP), which prevents immature oocytes from undergoing GVBD. The ovarian follicles were punctured to release the enclosed oocytes, and immature oocytes displaying a germinal vesicle (GV) were collected (oocytes from all strains used in this study have similar sizes). Groups of oocytes used to examine the synthesis of cyclin B1 were washed out of dbcAMP and cultured in either M2 medium alone under liquid paraffin oil at 37°C, or medium M2 containing 500 μCi/ml of [^{35}S]methionine (specific activity 1000 Ci/mM; SJ 1015 Amersham) and labeled for 4 hours. The labeling time and the number of oocytes collected were consistent for sample groups compared subsequently. Samples were then collected immediately. All samples were collected in M2 medium + 4 mg/ml polyvinylpirolidone (PVP) and frozen immediately at −70°C.

Metaphase II-arrested oocytes were recovered from mice superovulated by intraperitoneal injections of pregnant mare's gonadotrophin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet) 48 hours apart. Ovulated oocytes were released from the ampullae of oviducts 13.5 hours post-hCG. The cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma) and after careful washing, oocytes were subsequently labeled for 4 hours in medium containing 500 μCi/ml of [^{35}S]methionine.

Immunofluorescence

The fixation and labeling of oocytes were performed as described in Kubiak et al. (1992). We used the rat monoclonal antibody YL1/2 specific for tyrosinated α-tubulin (Kilmartin et al., 1982). As the second layer, we used a fluorescein-conjugated anti-rat antibody (Miles). The chromatin was visualized using Propidium Iodide (Molecular Probes; 5 mg/ml in PBS). Samples were observed with a Leica TCS4D confocal microscope.

Oocyte bisection and electrofusion

Oocytes were released from ovaries into M2 medium supplemented with dbcAMP (Sigma; 150 μg/ml). The zonae pellucidae were removed by treatment with 0.25% Pronase (B grade, Calbiochem) in M2 medium. The oocytes were bisected with a glass needle according to the method of Tarkowski (1977) in a medium containing 5 μg/ml cytochalasin B (Sigma). Individual oocytes were bisected into two equivalent halves, or into a smaller nuclear and a larger anuclear fragment (with a volume ratio of about 1:3). To obtain these fragments, the oocytes were first pipetted through a narrow pipette (with a diameter of about half that of an oocyte) before cutting. In the majority of the elongated oocytes obtained in this way, the GV was located close to one of the extremities and could be cut off easily with an appropriate portion of the cytoplasm.

After bisection, oocyte fragments were rinsed in M2 supplemented with dbcAMP, cultured for 1-3 hours and then agglutinated in phytohaemagglutinin (PHA-M; Gibco) diluted in M2 without BSA to a final concentration of 400 μg/ml for 5 minutes on 1% agarose at 37°C. Aggregated pairs were washed in 0.25 M glucose prepared in double-distilled water supplemented with 1 mg/ml BSA and electrofused in this solution (Kubiak and Tarkowski, 1985). Pairs were placed in a fusion chamber between platinum electrodes (1 mm apart) and subjected to two pulses of DC (80 V, duration 100 μs, 100 ms interval between pulses), then rinsed in M2 + dbcAMP and transferred to drops of medium under paraffin oil. The treatment was repeated for pairs that had failed to fuse after approximately 1 hour, with a maximum of two repeats for a given pair. Fused pairs (hybrids) were then rinsed out of dbcAMP and culture was continued in M2 medium. Hybrids were examined at hourly intervals (from 8 to 16 hours after the beginning of culture) for the presence of the first polar body. The timing of first polar body extrusion was not influenced by the timing of GVBD, which is very similar in both strains: 2.35±0.37 hours (n=65) for CBA and 2.33±0.64 hours (n=72) for KE (hours after release from the dbcAMP block).

Immunoprecipitation

[^{35}S]methionine-labeled samples were resuspended in RIPA buffer (Kubiak et al., 1993) and cyclin B1 was immunoprecipitated using a polyclonal antibody directed against cyclin B1 (Pines and Hunter, 1989). Immunoprecipitates were lysed in SDS-sample buffer and analyzed by 10% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and autoradiography. To assess overall protein synthesis, [^{35}S]methionine-labeled samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

The autoradiographs were digitized using an Agfa Arcus Plus scanner and the radioactivity present in each sample was quantified using the NIH image (v. 1.60) image analysis software.

Immunoblotting

Samples were collected in sample buffer (Laemmli, 1970) and heated to 100°C for 3 minutes. The proteins were separated by electrophoresis in 10% polyacrylamide (ratio acrylamide/bis-acrylamide: 100/1), containing 0.1% SDS and electrically transferred to nitrocellulose membranes (Schleicher and Schuell, pore size 0.45 μm). Following transfer and blocking for 2 hours in 3% skimmed milk in 10 mM Tris (pH 7.5), 140 mM NaCl (TBS) containing 0.1% Tween-20, the membrane was incubated overnight at 4°C with the primary antibody (either anti-ERK, #691 Santa Cruz Biotechnology, 1:200 or anti-cyclin B1, Serotec, 1:500) diluted in blocking solution. After three washes of 10 minutes each in 0.1% Tween-20/TBS, the membrane was incubated for 1 hour at room temperature with an anti-rabbit antibody conjugated to horseradish peroxidase (Amersham) diluted 1:300 in 3% skimmed milk in 0.1% Tween-20/TBS. The membrane was washed three times in TBS/Tween-20 and then processed using either the ECL (Amersham) or the Super Signal (Pierce) detection system.

The films were digitized using a BioRad GelDoc 1000 (white light source) and the amount of cyclin B1 protein present in each sample was quantified using the BioRad MultiAnalyst image analysis software.

