Selective degradation of cyclin B1 mRNA in rat oocytes by RNA interference (RNAi)

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Abstract

Cyclic adenosine monophosphate (cAMP) keeps oocytes in meiotic arrest, thereby preventing activation of the key regulators of meiosis, p34cdc2/cyclin B1, (known as maturation-promoting factor (MPF)) and Erk 1 and 2, members of the mitogen-activated protein kinase (MAPK) family. The activity of MAPK in oocytes is upregulated by Mos. We previously demonstrated that Mos translation in rat oocytes is negatively regulated by a PKA-mediated cAMP action, which inhibits c-mos mRNA polyadenylation and is associated with the suppression of p34 cdc2 kinase. The goal of the present study was to provide definitive evidence that Mos translation is subjected to MPF regulation. In order to inhibit MPF activity, we employed the double-stranded (ds) RNA interference (RNAi) of gene expression. We demonstrated that the introduction of cyclin B1 dsRNA into rat oocytes selectively depleted the corresponding mRNA, further ablating its protein product. These oocytes, which exhibit low MPF activity, failed to elongate the c-mos mRNA poly(A) tail, did not accumulate Mos and were unable to activate MAPK. We conclude that an active MPF in rat oocytes is necessary for c-mos mRNA polyadenylation and Mos translation.

Journal of Molecular Endocrinology (2004) **33,** 73–85

Introduction

Meiosis in oocytes of all animals is arrested at the prophase that corresponds to the late G2-phase of the cell-division cycle. The G2-arrested oocytes are characterized by a distinct nuclear structure known as germinal vesicle (GV). Oocytes that escape G2-arrest undergo GV breakdown (GVB) and chromosome condensation. These oocytes proceed to the first metaphase (MI), emit the first polar body (PBI) and are arrested again at the second metaphase (MII) of meiosis. This process, defined as oocyte maturation, is subjected to regulation by the maturation-promoting factor (MPF), which comprises a catalytic 34 kDa Ser/Thr kinase, p34 cdc2, and a regulatory 45 kDa cyclin B1 (reviewed by Dekel 1996). Members of the MAP kinase (MAPK) family, the 42 and 44 kDa Erk 1 and 2, also participate in the regulation of meiosis (for reviews, see Gotoh *et al.* 1995, Dekel 1996). The signal for MAPK activation is mediated by a MAPK kinase (MEK), which, in oocytes, is activated by Mos (for review, see Sagata 1997).

Activation of both p34 cdc2 and MAPK accompanies the reinitiation of meiosis. On the other hand, a cAMP-activated protein kinase A (PKA) maintains the oocytes in meiotic arrest (Maller & Krebs 1977, Dekel & Beers 1978, Bornslaeger *et al.* 1986). The negative effect of cAMP on the resumption of meiosis is associated with an inhibited activation of both MPF (Choi *et al.* 1991, Goren & Dekel 1994) and MAPK (Lazar *et al.* 2002), suggesting that these two enzymes act downstream to PKA. Their sequential order, however, is still a matter of intensive debate.

Simultaneous activation of both enzymes in *Xenopus* oocytes (Gotoh *et al.* 1991*a*,*b*, Nebrada & Hunt 1993) cannot disclose their sequence and, consequently, attempts to study their interrelationships in this animal model generated controversial results. A number of earlier studies suggested that a Mos-activated MAPK is necessary for MPF activation (Yew *et al.* 1992, Kosako *et al.* 1994), and the G2 to M transition (Freeman *et al.* 1989, Sagata *et al.* 1989, Gotoh *et al.* 1995, Haccard *et al.* 1995, Huang *et al.* 1995, Sheets *et al.* 1995, Gross *et al.* 2001). Later reports, however, denied a role for the

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Mos/MAPK pathway at the reinitiation of meiosis (Fisher *et al.* 1999, Gross *et al.* 2000, Dupre *et al.* 2002). On the other hand, other studies demonstrated that MAPK activation is dependent on an active MPF (Ferrell *et al.* 1991, Gotoh *et al.* 1991*a*, Nebrada *et al.* 1995, Culp & Musci TJ 1999, Frank-Vaillant *et al.* 1999).

A similar confusion regarding the interplay between MPF and MAPK has been presented by results generated by using murine oocytes (Zhao *et al.* 1990, O'Keefe *et al.* 1991, Verlhac *et al.* 1994). This confusion in the mouse has been partially resolved by demonstrations showing that the ability of oocytes derived from Mos knockout mice to activate MPF is not impaired (Araki *et al.* 1996, Choi *et al.* 1996, Verlhac *et al.* 1996). Elevation of MPF activity in rodent oocytes resuming meiosis occurs prior to MAPK activation (Gavin *et al.* 1994, Verlhac *et al.* 1994, Zernicka-Goetz *et al.* 1997). This further supports the idea that MPF is not subject to regulation by MAPK. Nevertheless, a reverse hierarchy between these two kinases is possible.

A recent study in our laboratory demonstrated that translation of Mos in rat oocytes is negatively regulated by a PKA-mediated cAMP action. This action that inhibits c-mos mRNA polyadenylation is associated with the suppressed activity of p34 cdc2 (Lazar *et al.* 2002). Interference with p34 cdc2 activity in that study was achieved by exposing the oocytes to roscovitine, a purine analog that potently inhibits the activity of this kinase (Meijer & Kim 1997). Roscovitine, the affinity of which to p34 cdc2, is relatively high, elicits its inhibitory action by competing with ATP binding in a variety of protein kinases (Meijer *et al.* 1997). Therefore, conclusive evidence for the role of MPF in regulating Mos expression required the use of a specific molecular strategy. This requirement has been fulfilled by disturbing p34 cdc2 activity through the double-stranded (ds) RNA interference (RNAi), which was targeted at the expression of cyclin B1 mRNA. Employing this technique, we effectively depleted cyclin B1 mRNA in rat oocytes, thereby further ablating its protein product. Consequently, the oocytes were unable to activate MPF and remained meiotically arrested.

We herein report that in the absence of an active MPF the oocytes failed to elongate the c-mos mRNA poly(A) tail, did not accumulate Mos and were unable to activate MAPK. These results provide definitive evidence that the Mos/MAPK signal transduction pathway in rat oocytes is regulated by MPF.

