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Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes

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Meiosis is characterized by the absence of DNA replication between the two successive divisions. In *Xenopus* eggs, the ability to replicate DNA develops during meiotic maturation, but is normally suppressed until fertilization. Here we show that development of the DNA-replicating ability depends on new protein synthesis during meiosis I, and that mere ablation of the endogenous c-*mos* product Mos allows maturing oocytes to enter interphase and replicate DNA just after meiosis I. Moreover, we demonstrate that during normal maturation cdc2 kinase undergoes precocious inactivation in meiosis I and then premature reactivation before meiosis II; importantly, this premature cdc2 reactivation absolutely requires Mos function and its direct inhibition by a dominant-negative cdc2 mutant also results in nuclear reformation and DNA replication immediately after meiosis I. These findings indicate that suppression of DNA replication during meiotic divisions in *Xenopus* oocytes is accomplished by the Mos-mediated premature reactivation of cdc2 kinase. We suggest that these mechanisms for suppressing DNA replication may be specific for meiosis in animal oocytes, and that the ultimate biological function, including the well known cytostatic factor activity, of Mos during meiotic maturation may be to prevent undesirable DNA replication or parthenogenetic activation before fertilization.

Key words: c-*mos*/DNA replication/meiosis/oocyte maturation/parthenogenesis/*Xenopus* oocyte

Introduction

From the standpoint of the cell cycle, meiosis is characterized by the absence of DNA replication between the two successive divisions, meiosis I (MI) and meiosis II (MII) (Hotta, 1988; John, 1990). In the eggs of many animal species, however, parthenogenetic activation can be induced either naturally or experimentally (Masui, 1985; John, 1990), indicating that the eggs already have the ability to replicate DNA for immediate use after fertilization. In *Xenopus* eggs, this ability develops during oocyte maturation (Gurdon, 1967; Benbow and Ford, 1975), which encompasses the MI/MII transition (Masui and Clarke, 1979). In these eggs, therefore, the DNA replication-inducing ability must somehow be suppressed during maturation for meiosis to be accomplished.

In *Xenopus*, meiotic maturation of prophase I-arrested oocytes is triggered by progesterone and is followed by activation of the maturation-promoting factor (MPF), a stoichiometric complex of cdc2 kinase and cyclin B (Nurse, 1990), which causes germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation (Masui and Markert, 1971; Masui and Clarke, 1979). The activity of MPF falls soon after GVBD, i.e. at a time believed to correspond to the end of MI, but soon rises again to induce MII (Gerhart *et al.*, 1984). During this MI/MII transition, nuclear membranes do not reform, chromosomes remain condensed and DNA replication does not occur (Huchon *et al.*, 1981; Gerhart *et al.*, 1984). In MII, mature oocytes are arrested again at metaphase II by the cytostatic factor (CSF), which appears only during maturation and stabilizes MPF (Masui and Markert, 1971; Newport and Kirschner, 1984).

The c-*mos* proto-oncogene product Mos, a protein kinase (Maxwell and Arlinghaus, 1985; Watanabe *et al.*, 1989), is also only expressed during *Xenopus* oocyte maturation (Sagata *et al.*, 1988) and disappears on fertilization (Watanabe *et al.*, 1989; Nishizawa *et al.*, 1993). Its synthesis is both required and sufficient for the initiation of MI (Sagata *et al.*, 1989a; Freeman *et al.*, 1989; Yew *et al.*, 1991). It is also required for the normal MI/MII transition (Daar *et al.*, 1991; Kanki and Donoghue, 1991) and in MII it functions as an essential component of CSF to cause metaphase II arrest (Sagata *et al.*, 1989b). Thus, although Mos appears to function during the MI/MII transition, possible relevance of this function to the suppression of DNA replication during this period is not known. In any event, these functions of Mos during maturation are all thought to be exerted via activation or stabilization of MPF (Sagata *et al.*, 1988, 1989a,b; Freeman *et al.*, 1989; Daar *et al.*, 1991; Kanki and Donoghue, 1991; Yew *et al.*, 1991). Essentially similar but less extensive results have been obtained on mouse Mos (O'Keefe *et al.*, 1989; Paules *et al.*, 1989).

In this work we examined when and how the DNA replication-inducing ability develops during *Xenopus* oocyte maturation and how it is suppressed during the MI/MII transition. We show that the DNA replication-inducing ability develops shortly after GVBD in a protein synthesis-dependent manner, and that realization of this ability is apparently suppressed via the function of Mos. Moreover, by precisely monitoring both cytological and biochemical events during maturation, and by specifically inhibiting either Mos function or cdc2 kinase activity in maturing oocytes, we obtain strong evidence that Mos functions to prematurely reactivate MPF (or cdc2 kinase) well before MII, thereby preventing entry into interphase and DNA replication that

would otherwise occur immediately after MI. We discuss that the Mos-mediated suppression of DNA replication may be specific for meiosis in animal oocytes, and that the ultimate biological function, including the CSF function (Sagata *et al.*, 1989b), of Mos during meiotic maturation may be the prevention of undesirable DNA replication or parthenogenetic activation before fertilization.