Kinase activity assays

The histone H1 kinase and myelin basic protein (MBP) kinase activities were measured as described previously (Kubiak et al., 1992; Verlhac et al., 1994). Histone H1 kinase activity was determined as described by Félix (1989) in HK buffer (80 mM β-glycerophosphate, 20 mM EGTA, pH 7.3, 15 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin) using exogenous histone H1 (HIII-S from calf thymus, Sigma) as the substrate. Samples containing the oocytes in medium M2 + BSA were lysed by freezing and thawing three times, diluted twice in two times concentrated HK buffer and incubated for 15 minutes at 37°C in the presence of 3.3 mg/ml histone H1, 1 mM ATP and 0.25 mCi/ml [γ-^{32}P]ATP (Kubiak et al., 1991).

MBP kinase activity was determined according to Boulton et al. (1991). 8-10 oocytes were transferred into 4 ml of M2 medium, frozen immediately in a mixture of dry ice and ethanol, and stored at −80°C. For the kinase assays, 2 μl of 4× reaction buffer (100 mM Hepes-NaOH, pH 8.0, 40 mM $MgCl_2$, 4 mM DTT, 4 mM benzamidine, 120 mM ATP, 20 mM NaF, 0.4 mM sodium orthovanadate) supplemented with protease inhibitors was added to the oocyte sample, which was then thawed and frozen twice to lyse the cells. The reaction was started by addition of 2 μl of a solution containing 1.5 mg/ml MBP, 4 mM ATP and 0.5 mCi/ml [γ-^{32}P]ATP, and lasted 30 minutes at 30°C.

For the combined histone H1/MBP kinase assays, 2 μl of twice-concentrated reaction buffer (180 mM EGTA, pH 7.3, 300 mM β-glycerophosphate, 40 mM $MgCl_2$, 4 mM DTT, 4 mM benzamidine, 20 mM NaF, 0.4 mM sodium orthovanadate, 4 mM ATP) supplemented with protease inhibitors was added to the sample, which was then thawed and refrozen once to lyse the cells. The reaction was started by the addition of 2 μl of a solution containing 1.5 mg/ml MBP and 66 μg/ml histone H1 and 0.5 mCi/ml [γ-^{32}P]ATP, and lasted 30 minutes at 30°C.

In all cases, the kinase reaction was stopped by adding 2× sample buffer (Laemmli, 1970) and boiling for 3 minutes. The samples were then analyzed by electrophoresis in 15% polyacrylamide gels containing 0.1% SDS followed by autoradiography.

The autoradiographs were digitized using an Agfa Arcus Plus scanner and the kinase activity present in each sample was quantified using the NIH image (v. 1.60) image analysis software.

mRNA quantification

To quantify the amount of cyclin mRNA present in CBA/Kw and KE oocytes, a competitive RT-PCR method was used (Wang et al., 1989; Gilliland et al., 1990). The primers used in our experiments, 5′ TTT GCC CCC AAG TCT CAC TAT 3′ (5′ specific primer) and 5′ TGG CAT TAC AAG ACA GGA GTG 3′ (3′ specific primer) were defined using the Amplify software package (v1.2, W.R. Engels, University of Wisconsin, Madison). They define a 365 bp fragment in the cyclin B1 cDNA between positions 1853 and 2217. The internal standard used corresponded to a 264 bp fragment identical to the fragment of interest except for a deletion between positions 1874 and 1974.

Oocytes were collected in H_2O treated with 0.1% diethyl pyrocarbonate (H_2O-DEPC) and stored at −80°C. Samples corresponding to 60 oocytes in 6 μl were lysed by freezing and thawing. The genomic DNA from 10 oocytes was digested by adding 2 units of RQ1 DNase (Promega) in 20 μl of the reverse transcriptase mix (10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 1 mM each of dNTPs (Promega), 2.5 μM random hexamer (pd(N)6, Pharmacia), 20 units of RNase inhibitor (RNasin, Promega) and incubating at 37°C for 10 minutes. The reaction was stopped by heating to 85°C for 5 minutes. Then, the samples were supplemented with 50 units of SuperScript RNase H^- reverse transcriptase (Gibco BRL) to synthesize the cDNA. After a 1 hour incubation at 37°C, the reaction was stopped by heating to 95°C for 5 minutes. PCR amplification was performed in the same tube in a final volume of 50 μl containing 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 1 μM of the 5′ specific primer, 1 μM of the 3′ specific primer, various amounts of the internal standard (ranging from 0.27 to 4.32 arbitrary units (AU)) and 2.5 units of Taq Polymerase (Perkin Elmer). Samples were denatured at 94°C for 3 minutes and then subjected to 40 cycles of amplification (94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) and finally incubated for 10 minutes at 72°C. 13 μl of the amplified mixture were run on 2% agarose gels. The gels were stained with ethidium bromide (5 μg/ml) and photographed under UV illumination.

The ethidium bromide-stained gels were digitized using a BioRad GelDoc 1000 (UV light source) and the amount of cyclin B1 mRNA present in each sample was quantified using the BioRad MultiAnalyst image analysis software. The amount of mRNA was measured as AU/oocyte in samples containing varying numbers of oocytes to determine the linearity and potential limitations of oocyte number on the assay. A linear relationship was observed between the values for 0, 5, 10, 15 and 20 oocytes (correlation coefficient r=0.990).

In vitro synthesis of capped RNA

The DNA of p18.2-cyclin B1 (Chapman and Wolgemuth, 1992) was digested with *Xho*I (Appligene). Transcripts were prepared using linearized plasmids and the T3 RNA polymerase (Stratagene). The cyclin B1 sense RNA, approximately 1570 nt long, was polyadenylated by incubation in a polyadenylation mixture (250 mM NaCl, 50 mM Tris, 10 mM $MgCl_2$, 100 mg/ml BSA, 2 mM DTT, 1 U/ml RNasin (Promega), 100 mM ATP, 2.5 mM $MnCl_2$ and 0.05 U/ml PolyApolymerase (Pharmacia Biotech) for 37°C as described in Vassalli et al. (1989). The reaction was stopped by adding 25 mM EDTA. Samples were extracted with the RNeasy Kit (Quiagen) and resuspended in 0.1 mM EDTA, 10 mM Tris, pH 7.4 (injection buffer) to a final concentration of 2 mg/ml. The transcripts were then stored in 0.5 ml portions at −80°C. The mRNAs were diluted further to 1 mg/ml in injection buffer before oocyte microinjection. Each portion was thawed only once.