Materials and methods

Reagents and antibodies

Leibovitz's L-15 tissue culture medium and Lipofectamine were purchased from Gibco BRL (Paisley, UK). Antibiotics were purchased from Bio-Lab Ltd (Jerusalem, Israel). PKI, isobutylmethylxantine (IBMX), N6, 2′-*o*-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP, dbcAMP), Nonident P-40, β-glycerophosphate, PMSF, leupeptine, aprotonin, DTT, ethidium bromide, agarose and fetal bovine serum were purchased from Sigma. Affinity-purified polyclonal goat anti-Mos antibody, mouse anticyclin B1 and mouse anti-p34 cdc2 were purchased from Santa Cruz, Inc. (Santa-Cruz, CA, USA). Polyclonal rabbit anti-general MAPK (G-MAPK, Sigma) and monoclonal mouse anti-double-phosphorylated MAPK (DP-MAPK) were kindly provided by Prof. Rony Seger, Weizmann Institute of Science, Rehovot, Israel. Donkey antigoat, goat anti-mouse and goat antirabbit peroxidase-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). [α-^{32}P]dATP, [γ-^{32}P]ATP and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham. Roscovitine was purchased from Calbiochem (La Jolla, CA, USA). MEGASCRIPT-*in vitro* T7 transcription kit for large-scale synthesis of RNAs was purchased from Ambion (Austin, TX, USA).

dsRNA preparation

Total oocyte RNA was extracted by the RNasole method (Chomczynski & Sacchi 1987) and reverse transcribed, using specific primers, followed by PCR amplification. RT reaction contained 50 U of MMLV-RT, 200 μM dNTP, 6·5 mM $MgCl_2$, 20 U of RNasine, 500 ng Oligo(dT) and 1·5 × RT buffer (Promega). The reaction was carried out at 37 °C for 2 h. The PCR template method was used to synthesize dsRNA. Primers were chosen in order to amplify 650 bases region of exon sequences within the coding part of rat cyclin B1. The following pair of primers were employed: 5′-TGATACTCCCTC

TCCAAG-3′ and 5′-AATGCACCATGTCGT ATG-3′. The 5′ ends of each primer corresponded to a 27 nucleotide T7 promoter sequence (TAATACGACTCACTATAGGGAGACCAC). PCR reaction was further performed in the same RT test tube that finally contained 250 ng of each primer, 200 μM dNTP, 2·5 mM $MgCl_2$, 1 × PCR buffer (Promega) and 2·5 U Taq polymerase. Thirty cycles were conducted after a 5-min incubation at 95 °C as follows: 95 °C for 5 min; 95 °C for 1 min, 62 °C for 1·5 min, and 72 °C for 2 min. This was followed by a final extension for 10 min. The PCR products were phenol/chloroform extracted and ethanol precipitated in NH_4OAc. The precipitate was dissolved in autoclaved, double-distilled water, and the concentration was measured spectrophotometrically. dsRNA synthesis reaction was performed with the MEGASCRIPT-*in vitro* T7 transcription kit for large-scale synthesis of RNAs (Ambion, Austin, TX, USA), according to the instruction manual. Following an overnight incubation, DNA template was removed with DNase 1 (2 U) for 15 min at 37 °C. To stop the reaction, LiCl precipitation solution (7·5 M) was added to the RNA for 30 min at − 20 °C. The precipitate was centrifuged at 4 °C for 15 min, at maximum speed, to obtain the RNA pellet. The supernatant was removed carefully. The pellet was washed with 70% EtOH and recentrifuged to maximize removal of unincorporated nucleotides. The wash solution was removed carefully, and RNA was resuspended in autoclaved, double-distilled water. The presence of dsRNAs was confirmed by ribonuclease protection assay.

Ribonuclease protection assay

Volumes of 350 μl digestion buffer (300 mM NaAc, 10 mM Tris, 5 mM EDTA), 4 μg RNase A and 4 U RNase T1 were added to each sample. After 1-h incubation at 30 °C, 10 μl of 20% SDS and 2·5 μl of 10 mg/ml proteinase K were added, and the samples were incubated for 20 min at 37 °C. The products were extracted with 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was transferred into 1 ml 100% EtOH and 10 μg of glycogen and incubated at − 70 °C for 30 min. Samples were centrifuged for 15 min, EtOH was aspirated and pellets were allowed to dry. The pellets were resuspended in 8 μl of formamide-based loading dye and electrophoresed on 1% ethidium bromide agarose gel. Single-strand RNA synthesized with the MEGASCRIPT T7 *in vitro* transcription kit from control template (linearized TRIPLEscript plasmid containing 1·85 kb *Xenopus* elongation factor 1α gene under transcriptional control of tandem T7 promoters, pTRI-Xef1) served as a positive control.

Oocyte recovery and treatment

The physiological trigger for reinitiation of meiosis in mammalian oocytes is provided by the preovulatory surge of luteinizing hormone (LH) (Tsafriri *et al.* 1972, Tsafriri & Dekel 1994). However, when oocytes are removed from the ovarian follicles and placed in culture, meiosis resumption occurs spontaneously in the absence of gonadotropins (Pincus & Enzmann 1935). These spontaneously maturing oocytes were employed in our study.

The oocytes were recovered from 23-day-old Wistar female rats injected subcutaneously with 10 IU pregnant mare's serum gonadotropin (PMSG, Chrono-gest Intervest, Oss, The Netherlands) and killed 48 h later. The ovaries were removed and preovulatory cumulus–oocyte complexes were isolated into Leibovitz L-15 tissue culture medium containing 10% fetal bovine serum. The cumulus–oocytes complexes were then incubated in an acidic L-15 medium (pH=6·0) to remove the cumulus cells and obtain cumulus-free oocytes.

For dsRNA transfection, the zona pellucida (ZP) was removed by incubation in acid Tyrode's solution (pH=3·5) for a few seconds, followed by several washings in a large volume of L-15 tissue culture medium. Meiotically arrested oocytes, incubated in the presence of dbcAMP (2 mM) and IBMX (20 μM) were transfected by purified dsRNA (33 ng/μl), using cationic liposomes (35 μg/ml). Cationic liposomes devoid of dsRNA were employed to generate the control oocytes. After 7 h, the oocytes were transferred to inhibitor-free medium for an additional incubation time. The oocytes were then subjected to Western blot analysis and RT-PCR.