Results

Development of DNA replication-inducing ability

During *Xenopus* oocyte maturation, the ability to induce DNA replication develops some time after GVBD (Gurdon, 1967; Benbow and Ford, 1975), but it is unknown whether this ability depends on new protein synthesis. On the other hand, if applied around the time of GVBD, cycloheximide (CHX; a protein synthesis inhibitor) prevents oocytes from reactivating MPF on entry into MII (Gerhart *et al.*, 1984), but it is unknown whether these oocytes have reformed interphase nuclei and undergone DNA replication. To answer these questions, first we subjected oocytes to continuous CHX treatment from various times either before or after GVBD. These oocytes, which had been preinjected with [α-^{32}P]dCTP, were harvested individually 4 h after GVBD and their DNAs were analyzed. In untreated oocytes or those treated with CHX 60, 30 or 0 min before GVBD, the nuclear DNA showed very weak radioactive signals, which turned out to be mostly due to repair synthesis (see the legend to Figure 1A). By contrast, the nuclear DNA of oocytes treated with CHX 30 or 60 min after GVBD showed strong signals. This marked DNA synthesis was sensitive to aphidicolin (APD; a specific inhibitor of the DNA replicative enzyme or DNA polymerase α) (Figure 1A) and occurred only transiently between 120 and 150 min after GVBD (i.e. at a time corresponding to the MI/MII transition in normally maturing oocytes; see later) (Figure 1B). Thus, this synthesis was almost certainly replicative, probably occurring only once in a semi-conservative manner [in fact, we confirmed

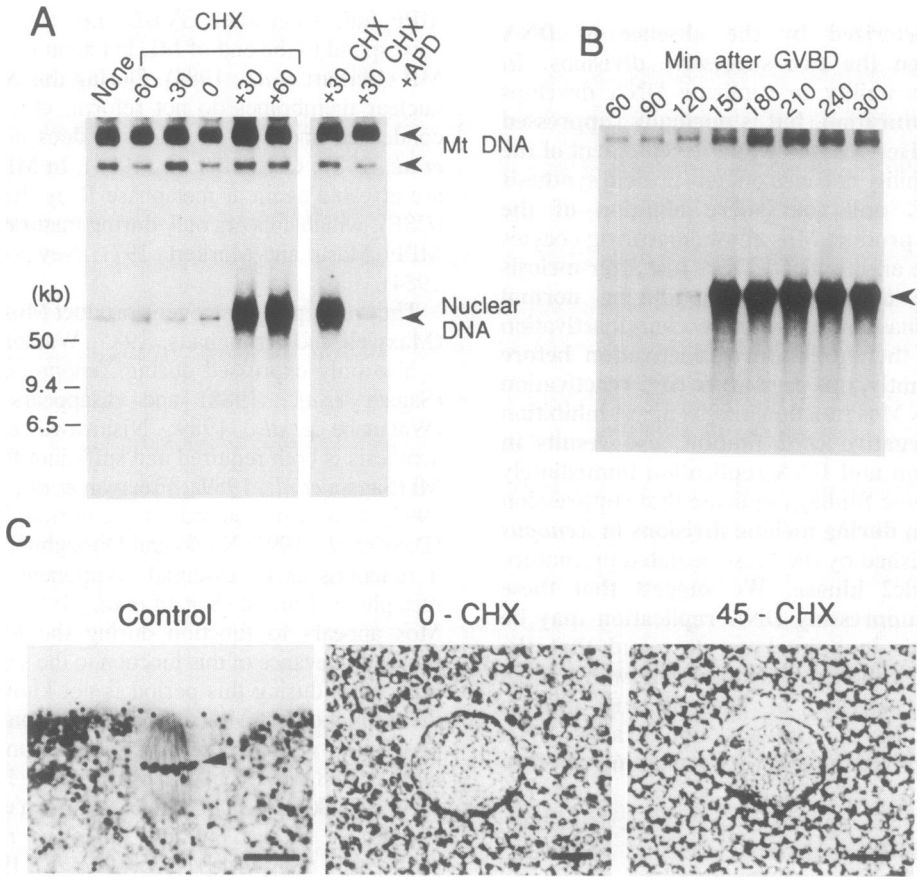

Fig. 1. DNA replication in CHX-treated oocytes. **(A)** Dependence of DNA replication on the time of CHX treatment. Maturing oocytes preinjected with [α-^{32}P]dCTP were untreated (None) or treated continuously with CHX from the indicated time (min) before (−) or after (+) GVBD. Oocytes were harvested individually 4 h after GVBD for analysis of DNA by agarose gel electrophoresis (~0.5 oocyte-equivalent per lane). Oocytes 30 min after GVBD were also treated with APD together with CHX. The upper bands represent closed and open circular mitochondrial (Mt) DNA, while the lower band represents sheared nuclear DNA fragments. The very weak [α-^{32}P]dCTP incorporation into the nuclear DNA of untreated oocytes or those treated with CHX before or at GVBD was largely sensitive to dideoxythymidine triphosphate (not shown), indicating that it was mainly due to repair synthesis (Jenkins *et al.*, 1992). DNA molecular size markers are indicated in kb on the left. **(B)** Time of DNA replication in CHX-treated oocytes. Oocytes preinjected with [α-^{32}P]dCTP were subjected to continuous CHX treatment from 45 min after GVBD, collected at the indicated times (after GVBD) and processed as for (A). The prominent increase in [α-^{32}P]dCTP incorporation into the nuclear DNA only between 120 and 150 min indicates that DNA replication probably occurred only once during this period (see also text). Arrowhead, nuclear DNA. **(C)** Cytology of CHX-treated oocytes. Untreated maturing oocytes (Control) or those treated with CHX at (0-CHX) or 45 min after (45-CHX) GVBD were cultured, harvested 4 h after GVBD and subjected to cytological examination. The control oocyte showed a metaphase II spindle with condensed chromosomes (arrowhead) and one expelled polar body (not shown here, but observed in another serial section; see Figure 3A for reference), while both CHX-treated oocytes showed an interphase nucleus with a clear nuclear membrane and decondensed chromosomes (as well as one expelled polar body; not shown here). Bars represent 10 μm.