Microinjection

The in vitro synthesized mRNAs were microinjected into the cytoplasm of GV oocytes using an Eppendorf pressure microinjector and siliconized sterile pipettes. About 10 pl of mRNA solution containing 25 pg of mRNA were injected per oocyte. The oocytes were kept in M2 medium supplemented with dbcAMP during the injection period. The resumption of meiotic maturation was triggered by removal of the injected oocytes from dbcAMP-containing medium. They were then cultured under paraffin oil at 37°C, in an atmosphere of 5% CO_2 in air, in M2 medium.

RESULTS

Timing of meiotic maturation in oocytes from the CBA/Kw and KE strains

Oocytes from CBA/Kw and KE females differ greatly in their speed of meiotic maturation (Polanski, 1986). In our culture conditions, germinal vesicle breakdown (GVBD) took place between 1.5 and 2.5 hours after release from the dbcAMP block in both strains. CBA/Kw oocytes extruded the first polar body about 6-7 hours after GVBD, whilst the KE oocytes did so approximately 9-12 hours after GVBD.

Spindle formation in oocytes from the CBA/Kw and KE strains

First, we checked whether this difference in the timing of polar body extrusion could be related to differences in the timing of meiotic spindle formation. The organization of the microtubule network during meiotic maturation in oocytes from both strains was studied by immunofluorescence. 1 hour after GVBD, microtubules formed an aster around the condensed chromosomes in the center of the oocyte, although they were smaller in KE oocytes (Fig. 1). 2 hours after GVBD, in both strains, microtubules became organized in bipolar structures with clearly defined poles and the chromosomes were randomly dispersed between these two poles. 4 hours after GVBD, the density of microtubules had increased to a maximum and the chromosomes were observed near the equatorial plane of the spindle. This organization remained until the end of MI (6 hours after GVBD in CBA oocytes, 10 hours in KE oocytes): the chromosomes were concentrated near the equatorial plane of the spindle but were rarely found sharply aligned on this plane. The spindle size differed in the two strains, being 30% shorter in KE oocytes. This was observed until the end of MI.

Kinase activities during meiotic maturation in CBA/Kw and KE oocytes

At least two major kinases are activated during meiotic maturation, the cdc2 kinase (responsible for MPF activity) and MAP kinase. Mouse oocytes possess two forms of MAP kinase, p42 and p44, which become phosphorylated and active during meiotic maturation (Verlhac et al., 1993). In mouse

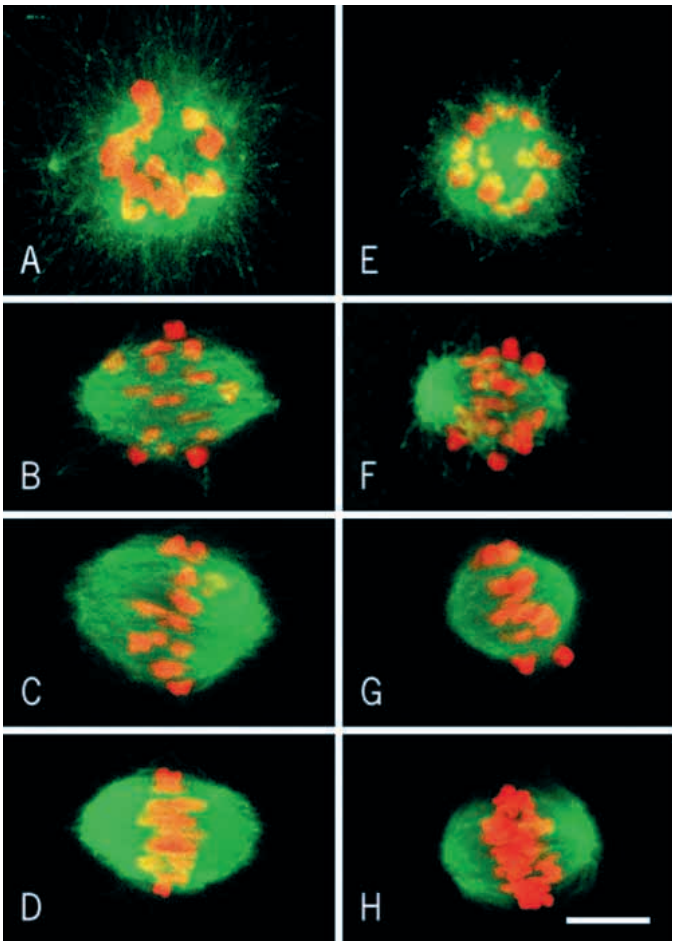

Fig. 1. Organization of the microtubules and chromosomes during the first meiotic M phase in CBA/Kw (A-D) and KE (E-H) mouse oocytes. Microtubules are shown in green and chromosomes in red. Oocytes were fixed 1 (A,E), 2 (B,F), 4 (C,G), 6 (D) and 10 (H) hours post-GVBD.