Other oocytes were incubated for the indicated times in a humidified incubator at 37 °C. These oocytes were examined morphologically for the presence of GV with a StereoZoom 5 Pod microscope (Bosh and Lomb, Rochester, NY, USA). For DNA staining, the oocytes were incubated with live

cells nucleic acid stain, syto-11 (Molecular Probes, Eugene, OR, USA, 1:1000) for 1 h at room temperature. These oocytes were visualized by a laser scanning confocal system (BioRad) connected to a Nikon (TE-300) inverted microscope.

The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory animals (National Research Council, National Academy of Science, Bethesda, MD, USA).

Western blot analysis

Samples of oocytes were extracted in a lysis buffer (β-glycerophosphate 50 mM, EGTA 1·5 mM, EDTA 1 mM, sodium-*o*-vanadate 0·1 mM, benzamidine 1 mM, aprotonine 10 μg/ml, leupeptine 10 μg/ml, pepstatine A 2 μg/ml, DTT 1 mM, PMSF 1 mM), and Laemmli sample buffer (Laemmli 1970) was added. The samples were boiled and loaded on 12% SDS–PAGE, followed by their transfer to a nitrocellulose membrane. Following blocking with TTBS, containing 10% skim milk, the membranes were incubated with the relevant antibodies. The following antibodies were used: affinity-purified polyclonal goat anti-Mos antibody (1:500 dilution), rabbit anti-general MAPK (G-MAPK, 1:5000 dilution), mouse anti-double-phosphorylated MAPK (DP-MAPK, 1:5000 dilution) (Yung *et al.* 1997), mouse anti-cyclin B1 (1:250 dilution) and mouse anti-p34 cdc2 (1:500 dilution). Donkey antigoat, goat antirabbit and goat anti-mouse HRP-conjugated second antibodies (1:5000 dilution) were used as second antibodies. The immunoreactive bands were detected by enhanced chemiluminescence (ECL) reagents. Quantitation of the autoradiograms was performed by densitometric analysis using the 420oe densitometer (Pdi, Huntington Station, NY, USA).

H1 kinase activity

Histone H1 activity was determined as described previously (Josefsberg *et al.* 2003). Briefly, lysates of 50 oocytes were prepared by freezing and thawing in 10 μl kinase buffer (15 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0·1 mM PMSF, 10 μg/ml leopeptin, 10 μg/ml aprotonin, 10 μg/ml PKI, a cAMP-dependent protein kinase inhibitor peptide). Kinase reactions were initiated by the addition of 10 μl substrate buffer (2 mg/ml histone H1, 2 mM dithiothreitol (DTT), 5 μCi [γ-^{32}P]ATP), and the reactions were carried out at 30 °C for 40 min. Kinase reaction products were subjected to SDS–PAGE and autoradiography.

RT-PCR

The p34 cdc2, S16, and c-mos mRNA levels were detected by RT-PCR. For this purpose, total oocyte mRNA was extracted by the acid-guanidinum-phenol/chloroform method (Chomczynski & Sacchi 1987) and reverse transcribed before PCR amplification by specific primers. RT reaction contained 50 U of Moloney murine leukemia virus-reverse transcriptase, 200 μM deoxynucleotide triphosphates (dNTPs), 6·5 mM $MgCl_2$, 20 U ribonuclease inhibitor, 500 mg Oligo(dT) and 1·5 × PCR buffer (Promega). The reaction was carried out at 37 °C for 2 h. Fragments of the reverse-transcribed indicated genes were amplified, and the following pairs of primers were employed: for c-mos gene amplification, 5′-GCACCACGACAACATAATCC-3′, and 5′-CAGCCGAAGTCACTTATCTTAC-3′; for S-16 gene amplification, 5′-cgttcaccttgatgagccc att-3′ and 5′-tccaagggtccgctgcagtc-3′; for cyclin B1 gene amplification, 5′-TGATACTCCCTCTCC AAGCC-3′ and 5′-AATGCACCATGTCGTAG TCC-3′; for p34 cdc2 amplification, 5′-AAA GCGAGGAAGAAGGAGTGCC-3′ and 5′-AGT CCAAGCCGTTTTCATCCAG-3′.

PCR reactions were further performed in the same RT test tube that finally contained 250 ng of each primer, 200 μM dNTP, 2·5 mM $MgCl_2$ 1 × PCR buffer and 2·5 U Taq polymerase (Promega). Thirty and 28 cycles for S-16 and c-mos respectively were performed after 2-min incubation at 94 °C as follows: 94 °C for 30 s; 60 °C for 30 s and 72 °C for 1 min. This was followed by a final extension for 5 min at 72 °C. Thirty cycles for cyclin B1 and p34 cdc2 were performed after a 5-min incubation at 95 °C as follows: 95 °C for 5 min; 62 °C for 1·5 min and 72 °C for 2 min. This was followed by a final extension for 10 min at 72 °C. The products were electrophoresed on 1% ethidium bromide agarose gel.

Analysis of c-mos mRNA polyadenylation

c-mos mRNA polyadenylation was detected by the polymerase chain reaction (PCR), as described by

Lazar *et al.* (2002). Briefly, total oocyte RNA was extracted by the RNasole method (Chomczynski & Sacchi 1987). An aliquot of RNA in H_2O (equivalent to 100–150 oocytes) was heat-denatured (65 °C for 5 min) in a 7 μl volume in the presence of 20 ng phosphorylated Oligo(dt) (Promega) and placed directly at 42 °C. Prewarmed mastermix (13 μl containing 4 μl of 5X Superscript RNase H^- reverse transcriptase (RT) buffer (Gibco BRL Paisley, UK), 2 μl of 0·1 M DTT, 1 μl of 10 mM dNTPs, 1 μl of 10 mM ATP, 4 μl of H_2O and 1 μl of 10 U/μl T4 DNA ligase)) was added, and the samples were incubated at 42 °C for 30 min. Subsequently, 1 μl Oligo(dt)-anchor (200 ng/μl, 5′-GCGAGCTCCG CGGCCGCGT12-3′) was added at 42 °C, and the reaction was transferred to a 12 °C water bath. After 2-h incubation, the samples were transferred back to 42 °C, 2 μl (200 U/μl) Superscript RNase H^- RT was added (Gibco BRL), and reverse transcription (RT) was performed for 1 h. cDNAs were diluted to 6 oocytes/μl, followed by 30-min incubation at 70 °C to inactivate the RT and the ligase. For PCR amplification, 1 μl cDNAs was added to a standard 50 μl PCR reaction spiked with 0·5 μl [α-^{32}P]dATP and containing 25 pmoles each of Mos mRNA specific primer (5′-GCACCACGACAACATAA TCC-3′) and the Oligo(dt)-anchor (amplification conditions: 93 °C for 5 min; 30 cycles at 93 °C for 30 s; 62 °C for 1 min; 72 °C for 1 min; with a final extension of 7 min at 72 °C). After amplification, PCR products were ethanol-precipitated with 2·5 M ammonium acetate to remove unincorporated label. To confirm the specificity of the amplification and demonstrate that the increase of the PCR fragments was attributable to elongation of the 3′ end of the mRNA, samples were digested with XmnI (Promega). This treatment showed heterogeneous 3′ ends. The radioactive products were electrophoresed on 5% nondenaturing polyacrylamide gel in 0·5X TBE buffer. Gels were dried, and radioactivity was determined by their exposure to radiographic film overnight.