this using a density-label protocol (Blow and Laskey, 1988); data not shown]. These analyses show that DNA replication does occur in CHX-treated oocytes, but only when CHX treatment is applied after GVBD (Table I). This conditional DNA replication indicates that accumulation of some new protein(s) around GVBD is required for the CHX-treated oocytes to undergo DNA replication. Thus, development of the DNA replication-inducing ability after GVBD (Gurdon, 1967) is almost certainly protein synthesis-dependent.

We then examined the cytology of oocytes that had been treated with CHX either at or 45 min after GVBD and then cultured for 4 h after GVBD. Control oocytes without CHX treatment showed a metaphase II spindle with condensed chromosomes, indicating the completion of maturation (Figure 1C). By contrast, oocytes treated with CHX at or 45 min after GVBD had a single nucleus (with decondensed chromosomes) and one expelled polar body, indicating that both had completed MI and reformed an interphase nucleus. However, the oocytes treated with CHX at GVBD were probably arrested in a G_1-like phase, whereas those treated with CHX after GVBD were in a G_2-like phase, because only the latter had undergone DNA replication, as described above. Thus, the new protein(s) that is accumulated shortly after GVBD and needed for DNA replication is likely to be a protein needed for the G_1/S transition.

Ablation of Mos allows DNA replication

The above analyses show that maturing oocytes shortly after GVBD acquire the ability to replicate DNA after MI in a protein synthesis-dependent manner. However, this ability is normally not realized until fertilization, implying that it is somehow suppressed during maturation. Since DNA replication did occur after protein synthesis inhibition, however, its suppression must also depend on a new or labile protein(s). One plausible candidate for this protein is the c-*mos* proto-oncogene product Mos, which is synthesized only during oocyte maturation (Sagata *et al.*, 1988, 1989b; Watanabe *et al.*, 1989), is very unstable (except in MII) (Nishizawa *et al.*, 1992), and is required for the normal MI/MII transition (Daar *et al.*, 1991; Kanki and Donoghue,

Table I. DNA replication and nuclear reformation in maturing oocytes treated with various reagents[a]

Treatment[b]	DNA replication		Nuclear reformation	
	Total No. of treated oocytes	No. of DNA replication-positive oocytes[c] (%)	Total No. of treated oocytes	No. of oocytes with an interphase nucleus[c,d] (%)
CHX (Figure 1)				
−60 min	25	0 (0)	ND	ND
−30 min	24	0 (0)	20	20 (100)
0 min	29	2 (7)	22	22 (100)
+30 min	34	34 (100)	26	26 (100)
+60 min	30	30 (100)	15	15 (100)
c-*mos* oligos (Figure 2)				
sense	37	8[e] (22)	25	6[e] (24)
antisense	40	31 (78)	26	21 (81)
Antibodies (Figures 2 and 3)				
control IgG	33	0 (0)	15	0 (0)
anti-Mos	30	29 (97)	15[f]	7[f] (47)
GST−Mos ± CHX (Figure 2)				
GST−Mos	18	0 (0)	ND	ND
GST−Mos + CHX	33	33 (100)	ND	ND
cdc2 mRNA (Figure 4)				
WT	40	2[g] (5)	17	1[g] (6)
K33R	43	41 (95)	18	18 (100)

[a]Summary of experiments described in Figures 1−4.
[b]For experimental conditions for each treatment, see the legend to the figure specified in parentheses. For each treatment, at least three independent experiments were performed.
[c]Most of these oocytes showed more or less surface (or pigment) changes ~2 h after GVBD, probably reflecting entry into interphase.
[d]Except for those in the −30 min CHX treatment group, most of these oocytes had a single expelled polar body (some without a polar body might have lost it during fixation or sectioning). As for the nuclear morphology, nuclei reformed in oocytes injected with c-*mos* antisense oligonucleotides or anti-Mos antibody often showed a lobed appearance (cf. Figure 2B), while those in oocytes treated with CHX or injected with K33R *cdc2* mRNA mostly showed a round appearance (cf. Figures 1C and 4D).
[e]Probably due to a cytotoxic effect(s), such as partial, non-specific inhibition of endogenous protein synthesis by the injected oligonucleotides (40 ng per oocyte) (Smith *et al.*, 1990; our unpublished data); at a higher dose of >80 ng per oocyte, higher proportions of oocytes entered interphase and replicated DNA, the proportion approaching that in oocytes injected with antisense oligonucleotides (not shown).
[f]Observed 150 min after GVBD. The oocytes without an interphase nucleus (eight out of 15 oocytes, or 53%) and all oocytes observed 3−4 h after GVBD had a 'monaster'-like structure (see Figure 3C). In contrast, most of the oocytes observed 120 min after GVBD were in apparently normal anaphase I (see Figure 3C). Thus, in the oocytes injected with anti-Mos antibody, interphase nucleus reformation was transient, probably occurring only during the period of DNA replication. The progression of interphase nuclei to 'monaster'-like structures in these oocytes may be attributed to synthesis of a new or labile protein(s) (presumably protein 'X'; see Discussion) during this period, since interphase nuclei reformed in oocytes whose protein synthesis was inhibited either totally (by CHX treatment) or partially (by injection with c-*mos* oligonucleotides or *cdc2* mRNA; see notes e and g) persisted even after DNA replication, at least up to 3−4 h after GVBD (see text).
[g]Probably due to partial, non-specific inhibition of endogenous protein synthesis by competition of the injected mRNA with endogenous mRNAs for translation factors (Laskey *et al.*, 1977; our unpublished data) (see also the legend to Figure 4B).
ND, not determined.