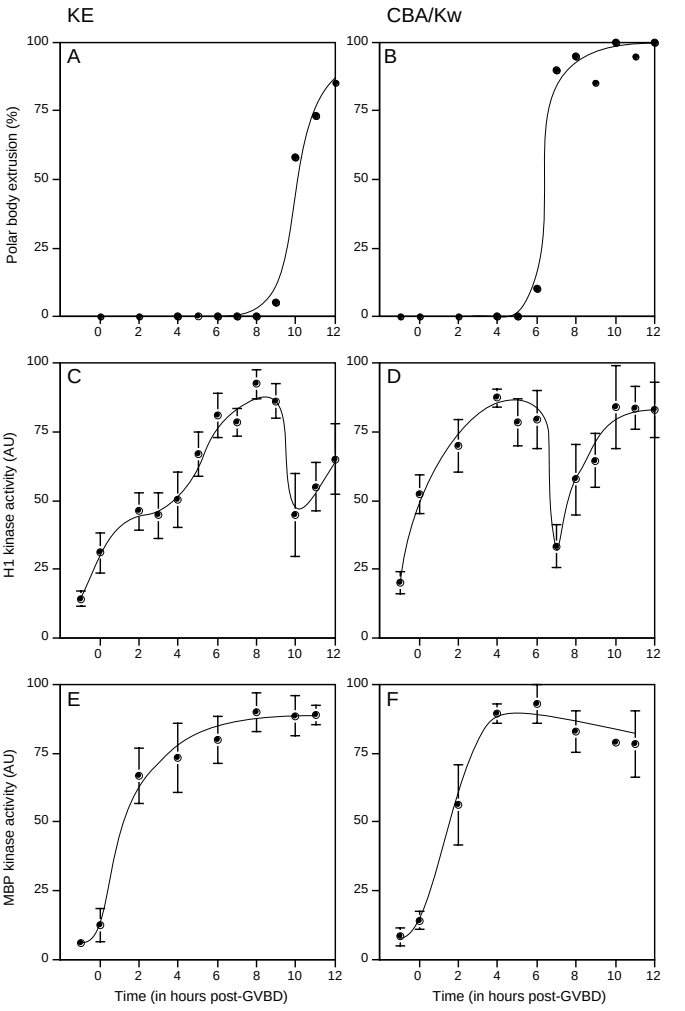

Fig. 2. Timing of polar body extrusion (A,B) and kinetics of histone H1 (C,D) and MBP (E,F) kinase activities during meiotic maturation in oocytes from KE (A,C,E) and CBA/Kw (B,D,F) mice. GVBD was considered as time 0. The bars show the mean±s.e.m. (3-8 experiments). Independent groups of 20 oocytes were scored at each time point for polar body extrusion.

oocytes, MAP kinase is activated after the cdc2 kinase and MAPK activity persists throughout maturation, even when the cdc2 kinase activity drops between the two meiotic M phases (Verlhac et al., 1994). We have compared the activities of histone H1 kinase (reflecting the cdc2 kinase activity) and MBP (myelin basic protein) kinase (reflecting the MAP kinase activity) during meiotic maturation of oocytes from the KE and CBA/Kw strains.

In both strains, the activity of histone H1 kinase started to increase at GVBD, reached a plateau and then dropped rapidly, concomitant with the extrusion of the first polar body. However, whereas the rise of histone H1 kinase activity was very rapid in CBA/Kw oocytes (Fig. 2D), it was much slower and diphasic in KE oocytes (Fig. 2C). The activity of MBP kinase increased steadily, beginning 1 hour after GVBD and reached a plateau that was maintained throughout metaphase I, extrusion of the first polar body and metaphase II (Fig. 2E,F). The rise in MBP kinase activity had a similar profile in oocytes from both strains, although it occurred slightly more slowly in KE oocytes. This observation was confirmed by assessing the phosphorylation state of ERK1 and ERK2 by analyzing their electrophoretic mobility on immunoblots. In both strains, oocytes taken at the GV stage displayed two bands of relative molecular mass (M_r) 42 and 44×10^3 (Fig. 3A). CBA/Kw oocytes collected 1 hour after GVBD displayed both the fast migrating (42 and 44×10^3) and the slow migrating (44 and 46×10^3) forms of ERK 1 and ERK 2, while KE oocytes only displayed the fast migrating forms. By 2 hours after GVBD, ERK 1 and ERK 2 proteins had shifted to their slow migrating forms in both strains. When MAP kinase activity was assayed in vitro using MBP as a substrate, we observed a rise in MBP kinase activity after 30 minutes in CBA/Kw oocyte and after 60 minutes in KE oocytes (Fig. 3B). Thus, using both criteria, MAP kinase activation is delayed in KE oocytes by about 30 minutes when compared to CBA/Kw oocytes.

Protein synthesis is required for maximum H1 kinase activation and spindle formation in both strains

In the presence of the protein synthesis inhibitor puromycin,

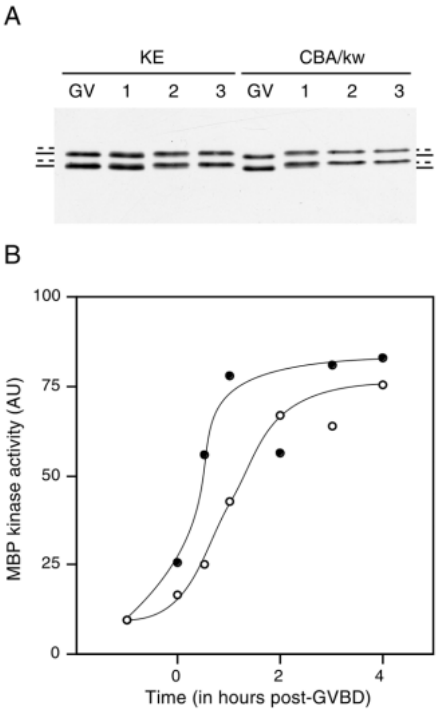

Fig. 3. (A) MAP kinase phosphorylation during meiotic maturation of oocytes from KE and CBA/Kw mice. Lysates of GV-stage or maturing oocytes (1, 2 and 3 hours after GVBD) were analyzed by immunoblotting using an anti-ERK antiserum (all samples contained 20 oocytes). Number of experiments: 2. (B) MBP kinase activities during meiotic maturation in oocytes from KE (○) and CBA/Kw (●) mice. GVBD was considered as time 0. (2 experiments).

histone H1 kinase activity increased but arrested shortly after GVBD, whilst MBP kinase was not activated in either strain (Fig. 4). Furthermore, the level of histone H1 kinase activity was slightly higher in CBA/Kw oocytes than in KE oocytes, suggesting that the pool of cyclin B1 already present in GV-arrested oocytes (Hampl and Eppig, 1995b) may be slightly larger in GV-arrested CBA/Kw oocytes

In both strains, the spindle did not form although the chromosomes were condensed (Fig. 4). Microtubules were longer and formed a single aster nucleated in the centre of the chromosome area in CBA oocytes while they were shorter and formed multiple asters in KE oocytes.