Results

Preparation of a cyclin B1 dsRNA

Roscovitine, a selective potent inhibitor of p34 cdc2 kinase (Meijer & Kim 1997), has been recently shown to prevent c-mos mRNA polyadenylation (Lazar *et al.* 2002) and abolish Mos expression

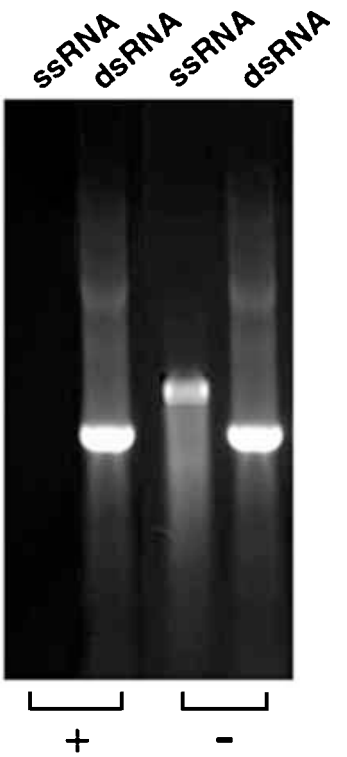
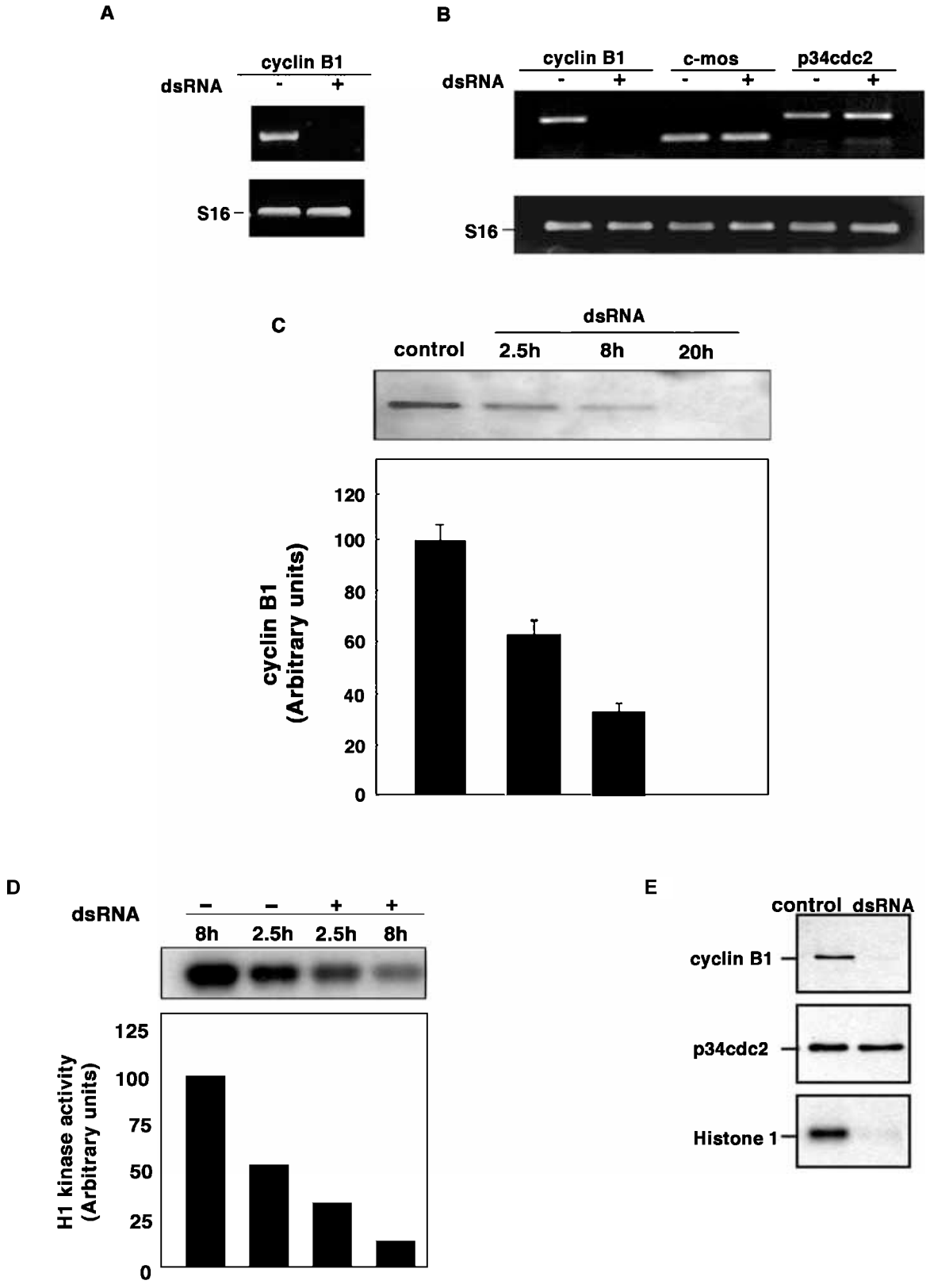


Figure 1 Ribonuclease protection assay. The purified cyclin B1 dsRNA was tested by the RNase protection assay. ssRNA (linearized TRIPLEscript plasmid containing 1·85 kb *Xenopus* elongation factor 1α gene under transcriptional control of tandem T7 promoters, pTRI-Xef1), which was synthesized with the *in vitro* transcription kit, served as positive control for this assay. The results of one representative experiment out of three repetitions are presented.