1991). We therefore tested whether oocytes depleted of Mos reform interphase nuclei and undergo DNA replication like oocytes treated with CHX after GVBD. For this, we injected [α-^{32}P]dCTP (Newport and Kirschner, 1984) and either c-*mos*-specific sense or antisense oligonucleotides (Sagata *et al.*, 1988) into oocytes undergoing GVBD. When examined 4 h after GVBD, most (>75%) of the oocytes injected with control sense oligonucleotides exhibited a normal metaphase II spindle (Figure 2B) and no sign of DNA replication (Figure 2A), although a small proportion showed an interphase nucleus and DNA replication (Table I). However, this situation was reversed substantially in oocytes injected with antisense oligonucleotides; as many as 80% of these oocytes reformed an interphase nucleus (often with a lobed appearance) after expelling a polar body and replicated DNA (Figure 2A and B and Table I).

As above, c-*mos* antisense oligonucleotides at the dose used (40 ng/oocyte) gave significant, but still not convincing, results (for the reason for this and for the results obtained with a higher dose of oligonucleotides; see the legend to Table I). To confirm these results, therefore, we used a polyclonal anti-Mos antibody that has neutralizing activity against Mos both *in vivo* and *in vitro* (see Materials and methods). We injected [α-^{32}P]dCTP and either the anti-Mos antibody or control preimmune rabbit IgG into oocytes just undergoing GVBD and examined whether these oocytes replicated DNA. We found that virtually all (>95%) oocytes injected with anti-Mos antibody underwent DNA replication, while none of the oocytes injected with control IgG did so (Figure 2A and Table I). Moreover, the oocytes injected with anti-Mos antibody reformed interphase nuclei, albeit transiently just after MI, while those injected with control IgG did not (for details see later and Table I). These results and those obtained with antisense oligonucleotides strongly indicate that mere and specific inhibition of Mos function around GVBD is sufficient to allow maturing oocytes to enter interphase (after MI) and replicate DNA. Thus, Mos indeed seems to be the new protein required for suppressing the DNA replication-inducing ability that develops soon after GVBD.