The rate of cyclin B1 synthesis differs in CBA and KE oocytes during meiotic maturation

Since cyclin B1 does not turn over during the first meiotic cell cycle (Winston, 1997), we only examined the synthesis of cyclin B1 in CBA/Kw and KE oocytes. Oocytes from the two strains were labeled with [^{35}S]methionine before and after GVBD and cyclin B1 was then immunoprecipitated with an anti-cyclin B1 antibody (Fig. 5A). First we observed that at the GV stage, the level of cyclin B1 synthesis was lower in KE oocytes. During maturation, from 2-6 hours post-GVBD, the level of cyclin B1 synthesis increased about 4 times in both strains. However, as a result of the differing initial levels of cyclin B1 synthesis, the measured value after 4 hours in culture was higher (about 50%) in CBA/Kw than in KE oocytes, whilst the overall level of protein synthesis was identical in oocytes from both strains (data not shown). Finally, during the metaphase II arrest, the level of cyclin B1 synthesis was identical in oocytes from both strains (Fig. 5A).

This difference in the rate of cyclin B1 synthesis during MI was correlated with differences in the amount of cyclin B1 detected by immunoblotting in oocytes 4 hours post-GVBD, about twice the amount of cyclin B1 being present in CBA oocytes (1.94±0.13; n=3; Fig. 5B).

The amount of cyclin B1 mRNA is similar in CBA and KE oocytes at the GV stage

Since the rate of cyclin synthesis was higher in CBA than in KE oocytes, we checked whether this difference was due to differences in the amount of cyclin B1 mRNA in oocytes from both strains. We used a quantitative RT-PCR approach to measure the amount of mRNA present in oocytes. When we compared the quantity of cyclin B1 mRNA found in GV oocytes, we did not observe a significant difference between the two strains (Table 1, group 2 versus 3; P=0.097). This amount was also similar to the one observed in our usual working strain, Swiss (Table 1, group 1).

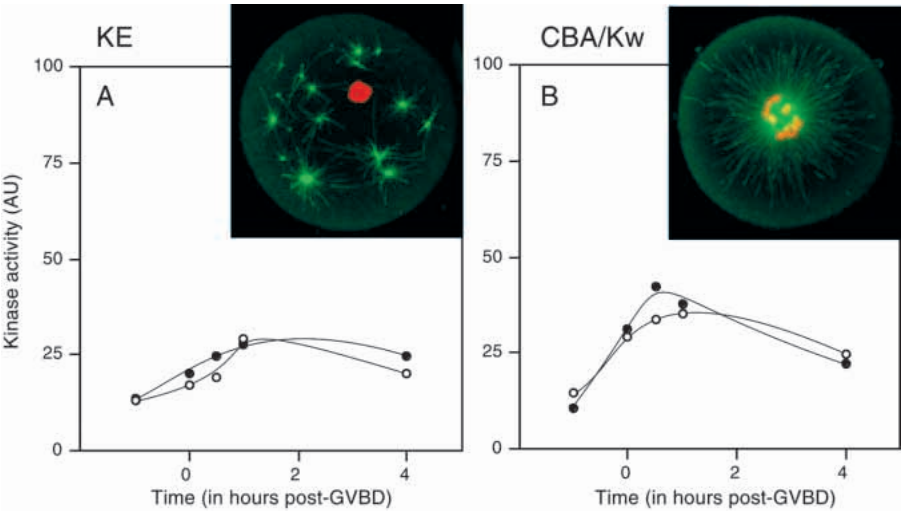

Fig. 4. Effect of protein synthesis inhibition on kinase activation during meiotic maturation in oocytes from KE (A) and CBA/Kw (B) mice. Histone H1 (●) and MBP (○) kinase activities were estimated in oocytes from KE and CBA/Kw mice in the presence of 10 μg/ml puromycin. 10 oocytes were used per assay, 2 experiments. (Insets) Organization of the microtubules and chromosomes in puromycin-treated KE (A) and CBA/Kw (B) mouse oocytes. Microtubules are shown in green and chromosomes in red. Oocytes were fixed 3 hours post-GVBD.

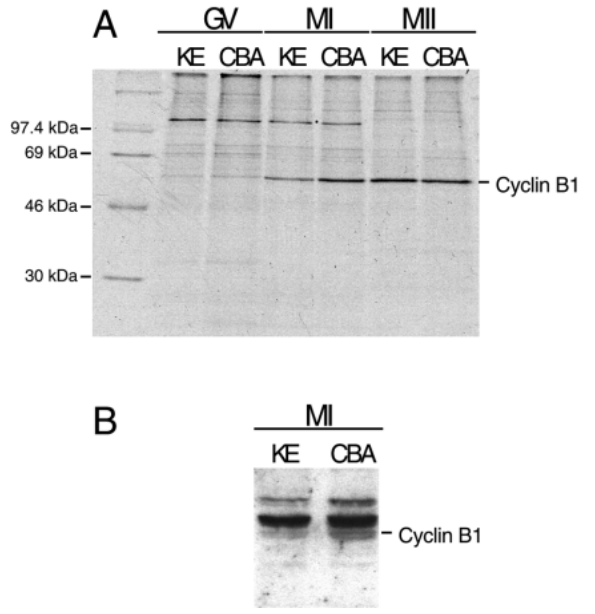

Fig. 5. (A) Cyclin B1 synthesis in oocytes from CBA/Kw and KE oocytes. GV lanes: oocytes were labeled for 4 hours in the presence of dbcAMP; MI lanes: oocytes were labeled from 2 to 6 hours post-GVBD; MII lanes: oocytes were labeled from 15 to 19 hours post-hCG. 2 experiments. The positions of molecular mass markers are shown. (B) Cyclin B1 accumulation in oocytes from CBA/Kw and KE oocytes. Lysates of CBA/Kw and KE oocytes (4 hours post-GVBD) were analyzed by immunoblotting using an anti-cyclin B1 antiserum (both samples contained 137 oocytes). 3 experiments.