(Josefsberg *et al.* 2002). Roscovitine does not interfere solely with p34 cdc2 kinase activity, but rather competes with ATP binding in a variety of protein kinases (Meijer *et al.* 1997). In the present study, to prove that MPF participates in regulation of Mos expression, we employed dsRNAi, which was targeted at the expression of cyclin B1 mRNA. For this purpose, cyclin B1 dsRNA was generated by *in vitro* transcription of cyclin B1 template containing a T7 promoter sequence, and the products were subjected to ribonuclease protection assay. This assay confirmed that the *in vitro* transcription output is indeed dsRNA (Fig. 1).

Specificity and efficiency of cyclin B1 RNAi in rat oocytes

Cationic liposomes were employed for the introduction of the cyclin B1 dsRNA into oocytes that


A
cyclin B1
dsRNA
-
+
S16
B
cyclin B1
c-mos
p34cdc2
dsRNA
-
+
-
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-
+
S16
C
dsRNA
control
2.5h
8h
20h
cyclin B1
(Arbitrary units)
120
100
80
60
40
20
0
D
dsRNA
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Histone 1

were maintained in meiotic arrest by dbcAMP and IBMX. To test the efficiency of transfection, these oocytes were examined for the expression of cyclin B1 mRNA at different time points, after removal of the above-mentioned inhibitors. We found that cyclin B1 mRNA was totally eliminated in oocytes incubated for 2·5 h in an inhibitor-free medium (Fig. 2A). No detectable changes were observed in the c-mos and p34 cdc2 mRNAs, indicating that the destruction of cyclin B1 mRNA was highly specific (Fig. 2B). The consequences of interference of cyclin B1 dsRNA with its corresponding mRNA was further confirmed by Western blot analysis. This experiment revealed a substantial reduction in cyclin B1 at 2·5 h, with a further decrease of the protein levels at 8 h (Fig. 2C). Full ablation of the cyclin B1 protein was observed after an additional 12 h of incubation (Fig. 2C).

Cyclin B1 is an essential component of an active MPF. Therefore, the absence of cyclin B1 was functionally examined by the H1 kinase assay. As shown in Fig. 2D, the ability of MPF to phosphorylate histone H1 at 2·5 h of incubation was lower in oocytes transfected with cyclin B1 dsRNA than in controls. The effect of dsRNA transfection on MPF activity was more pronounced at 8 h, a time point at which MPF activity reaches its maximum (Josefsberg *et al.* 2003). Interestingly, activity of MPF could not be detected in oocytes transfected with cyclin B1 dsRNA, despite the comparable amounts of p34 cdc2 demonstrated in the experimental and control groups (Fig. 2E).

MPF activation, upon reinitiation of meiosis, induces GVB and chromosome condensation. The presence of GVB was monitored microscopically in both the experimental ($n=68$) and control ($n=44$) oocytes, and served as an additional functional parameter for the evaluation of the efficiency of cyclin B1 dsRNA transfection. This experiment was complemented by DNA staining. As expected, in the absence of an active MPF, 81% of the the cyclin B1 dsRNA-transfected oocytes failed to undergo GVB, and their chromatin stayed in its diffused configuration (Fig. 3). On the other hand, disappearance of the nuclear structure accompanied by chromosome condensation was

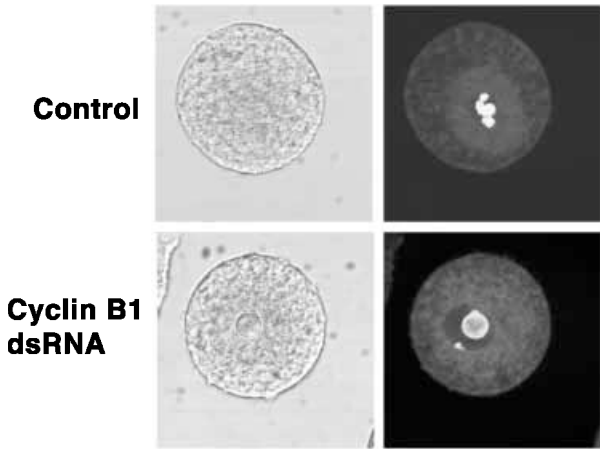

Figure 3 Effect of cyclin B1 dsRNA on the meiotic status of the oocyte. Oocytes incubated in the presence of dbcAMP (2 mM) and IBMX (20 μM) for 7 h were transfected with or without dsRNA (33 ng/μl), using cationic liposomes. After an additional incubation time of 8 h in inhibitor-free medium, DNA was stained with cyto 11 (1/1000), and the oocytes were examined by confocal microscopy. The results of one representative experiment out of three repetitions are presented.

Figure 2 The effect of cyclin B1 dsRNA in rat oocytes. Oocytes incubated in the presence of dbcAMP (2 mM) and IBMX (20 μM) for 7 h were transfected with dsRNA (33 ng/μl), using cationic liposomes. (A) Efficiency of cyclin B1 dsRNA transfection. RNA was extracted from oocytes (20 oocytes/lane) at 2·5 h after their incubation in inhibitor-free medium. RT-PCR was performed with specific primers for cyclin B1. The PCR product was analyzed by ethidium bromide agarose gel electrophoresis. The results of one representative experiment out of three repetitions are presented. (B) Specificity of cyclin B1 dsRNA transfection. RNA was extracted from oocytes (20 oocytes/lane) 8 h after their incubation in inhibitor-free medium. RT-PCR was performed with specific primers for cyclin B1, c-mos, p34 cdc2 and S16 mRNA. PCR products were analyzed by ethidium bromide agarose gel electrophoresis. The results of one representative experiment out of three repetitions are presented. (C) Effect of cyclin B1 dsRNA transfection on cyclin B1 protein. Western blot analysis was performed after 2·5 and 8 h of incubation in inhibitor-free medium, using specific antibodies against cyclin B1. The results of one representative experiment out of three repetitions are presented. (D) Effect of cyclin B1 dsRNA transfection on the activity of MPF. MPF activity was assayed in oocytes collected at 2·5 and 8 h of incubation in inhibitor-free medium. The results of one representative experiment out of three repetitions are presented. (E) Effect of cyclin B1 dsRNA transfection on the presence of p34 cdc2. Western blot analysis was performed after 20-h incubation in inhibitor-free medium, using specific antibodies against cyclin B1 and p34 cdc2. MPF activity was assayed by H1 kinase activity. The results of one representative experiment out of three repetitions are presented.

clearly observed in 95% of the control oocytes transfected by cationic liposomes devoid of dsRNA.