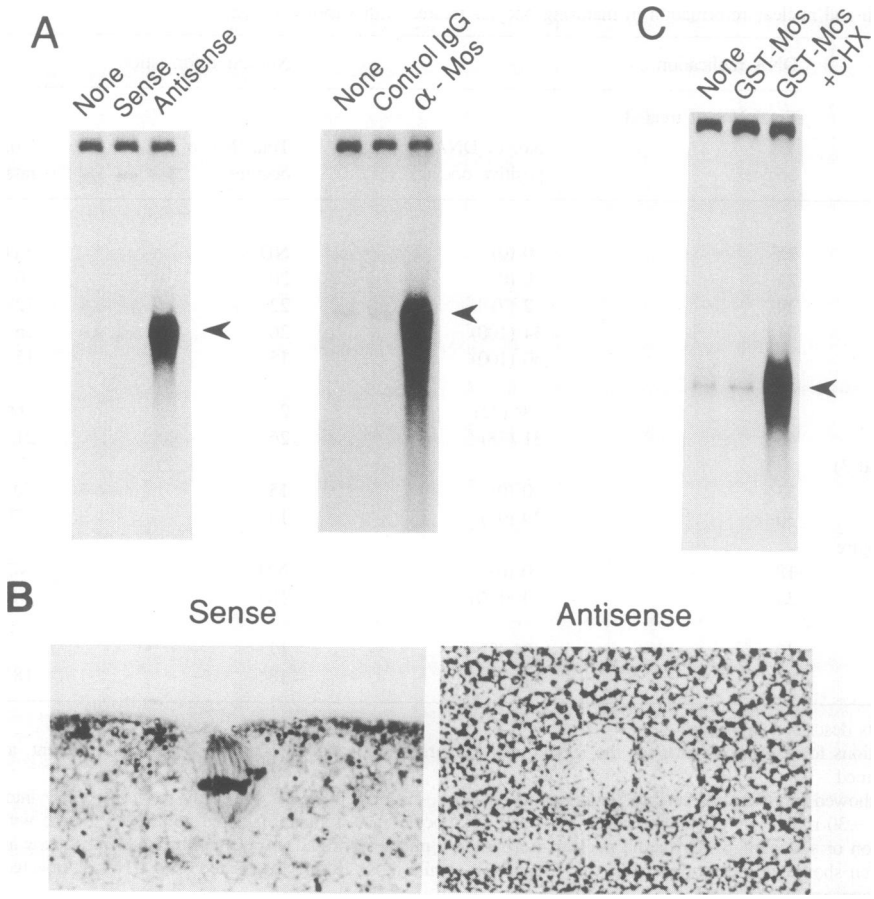

Fig. 2. Requirement of Mos for suppression of DNA replication during maturation. (**A**) DNA replication in maturing oocytes depleted of Mos. Oocytes prelabeled with [α-^{32}P]dCTP were not injected (None) or injected at GVBD with either c-*mos*-specific sense or antisense oligonucleotides (left panel). Prelabeled oocytes were also injected at GVBD with either control preimmune rabbit IgG (Control IgG) or affinity-purified polyclonal anti-Mos antibody (α-Mos) (right panel). These oocytes were harvested individually 4 h after GVBD and processed as described in Figure 1A. Each lane represents a typical result from a single oocyte (see Table I for detailed results). Arrowhead, nuclear DNA. (**B**) Cytology of oocytes injected with c-*mos*-specific oligonucleotides. Oocytes undergoing GVBD were injected with c-*mos*-specific sense or antisense oligonucleotides, cultured and harvested 4 h after GVBD for cytological examination. More than 80% of the oocytes injected with antisense oligonucleotides reformed an interphase nucleus, often with a lobed appearance (and with one expelled polar body; not shown here), while >75% of oocytes injected with sense oligonucleotides showed an essentially normal metaphase II spindle (see Table I for detailed results). (**C**) DNA replication in oocytes without new protein synthesis except for Mos. At GVBD, oocytes preloaded with [α-^{32}P]dCTP were not injected (None) or injected with GST−Mos fusion protein, and half the injected oocytes were treated with CHX from 45 min after GVBD (GST−Mos + CHX). Oocytes were cultured, collected 4 h after GVBD and analyzed as described in Figure 1A. See also Table I. Arrowhead, nuclear DNA.

We then tested whether Mos is the only new protein needed for suppressing DNA replication during maturation. To do this, we injected maturing oocytes (at GVBD) with [α-^{32}P]dCTP and glutathione *S*-transferase (GST)−Mos fusion protein, which is stable and active in oocytes (see Materials and methods), and treated them with CHX 45 min after GVBD (i.e. shortly after the development of DNA replication-inducing ability; see Figure 1A). Results showed that despite the presence of GST−Mos, the oocytes replicated DNA (Figure 2C and Table I). This suggests that synthesis of another new or labile protein(s), besides Mos, is also required to suppress DNA replication during maturation.

Premature reactivation of MPF and involvement of Mos in it

As above, Mos functions to suppress DNA replication during maturation (after MI), but probably does not act directly. A likely mediator for this Mos function is then MPF itself, since Mos is required for reactivation of MPF on entry into MII (Daar *et al.*, 1991; Kanki and Donoghue, 1991; Yew *et al.*, 1991; see below) and this reactivation could prevent maturing oocytes from entering interphase and replicating DNA after MI. If so, during the MI/MII transition, MPF, under the influence of Mos, might be in some specific state that is accessible to the reactivation. We therefore examined precisely both the cytology and the activity and molecular state of MPF in normally maturing oocytes as well as in Mos-ablated oocytes.

Cytology. First, we examined the cytology of normally maturing oocytes every 15 min after GVBD to determine the normal time of the MI/MII transition, which has so far been only poorly analyzed (Huchon *et al.*, 1981; Gerhart *et al.*, 1984). The morphology and configuration of the spindle and chromosomes changed during maturation as shown in Figure 3A. The MI/MII transition (defined here as anaphase I to prometaphase II) occurred 120−150 min after GVBD, during which time the chromosomes remained condensed and no interphase nuclei were observed, as noted previously (Huchon *et al.*, 1981; Gerhart *et al.*, 1984). This time of the transition was highly reproducible and was essentially similar to that determined recently by quite different cytological methods (Gard, 1992).

MPF activity (histone H1 kinase activity). The MPF activity or its histone H1 kinase activity falls and rises again soon after GVBD (Gerhart *et al.*, 1984; Daar *et al.*, 1991; Kanki and Donoghue, 1991; Yew *et al.*, 1991). In parallel with the cytological examination described above, we monitored the H1 kinase activity of maturing oocytes after GVBD. Results revealed reproducibly that the activity began to fall 30−45 min after GVBD (prometaphase I), reached its lowest level at 70−80 min (early metaphase I) and then rapidly rose at 105−120 min (late metaphase I to anaphase I) (upper panel in Figure 3B). Thus, the fall and rise of MPF activity after GVBD occurred much earlier than the cytologically staged MI/MII transition, which occurred 120−150 min after GVBD (Figure 3A). This finding was surprising, because the transient MPF inactivation after GVBD has been widely believed to coincide exactly with the MI/MII transition (Gerhart *et al.*, 1984; Daar *et al.*, 1991; Kanki and Donoghue, 1991; Yew *et al.*, 1991; Gabrielli *et al.*, 1993).

Phosphorylation of cdc2 Thr161. The MPF activity is positively regulated by the phosphorylation at a threonine residue (Thr161) of its catalytic subunit cdc2 kinase (Draetta, 1993). Western blot analysis of cdc2 showed that during maturation (after GVBD) it consisted of apparently three forms (in terms of electrophoretic mobility) (middle panel in Figure 3B). However, the form with the highest mobility, which is known to be phosphorylated only at Thr161 and to be active (Lorca *et al.*, 1992; our unpublished data), was more or less absent between 45 and 105 min after GVBD. Thus, dephosphorylation of Thr161 coincided well with the transient MPF inactivation, occurring substantially before the MI/MII transition (see above).