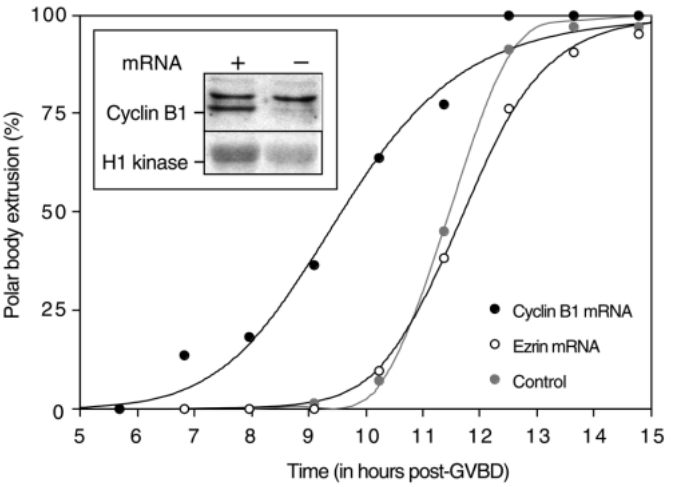

Fig. 6. Effect of cyclin B1 mRNA micro-injection on polar body extrusion in KE oocytes. Cyclin B1 mRNA injected (●; *n*=22), ezrin mRNA injected (○; *n*=21) and control non-injected (◐; *n*=69) oocytes from KE mice. GVBD was considered as time 0. Sigmoids were fitted using the least square fit. 2-4 experiments (see Table 2). (Inset) Cyclin B1 overexpression and H1 kinase activity in oocytes from KE mice micro-injected with cyclin B1 mRNA. Lysates of injected or control non-injected oocytes taken 4 hours post-GVBD were either analyzed by immunoblotting using an anti-cyclin B1 antiserum (both samples contained 54 oocytes) or used for an H1 kinase assay (both samples contained 5 oocytes).

Cyclin B1 mRNA injection in KE oocytes speeds up polar body extrusion

Our data suggested that the duration of the first meiotic M phase was dependent upon the level of cyclin B synthesis, which in turn controls the time required for the cdc2 kinase to reach its maximum activity. Thus, we attempted to speed up polar body extrusion by micro-injecting the mRNA coding for the mouse cyclin B1 into KE oocytes at the GV stage. First we checked that the injection led to an increase in the amount of cyclin B1 protein present in the oocyte (Fig. 6, inset). This increase in the amount of cyclin B1 was associated with a raise in H1 kinase activity, being 2.11±0.49 (mean±s.d.; *n*=5) times higher in injected oocytes than in control oocytes 4 hours post-GVBD. Then, we verified that micro-injection of an unrelated mRNA (coding for ezrin) did not influence significantly the timing of polar body extrusion (Table 2, group 1 versus 2; *P*=0.398). Finally, injection of the mRNA coding for the mouse cyclin B1 sped up polar body extrusion (Fig. 6), the polar bodies being extruded about 1.9 hours earlier in the oocytes injected with the cyclin B1 mRNA (Table 2, group 1 versus 3; *P*<0.001). This effect was even more pronounced when compared to the oocytes injected with the ezrin mRNA (2.25 hours, Table 2, group 1 versus 2; *P*=0.005).

The length of the first meiotic M phase is controlled by factor(s) present both in the germinal vesicle and in the cytoplasm of prophase oocytes

Since the synthesis of cyclin B seems to be under the control of a nuclear factor in starfish oocytes (Galas et al., 1993), we performed the following experiments. Oocytes from CBA/Kw and KE mice were bisected either into equivalently sized halves, or to give small nuclear and large anuclear fragments. The oocyte fragments obtained were then fused in varying

Table 1. Quantification of cyclin B1 mRNA levels in germinal vesicle stage oocytes

Strain	Number of oocytes in assay	mRNA content (AU/oocyte) mean±s.d.	*n*	*P** CBA	*P** KE
(1) Swiss	10	228±45	5	0.3767	0.4005
(2) CBA/Kw	5-10	251±20	3		0.0964
(3) KE	5-10	205±26	3	0.0964	

*Using the unpaired Welch *t*-test.
‡mRNA is determined as AU (arbitrary units)/oocyte.

Table 2. Effect of cyclin B1 mRNA injection on polar body extrusion in KE oocytes

Microinjected mRNA	Time of polar body extrusion (hours post-GVBD) mean±s.d.	*n* (expt)	*P** Cyclin B1	*P** Ezrin
(1) Control (none)	11.38±1.32	69 (4)	<0.0001	0.3982
(2) Ezrin	11.74±1.42	21 (2)	0.0005	
(3) Cyclin B1	9.49±1.86	22 (3)		0.0005

*Using the non-parametric Mann-Whitney test.
n values are numbers of oocytes. Number of experiments in each group are given in parentheses.