Effect of cyclin B1 mRNA depletion on c-mos mRNA polyadenylation, Mos accumulation and MAPK activity

Having established the successful depletion of the cyclin B1 mRNA, we further examined the oocytes for the length of their c-mos mRNA poly(A) tail. We previously reported that the c-mos mRNA in rat meiotically arrested oocytes possesses a poly(A) tail of 50 bp that was elongated by about 300 bases towards the MI of meiosis (Lazar *et al.* 2002). Similarly, control oocytes that were allowed to resume meiosis in the present study expressed a c-mos mRNA poly(A) tail of about 350 bp (Fig. 4A). On the other hand, the c-mos mRNA in the cyclin B1 dsRNA-transfected oocytes exhibited a poly(A) tail of only 50 bp, suggesting failure of polyadenylation under these conditions.

We further examined the oocytes for their capacity to translate the mos-mRNA and accumulate its protein product by Western blot analysis. This experiment could not detect Mos in the cyclin B1 dsRNA-transfected oocytes (Fig. 4B). Since Mos is the upstream regulator of MAPK, our assumption was that the absence of Mos would be further manifested by the failure of the oocytes to activate MAPK. Indeed, depletion of cyclin B1 resulted in the absence of the phosphorylated, active form of MAPK (Fig. 4C).

The MPF-sensitive window for Mos translation

Activation of MPF in rat oocytes is intially observed at 2 h after the onset of meiosis and reaches its maximum at 8 h (Josefsberg *et al.* 2003). However, the presence of Mos in these oocytes could not be detected until 6 h after the reinitiation of meiosis (Lazar *et al.* 2002). In order to define the MPF-sensitive window for c-mos mRNA polyadenylation and Mos accumulation, we used the following experimental strategy: freshly isolated oocytes were incubated for different time periods, which allowed for the initial stages of MPF activation. The oocytes were then transferred into medium containing roscovitine for cessation of p34 cdc2 kinase activity. After the total incubation time of 8 h, the oocytes were analyzed for c-mos mRNA polyadenylation and the presence of Mos. We

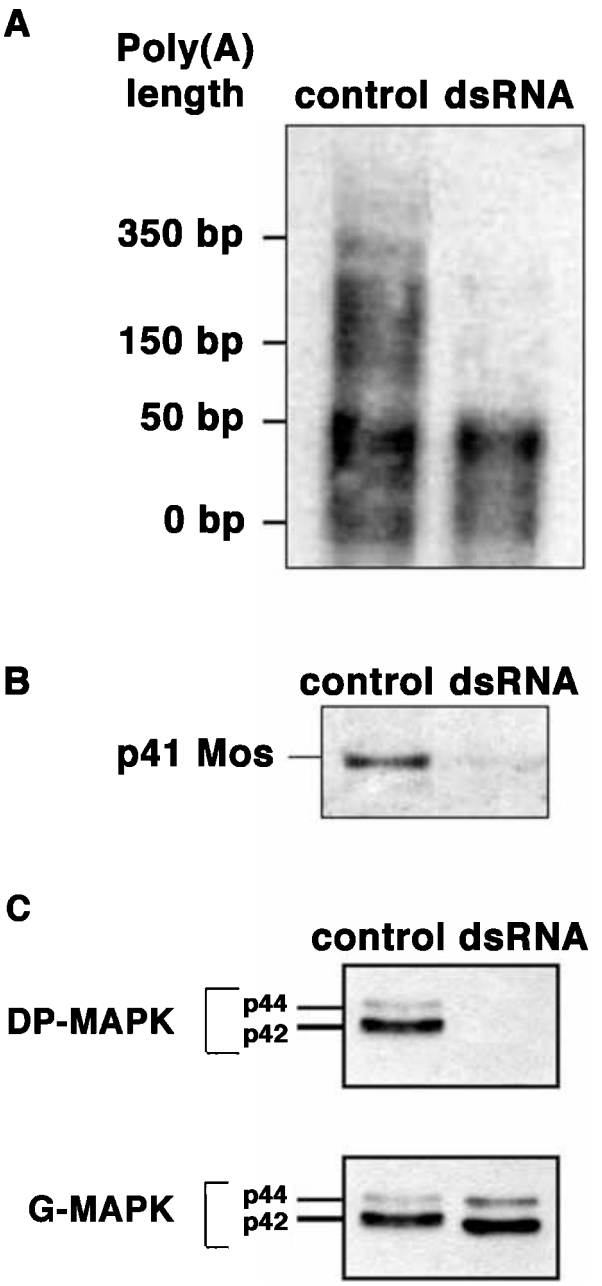

Figure 4 Effect of cyclin B1 dsRNA on c-mos mRNA polyadenylation, Mos expression and MAPK activation. Oocytes incubated in the presence of dbcAMP (2 mM) and IBMX (20 μM) for 7 h were transfected with or without dsRNA (33 ng/μl), using cationic liposomes. After an additional 20 h of incubation in inhibitor-free medium, the oocytes were subjected to poly(A) tail analysis (PAT) of c-mos mRNA (A), Western blot analysis with anti-Mos antibodies (B), and Western blot analysis with specific antibodies against DP-MAPK and G- MAPK (C). The results of one representative experiment out of three repetitions are presented.

found that at least 3 h of incubation in roscovitine-free medium are required for the elongation of the c-mos mRNA poly(A) tail (Fig. 5A) and the subsequent expression of Mos (Fig. 5B). Elevated activity of MPF, generated for a shorter time interval, is apparently insufficient to allow mos translation (Fig. 5B). A substantial elevation in the fraction of oocytes with an intact GVB, monitored between 2 and 3 h of incubation in roscovitine-free medium (Fig. 5C), suggests that a similar MPF-sensitivity window applies to the dissolution of the nuclear structure; this is the first morphologic marker for oocyte maturation.