Phosphorylation of cdc2 Tyr15. Contrary to Thr161, phosphorylation at Tyr15 of cdc2 kinase negatively controls the MPF activity and only occurs in interphase of the cell cycle (Nurse, 1990; Krek and Nigg, 1991a). Western blot analysis of cdc2 with anti-phosphotyrosine antibodies revealed that Tyr15 phosphorylation did not occur either during the transient MPF inactivation or during the MI/MII transition, whereas it was readily detected in immature oocytes arrested in G2 as described previously (Ferrell *et al.*, 1991) (lower panel in Figure 3B). Thus, interestingly, Tyr15 of cdc2 kinase remained dephosphorylated even during its transient inactivation after GVBD, indicating unusual regulation of the kinase during this specific period.

Events occurring in Mos-ablated oocytes. We also examined the cytology and the activity and phosphorylation state of cdc2 kinase in oocytes that had been injected at GVBD with the neutralizing anti-Mos antibody. These oocytes were apparently in normal anaphase I at 120 min after GVBD, as were oocytes injected with control IgG, but soon reformed an interphase nucleus transiently at 130−150 min and then formed a 'monaster'-like structure (Figure 3C, upper panel and Table I) (for the probable reason of transient nuclear reformation, see the legend to Table I). On the other hand, the H1 kinase activity in these oocytes was low at 120 min and remained so thereafter (Figure 3C, lower panel), indicating that the rapid MPF reactivation before MII did not occur in these oocytes. In addition, when examined at 150 min after GVBD, Thr161 of cdc2 kinase in these oocytes was dephosphorylated and Tyr15 was phosphorylated, though very weakly (last lanes of the middle and lower panels, respectively, in Figure 3B). Thus, the oocytes injected with anti-Mos antibody failed to enter MII because of their inability to rapidly reactivate MPF before MII.

In summary, during normal maturation, the inactivation and reactivation of MPF (or Thr161 dephosphorylation/phosphorylation of cdc2 kinase) after GVBD occurred much earlier (by ~60 min) than the cytologically staged MI/MII transition and was not accompanied by Tyr15 phosphorylation. In Mos-ablated oocytes, however, the rapid reactivation of MPF before MII was abolished and the cytology and the phosphorylation state of cdc2 kinase became interphasic (and even DNA replication occurred; Figure 2). These findings indicate that Mos somehow functions to prematurely reactivate MPF well before MII.

A dominant-negative cdc2 mutant allows DNA replication

The above results indicate the possibility that the Mos-mediated premature reactivation of MPF directly induces