Table 3. Effects of cytoplasmic and nuclear factors on the timing of polar body extrusion (PBE) in hybrids between KE and CBA/Kw oocytes

Hybrid	Size ratio	Number of oocytes	Time of PBE (hours)* mean ±s.d.	Median
Whole oocytes				
(1) KE/KE	1:1	12	13.75±0.87	13.5
(2) CBA/CBA	1:1	9	8.56±0.88	8
(3) CBA/KE	1:1	9	10.33±0.87	10
Oocyte fragments‡				
(4) KEn/KE	1:1	10	14.60±1.71	14.5
(5) CBAn/CBA	1:1	13	9.85±0.99	10
(6) KEn/CBA	1:1	17	12.18±2.01	12
(7) KEn/CBA	1:3	21	10.62±1.32	11
(8) CBAn/KE	1:1	20	10.30±1.45	10
(9) CBAn/KE	1:3	16	10.44±1.79	10

*GVBD took place 2.35±0.37 hours (n=65) in CBA/Kw and 2.33±0.64 hours (n=72) in KE after release from the dbcAMP block.
‡n, nucleated fragment.

combinations, as shown in Table 3. Statistical analysis of the data using the Welch t-test showed that the fusion process itself did not modify the relative differences in the timing of oocyte maturation between the two strains (Table 3, group 4 versus 5, P<0.001). When whole oocytes were fused, the timing of polar body extrusion in CBA/Kw:KE hybrids was significantly faster than that in KE:KE hybrids (Table 3, group 3 versus 1, P<0.001), but slower than in hybrids containing CBA/Kw material alone (Table 3, group 3 versus 2, P<0.001) suggesting that in the CBA/Kw:KE group the effect of CBA/Kw factor(s) may be suboptimal since they are required to act in a double quantity of cytoplasm.

A fused oocyte containing a KE nucleus is always slower to mature than an oocyte containing a CBA nucleus, regardless of the cytoplasmic environment (Table 3, group 4 versus 8, P<0.001 and group 5 versus 6, P<0.001). Therefore, there is a nuclear factor. The influence of the nucleus on the rate of polar body extrusion may also be detected when comparing groups with the same cytoplasmic composition (50% KE/50% CBA/Kw). The polar body was extruded significantly earlier in the hybrids containing a CBA/Kw nucleus (Table 3, group 6 versus 8, P<0.003).

A KE nucleus matures faster in CBA cytoplasm than in KE cytoplasm (Table 3, group 6 versus 4, P≤0.02 and group 7 versus 4, P<0.001). Therefore, there is a cytoplasmic factor. However, the presence of KE cytoplasm does not influence the maturation of a CBA/Kw nucleus (Table 3, group 8 versus 5, P=0.298 and group 9 versus 5, P=0.272).

DISCUSSION

The period of meiotic maturation leading to the extrusion of the first polar body is completed in approximately 7 hours post-GVBD in CBA/Kw oocytes, whilst oocytes from KE mice require a further 3-4 hours to undergo the same process. One of the main events of M phase is spindle formation. We first tried to determine if the difference in MI duration between CBA/Kw and KE strains might be correlated with any difference in MI spindle assembly, but the kinetics of spindle assembly were similar in both strains. Then, we examined the kinetics of activation of the two major meiotic kinases in both strains. While there was very little difference in the kinetics of activation of the MAP kinases (about 30 minutes earlier in CBA/Kw), the rise in H1 kinase activity was found to differ between the two strains, being both slower and diphasic in KE oocytes. However, at the end of metaphase I, the H1 kinase activity reached a plateau that lasts about 3-4 hours before polar body extrusion in both strains (this plateau is observed likewise in oocytes from other mouse strains; Verlhac et al. 1994, 1996). We also noticed that protein synthesis was required for the second phase of H1 kinase activation. In the presence of puromycin, the cdc2 kinase activity rises but arrests shortly after GVBD, the spindle does not form and MAP kinase is not activated in either strain, suggesting that the transient activation of the cdc2 kinase activity, independent of protein synthesis, is due to a limited pool of preexisting inactive cyclin-cdc2 kinase complexes, which become activated by dephosphorylation of the cdc2 subunit (Fulka et al., 1988; Choi et al., 1991, 1992; Hampl and Eppig, 1995b; Winston, 1997). Furthermore, the level of histone H1 kinase activity was slightly higher in CBA/Kw oocytes than in KE oocytes, possibly because the pool of cyclin B1 is slightly larger in CBA/Kw oocytes. Finally, these data show that spindle formation does not require full MPF activation in KE oocyte. However, a threshold level of activity is required since a spindle does not form after GVBD in a puromycin-treated KE oocyte.

These observations suggested to us that the prolonged period of meiotic maturation in KE oocytes may be attributed directly to the slower rise in MPF activity. This rise in MPF activity is a consequence of the synthesis of cyclin B during maturation (Hampl and Eppig, 1995a; Winston, 1997). After GVBD, the rate of cyclin B1 synthesis increases progressively to reach a maximum in metaphase II (Winston, 1997). Cyclin B1 turns over continuously during the metaphase II-arrest (Kubiak et al., 1993); however, during metaphase I, the level of cyclin B1 degradation is extremely low (Hampl and Eppig, 1995b; Winston, 1997). Thus the rise in MPF activity during metaphase I is only controlled at the level of cyclin B1 synthesis.

When we studied the level of cyclin B1 synthesis in maturing oocytes from the CBA/Kw and KE strains, we observed that it was higher in CBA/Kw than in KE oocytes, whilst the overall level of protein synthesis was otherwise identical in oocytes from both strains. This difference disappeared during the metaphase II arrest, cyclin B1 synthesis being similar in both strains. Thus, the diphasic activation of MPF in maturing KE oocytes could be due to the rapid activation of $p34^{cdc2}$-cyclin B complexes stored in the GV oocyte followed by the recruitment of newly synthesized cyclin B, the rate of synthesis of this protein being low during the beginning of the first meiotic M phase. Finally, we observed that the amount of cyclin B1 mRNA was similar in immature oocytes from both strains, suggesting that the differences in cyclin B1 synthesis are controlled specifically at the level of the protein synthesis machinery rather than by the amount of cyclin B1 mRNA present in the oocyte.