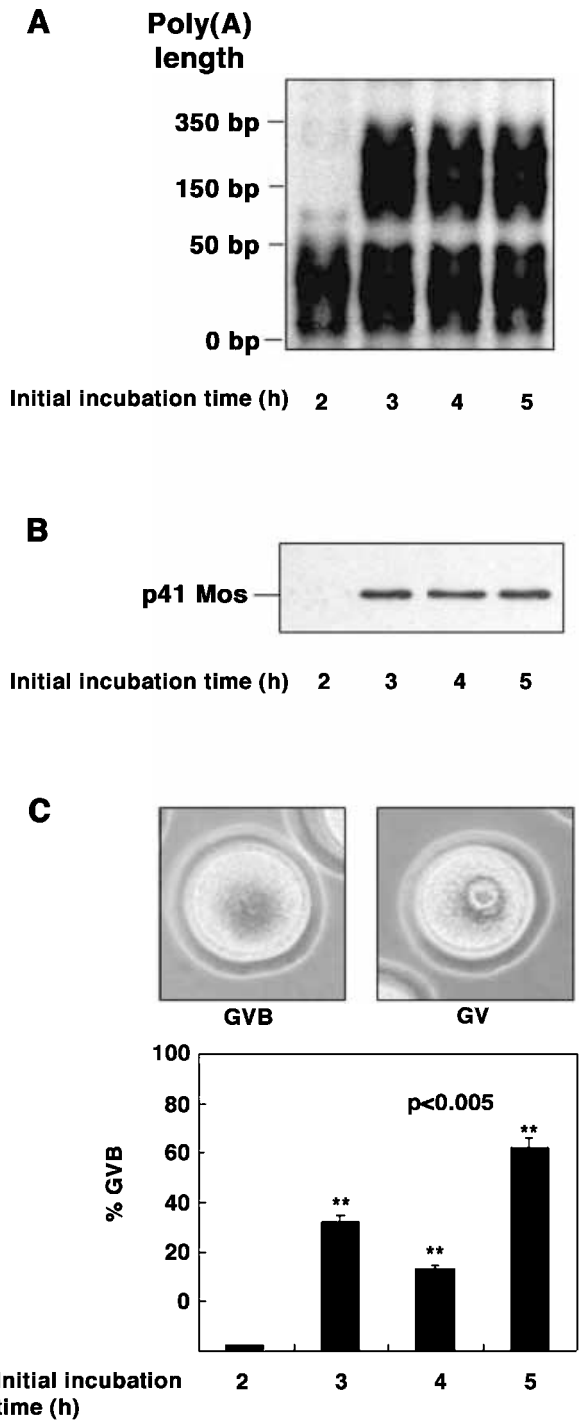

Figure 5 The MPF-sensitive window for c-mos mRNA polyadenylation, Mos translation and GVB. Freshly isolated oocytes (500/lane) were exposed to roscovitine-free medium for the indicated periods of time. The oocytes were then transferred into medium containing roscovitine (100 μM) for further incubation. After a total incubation time of 8 h, poly(A) tail analysis (PAT) of c-mos mRNA (A) and Western blot analysis, using anti-Mos antibodies (B), were performed. The results of one representative experiment out of three repetitions are presented. Another group of the above oocytes were monitored for the presence of GVB (C). Means±standard errors of quantitative analysis of three experiments are presented.

Discussion

We herein report the first successful dsRNA-mediated interference with cyclin B1 mRNA. This efficient and specific ablation of cyclin B1 gene expression was followed by a time-dependent elimination of the cyclin B1 protein. As a result, the oocytes failed to activate MPF and remained meiotically arrested. We employed this experimental model to prove that rat oocytes unable to activate MPF will not promote c-mos mRNA cytoplasmic polyadenylation and its subsequent translation. As anticipated, these oocytes failed to activate MAPK.

In a previous attempt to shed light on the specific role of cyclin B1 in vertebrate cells, the classical technique of gene 'knockout' was employed (Brandeis *et al.* 1998). However, this experimental approach that eliminated the gene function universally resulted in embryonic lethality, failing to generate the information of interest. A later study, which used the antisense strategy specifically to ablate cyclin B1, demonstrated its dispensability at entry into M-phase in *Xenopus* oocytes (Hochegger *et al.* 2001). The dsRNA-mediated RNAi, directed at destruction of cyclin B1 mRNA in individual rat oocytes employed in the present

study, allowed full clarification of the role of this gene at the G2/M transition of meiosis in mammals.

Genetic and biochemical studies suggest that the RNAi pathways evolved as a defense against RNA viruses or transposones, possibly before plants and animals diverged. This phenomenon, first described less than two decades ago (Marcus 1983), allows targeted genes to be efficiently silenced, revolutionizing functional genomics. This experimental approach has been previously shown to ablate targeted mRNA in lower organisms such as *Caenorhabditis elegans* (Fire *et al.* 1998, Montgomery *et al.* 1998, Fire 1999), zebrafish (Li *et al.* 2000) and *Drosophila* (Kenerdell & Carthew 1998, Tuschl *et al.* 1999, Hammond *et al.* 2000). Specific interference of gene function by dsRNA treatment has only recently been achieved in mice (Svoboda *et al.* 2000, Wianny & Zernicka-Goetz 2000). In view of the fact that mammalian cells typically respond to dsRNA accumulation after viral infection, by an overall block of translation (Lee & Esteban 1994), the successful demonstration of RNAi in the mouse was somewhat surprising. This success may be attributed, at least in part, to the lack of such a response in oocytes and the early embryonic stages that served as experimental models in these studies.

Maturing oocytes are known to be transcriptionally dormant. Protein synthesis in these oocytes is enhanced by recruitment of pooled mRNA and its selective cytoplasmic polyadenylation (reviewed by Colgan & Manley 1997). Unlike previous studies that chose to ablate dormant mRNAs prior to their translation (Svoboda *et al.* 2000, Wianny & Zernicka-Goetz 2000), the cyclin B1 mRNA that was targeted in our study was translated, and the G2-arrested oocytes expressed a certain amount of its protein product. Our strategy took into account the oscillatory pattern of cyclin B1 accumulation that is regulated by a modulated balance between its synthesis and degradation (Winston 1997). We had postulated that interference with cyclin B1 translation would shift this balance, eventually resulting in protein elimination. Our experimental protocol was designed to allow the degradation of the pre-existing cyclin B1 in oocytes, maintained throughout transfection in a state of meiotic arrest by their incubation in dbcAMP/MIX. The removal of these inhibitors allowed control oocytes to resume meiosis spontaneously. However, the cyclin B1 dsRNA-transfected oocytes that had their corresponding mRNA ablated did not translate cyclin B1, failed to activate MPF and remained meiotically arrested.