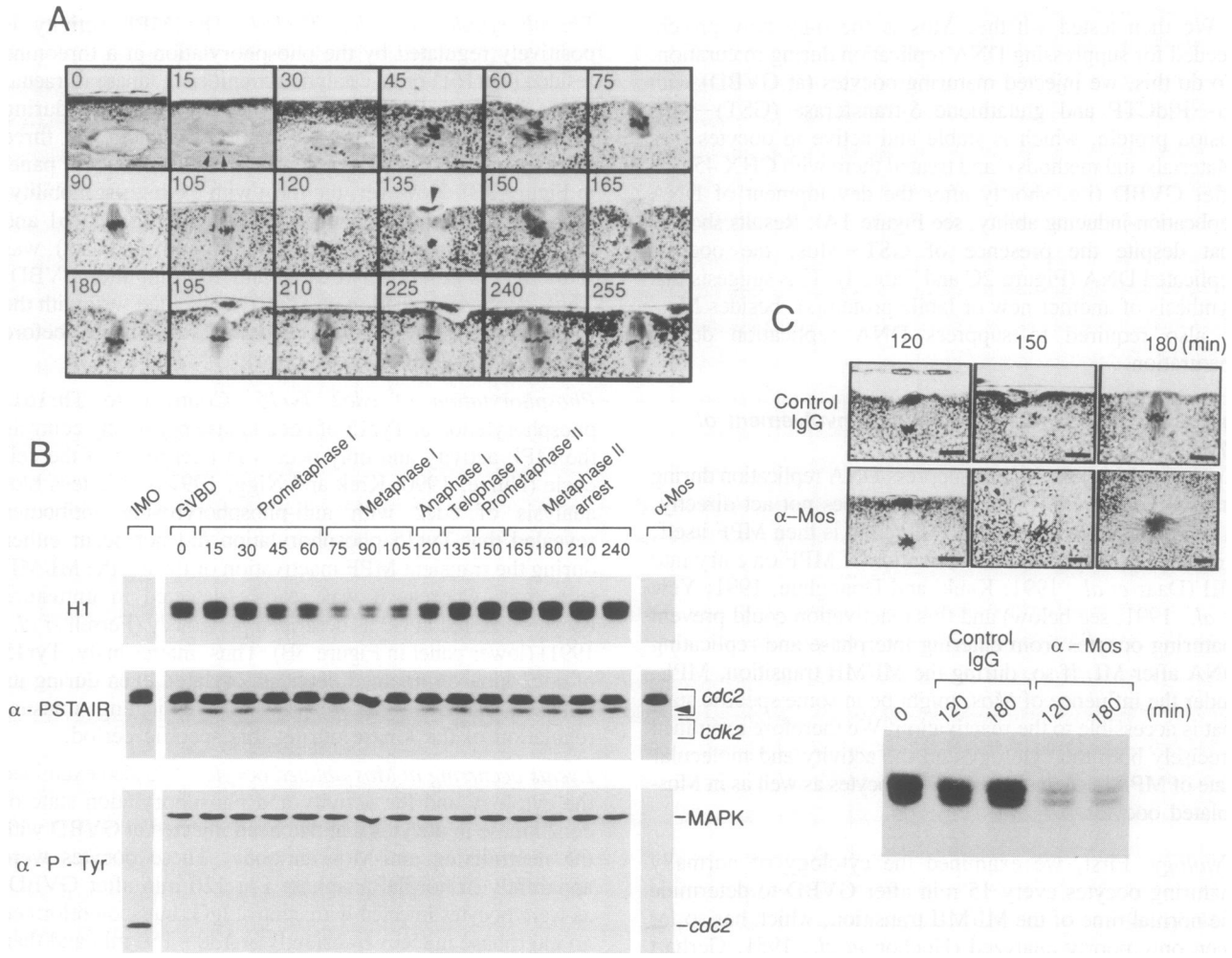

Fig. 3. Changes in the cytology and the activity and molecular form of MPF (or cdc2 kinase) during maturation. (**A**) Maturing oocytes were examined cytologically at the indicated times (in min) after GVBD. Morphological changes were as follows: 0 min, breakdown of the germinal vesicle; 15 min, formation of dot-like condensed chromosomes (arrowheads) at the basement of the broken-down germinal vesicle; 30 min, formation of a transversely (with respect to the animal−vegetal axis)-oriented bipolar spindle with condensed chromosomes dispersed on it (early prometaphase I); 45−75 min, gradual rotation of the spindle to a perpendicular orientation and movement of chromosomes to the spindle center (mid-prometaphase I to early metaphase I); 90−105 min, maintenance of the perpendicularly oriented bipolar spindle with chromosomes aligned on its center (metaphase I); 120 min, separation of chromosomes (anaphase I); 135 min, clustering of compacted chromosomes (telophase I) and elimination of the first polar body (arrowhead); 150 min, reformation of a (diagonally oriented) bipolar spindle with highly condensed chromosomes dispersed on it (prometaphase II); 165 min, rotation of the spindle to a perpendicular orientation and chromosome movement to the spindle center; 180 min and thereafter (up to 255 min, the final time point of observation), maintenance of the perpendicularly oriented spindle with chromosomes aligned on its center (metaphase II arrest). The bar represents 50 μm (for panels 0 and 15), 20 μm (for panel 30) or 10 μm (for panel 45, which has the same magnification as those of panels 60−255). (**B**) Time courses of changes in the histone H1 kinase activity and the phosphorylation state of cdc2 kinase during maturation. Maturing oocytes at the indicated times (min) after GVBD were subjected to *in vitro* histone H1 kinase assays (H1) or Western blot analysis with either anti-PSTAIR antibody (α-PSTAIR) or anti-phosphotyrosine antibody (α-P-Tyr). Cell cycle stages determined in parallel [see (A)] are indicated at the top. Immature oocytes (IMO; first lanes), as well as oocytes injected with anti-Mos antibody at GVBD and then cultured for 150 min (α-Mos; last lanes), were also analyzed similarly. In the middle panel (α-PSTAIR), the band indicated by the arrowhead corresponds to a Thr161-phosphorylated form of cdc2 kinase (Lorca *et al.*, 1992; our unpublished data); on long exposure of the film, two fast-migrating bands of cdk2 kinase, a cognate kinase of cdc2 (Pines, 1993), were also detected in the region indicated. In the lower panel (α-P-Tyr), the identity of cdc2 was confirmed by its abilities to react with anti-PSTAIR antibody and to bind to $p13^{suc1}$ beads (data not shown); a band of MAP kinase (MAPK) was also detected as indicated. (**C**) Cytology and H1 kinase activity of oocytes injected with anti-Mos antibody (α-Mos). Oocytes undergoing GVBD were injected with either α-Mos or control preimmune rabbit IgG, harvested at the indicated times (after GVBD or injection) and then subjected to either cytological examination (upper panel) or *in vitro* H1 kinase assay (lower panel). Bars in the upper panel represent 10 μm.

MII, thereby preventing entry into interphase and DNA replication that would otherwise occur immediately after MI. [Generally, the M phase is dominant over interphase; Johnson and Rao (1970).] If this is true, direct inhibition of the premature MPF reactivation would allow maturing oocytes to enter interphase and replicate DNA after MI. We tested this possibility by injecting oocytes at GVBD with mRNA (60 ng) encoding either wild-type cdc2 kinase or a kinase-defective (Lys33 → Arg) cdc2 mutant (Solomon *et al.*, 1992). (This K33R mutant was found to act in a dominant-negative manner, apparently by competing with endogenous cdc2 kinase for cyclin B; see also Materials and methods). After the injections, these oocytes expressed 3- to 15-fold higher levels of wild-type or K33R cdc2 protein than the endogenous protein 1−4 h after GVBD (Figure 4A). Under these conditions, almost all oocytes expressing wild-type cdc2 matured essentially normally, as evidenced by oscillation of the H1 kinase activity, formation