To test directly our hypothesis (the duration of the first meiotic M phase is controlled by the kinetics of MPF activity, which in turn is dependent upon the synthesis of cyclin B1), we tried to increase the level of cyclin B in KE oocytes.

Attempts to augment the cdc2 kinase activity by microinjecting the human cyclin B1 protein were unsuccessful (data not shown). This may be due to the requirement of a homologous protein, as suggested by the work of Sigrist et al. (1995) in *Drosophila*, or to an abnormal conformation of the bacterially produced protein. To overcome these problems, we injected the mouse cyclin B1 mRNA into immature oocytes from the KE strain. This led to an overexpression of the cyclin B1 protein, an increase in H1 kinase activity and to a shortening of the time required for first polar body extrusion, thus validating our hypothesis.

In starfish oocytes, it has been shown that a nuclear factor, required for specific translation of cyclin B, may control the timing of first meiotic cleavage (Galas et al., 1993). However, in starfish this control works through an increased synthesis of cyclin B1 after GVBD that balances out cyclin degradation and increases the time required for cyclin B-cdc2 kinase to fall below the critical level that is required for the metaphase/anaphase transition. In the maturing mouse oocyte, no marked cyclin B1 degradation was observed before the MI/MII transition (Winston, 1997).

Analysis of the rate of maturation in hybrids obtained after fusion of nuclear and cytoplasmic fragments of oocytes from both strains suggests that there is no inhibiting factor(s) in the KE nucleus (Table 3, group 7 versus 5; P=0.063), but rather that the CBA/Kw nucleus may contain factor(s) increasing the speed of maturation (Table 3, group 9 versus 4; P<0.001). However, cytoplasmic factor(s) that is (are) able to speed up maturation is (are) also present in CBA/Kw oocytes (Table 3, group 6 versus 4; P=0.003). Thus, nuclear and cytoplasmic factor(s), influence the timing of the first meiotic division. It also appears that in CBA/Kw oocytes, the nuclear and cytoplasmic effects are not synergistic since hybrids containing a CBA/Kw nucleus and CBA/Kw cytoplasm do not mature significantly faster than those containing a CBA/Kw nucleus and KE cytoplasm (Table 3, group 5 versus 9; P=0.272). This may be due to the same factor(s) being present both in the nucleus and the cytoplasm of CBA/Kw oocytes, or different factors interacting with the same target. If this (these) factor(s) is (are) absent in KE oocytes, this would explain why a CBA/Kw nucleus can have a maximal effect on hybrids containing a three times greater volume of KE cytoplasm and why the cytoplasm from CBA/Kw oocytes titrates the KE cytoplasm (Table 3, group 7 versus 6; P=0.011).

Taking into account the experimental data (equal amounts of cyclin B mRNA in oocytes from both strains), we made the simple model described in Table 4. A nuclear factor, able to speed up cyclin B synthesis and present in excess in the germinal vesicle of CBA/Kw oocytes, is loaded on cyclin B mRNAs before their export into the cytoplasm. In hybrids containing a CBA/Kw nucleus, this factor is released into the cytoplasm at GVBD and associates with cyclin B mRNAs from KE oocytes. This model does not exclude the possibility that a smaller amount of factor, not sufficient to saturate all the mRNAs, is present in the cytoplasm of KE oocytes.

Finally Sheets et al. (1995) have shown that the polyadenylation of the mRNA of the proto-oncogene c-mos is a pivotal regulatory step in *Xenopus* meiotic maturation. Furthermore, translational control by cytoplasmic polyadenylation of c-mos mRNA is necessary for oocyte maturation in the mouse (Gebauer et al., 1994). The mRNA of cyclin B must also be polyadenylated for normal function/activity (Sheets et al., 1994) and may share a common regulatory mechanism as for c-mos. The small delay in MAP kinase activation that we observed in KE oocytes may suggest that the synthesis of c-mos, which controls MAP kinase activation in the mouse oocyte (Verlhac et al., 1996), is also affected in this strain of mice. In our experiments, we used a polyadenylated version of the cyclin B1 mRNA to speed up polar body extrusion. It is possible that the CBA/Kw factor(s) may be involved in polyadenylation and that the rate of polyadenylation may differ between the two strains. Alternatively, the CBA/Kw factor(s) may be regulating cyclin B1 (and perhaps c-mos) translation at a different level, like the translation machinery itself. We are now investigating these possibilities further.

Table 4. Interpretation of the data obtained in the fusion experiments described in Table 2

			Calculated acceleration in the timing of PBE§		
	RNA population in hybrids		in	in	expected time
Hybrids‡	before GVBD	after GVBD	%	hours	of PBE¶
(4) KEn/KE	2RNA/2RNA	2RNA/2RNA	0	0	14
(5) CBAn*/CBA	2RNA*/2RNA*	2RNA*/2RNA*	100	4	10
(6) KEn/CBA	2RNA/2RNA*	2RNA/2RNA*	50	2	12
(7) KEn/3CBA	1RNA/3RNA*	1RNA/3RNA*	75	3	11
(8) CBAn*/KE	2RNA*/2RNA	2RNA*/2RNA*	100	4	10
(9) CBAn*/3KE	1RNA*/3RNA	1RNA*/3RNA*	100	4	10

‡The group numbers correspond to those in Table 1. A total amount of 4 copies mRNA/intact oocyte was used in all cases.

Factor(s) able to accelerate cyclin B synthesis present in the germinal vesicle of CBA/Kw oocytes (n) and associated with cyclin mRNAs (RNA*) in the cytoplasm.

§CBA/Kw=100%.

¶Compare with observed time of PBE in Table 3.

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