We have previously shown that MPF activity in rat oocytes is elevated immediately after meiosis reinitiation (Josefsberg *et al.* 2003), whereas the accumulation of Mos is substantially delayed (Lazar *et al.* 2002). The relatively extended time interval that elapses between the initial increase in MPF action and c-mos mRNA translation may raise some concerns with regard to the direct correlation between these two events. By transiently exposing rat oocytes to a roscovitine-free medium, followed by their transfer into medium containing this p34 cdc2 inhibitor, we defined the MPF-sensitive window for Mos translation. This experiment revealed that the level of MPF activity generated at 3 h, rather than at 2 h after meiosis reinitiation, allowed the elongation of the c-mos mRNA poly(A) tail and the subsequent expression of Mos. There was a substantial increase in the fraction of oocytes undergoing GVB after 3 h of incubation in roscovitine-free medium; this suggested that the dissolution of the nuclear membrane, which is the hallmark of the resumption of meiosis, exhibited a similar range of sensitivity to MPF. The kinetic analysis of cyclin B1 depletion in the dsRNA-transfected oocytes demonstrated that this experimental modification did indeed take place within the MPF-sensitive window for c-mos mRNA polyadenylation and Mos expression.

The inhibitory action of cAMP on the resumption of meiosis is well established (Cho *et al.* 1974, Dekel & Beers 1978, 1980). This negative effect of cAMP, shown to inhibit the activation of the two key regulators of meiosis, MPF (Choi *et al.* 1991, Goren & Dekel 1994) and MAPK (Lazar *et al.* 2002), raises the question of whether these enzymes operate independently, in parallel, or sequentially. Attempts to disclose the sequential order of these two enzymes, made in amphibians as well as murine oocytes, produced conflicting results (reviewed by Castro *et al.* 2001). Generation of the Mos-deficient mouse model (Colledge *et al.* 1994, Hashimoto *et al.* 1994) suggested that, at least in rodent oocytes, MPF activation is independent of the Mos/MAPK signaling pathway (Hashimoto 1996), leaving the option of a reverse sequential order open. We clearly show that MPF activation is an essential precondition for c-mos mRNA polyadenylation, Mos translation and MAPK activation. A different experimental

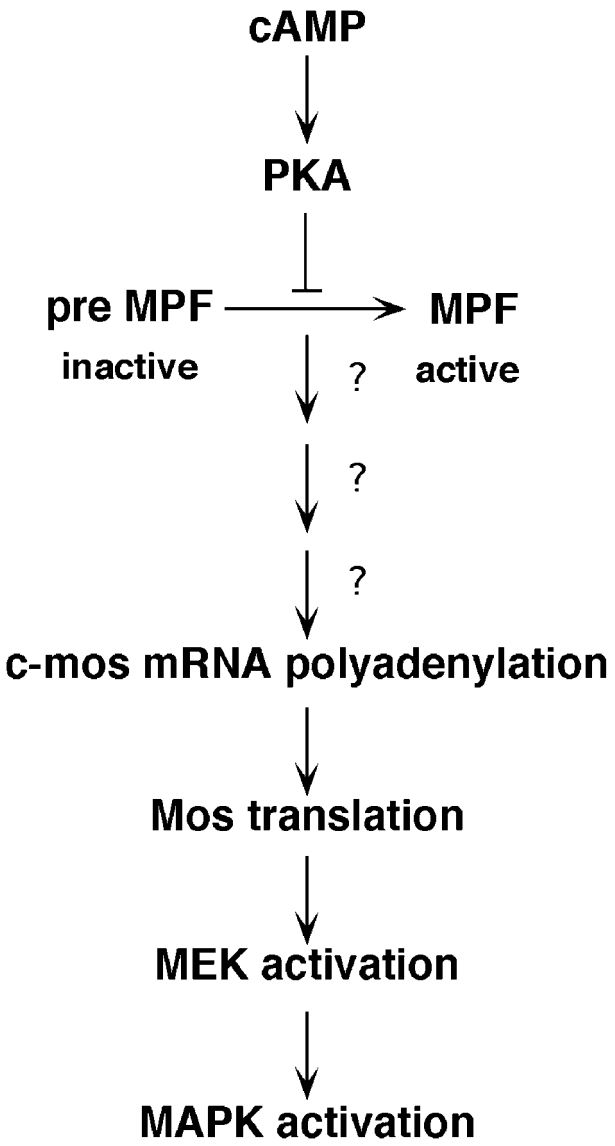

Figure 6 A proposed model for regulation of Mos expression in rat oocytes resuming meiosis. The transition of the inactive pre-MPF into the active MPF is suppressed by active PKA. Upon the drop of intraoocyte concentration of cAMP at reinitiation of meiosis, PKA becomes inactive and MPF is activated. The active MPF induces c-mos mRNA polyadenylation and subsequent Mos translation. The signal is transmitted to MEK, which activates MAPK.

approach that we previously employed also suggested that MAPK activation is governed by MPF (Josefsberg *et al.* 2003). These findings, combined with our previous report of a PKA-mediated cAMP inhibition of c-mos mRNA polyadenylation (Lazar *et al.* 2002), establish the hirearchy of the molecular events that reinitiate meiosis in oocytes as follows: the meiosis reinitiation-associated drop in intraoocyte concentrations of cAMP leads to an inactivation of PKA that allows the transition of the inactive pre-MPF into active MPF (Duckworth *et al.* 2002). Elevated activity of MPF switches on the cascade of events, although yet unresolved, that recruit c-mos mRNA for the polyadenylation inducing Mos translation. This accumulated Mos further stimulates MEK, which, in turn, upregulates the activity of MAPK (Fig. 6).

Acknowledgements

This work was supported by the Dwek Fund for Study of Fertility. ND is the incumbent of the Phillip M. Klutznick Professorial Chair for Developmental Biology. We thank Ms Martie Spiegel for editing the manuscript of this study.

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Received in final form 17 March 2004
Accepted 31 March 2004
Made available online as an Accepted Preprint 14 April 2004