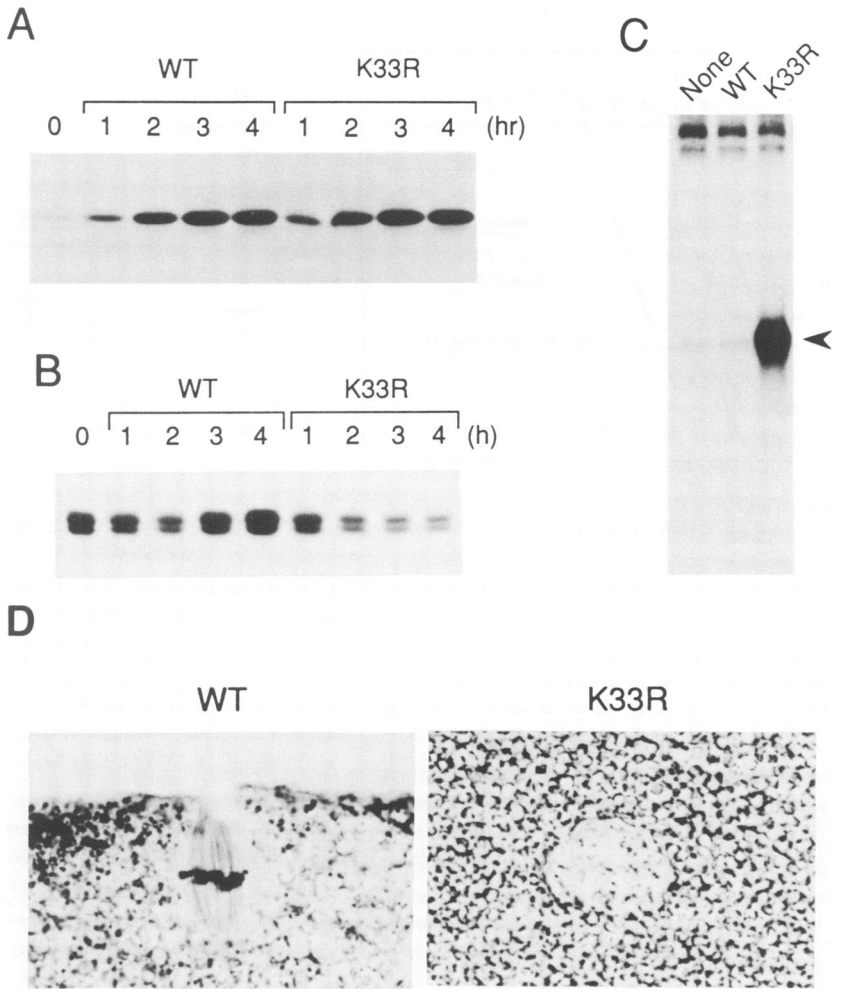

Fig. 4. DNA replication in oocytes expressing a dominant-negative cdc2 mutant. (**A**) Levels of wild-type cdc2 kinase and a dominant-negative cdc2 mutant ectopically expressed in maturing oocytes. Oocytes undergoing GVBD were injected with mRNA (60 ng) encoding either wild-type cdc2 kinase (WT) or a dominant-negative (Lys33 → Arg) cdc2 mutant (K33R). At the indicated times (in h) after GVBD (or injection), they were subjected to Western blot analysis using anti-PSTAIR antibody. In uninjected control oocytes, the level of the endogenous cdc2 kinase (cf. 0 time) did not change appreciably during maturation (cf. Figure 3B). (**B**) The time course of change in the histone H1 kinase activity in maturing oocytes expressing either WT cdc2 kinase or a K33R cdc2 mutant. Oocytes undergoing GVBD were injected as above. At the indicated times (in h) after GVBD (or injection) they were subjected to histone H1 kinase assay. The slower reincrease in the H1 kinase activity in WT mRNA-injected oocytes than that in uninjected normally maturing oocytes (cf. Figure 3B) is probably due to partial, non-specific inhibition of endogenous protein synthesis by the injected mRNA (data not shown). (**C**) DNA replication in oocytes expressing a K33R cdc2 mutant. Oocytes prelabeled with [α-^{32}P]dCTP were not injected (None) or injected at GVBD with mRNA encoding either WT cdc2 kinase or a K33R cdc2 mutant. They were harvested 4 h after GVBD and analyzed as described in Figure 1A. See also Table I. Arrowhead, nuclear DNA. (**D**) Cytology of oocytes expressing WT cdc2 kinase or a K33R cdc2 mutant. Unlabeled oocytes injected as described above were examined cytologically 4 h after GVBD.

of a normal metaphase II spindle and no DNA replication (Figure 4). In sharp contrast, virtually all oocytes expressing the dominant-negative K33R cdc2 mutant did not show the reincrease in the H1 kinase activity (Figure 4B), entered interphase after MI (Figure 4D) and even replicated DNA (Figure 4C) (Table I). These results clearly show that mere inhibition of cdc2 reactivation is sufficient to allow maturing oocytes to enter interphase and replicate DNA just after MI. Thus, the Mos-mediated premature reactivation of cdc2 kinase or MPF before MII indeed seems to be the cause of suppression of DNA replication after MI.

Discussion

In this work we examined the mechanisms of suppression of DNA replication during meiotic divisions in *Xenopus* oocytes. Figure 5 summarizes the conclusions and implications drawn from our study.

Dependence of DNA replication-inducing ability on new protein synthesis

During *Xenopus* oocyte maturation, the ability to induce DNA replication develops some time after GVBD (Gurdon, 1967; Benbow and Ford, 1975). This ability has been ascribed to a cytoplasmic factor(s) designated 'DNA synthesis inducer' (Gurdon, 1967) or 'initiation factor (I-factor)' (Benbow and Ford, 1975), but its molecular nature is unknown. We have shown that maturing oocytes can enter interphase and even replicate DNA just after MI if they are treated continuously with CHX from 30 min after, but not at or before GVBD (Figure 1). This indicates that development of the DNA replication-inducing ability depends on a new protein(s) that is synthesized and accumulated

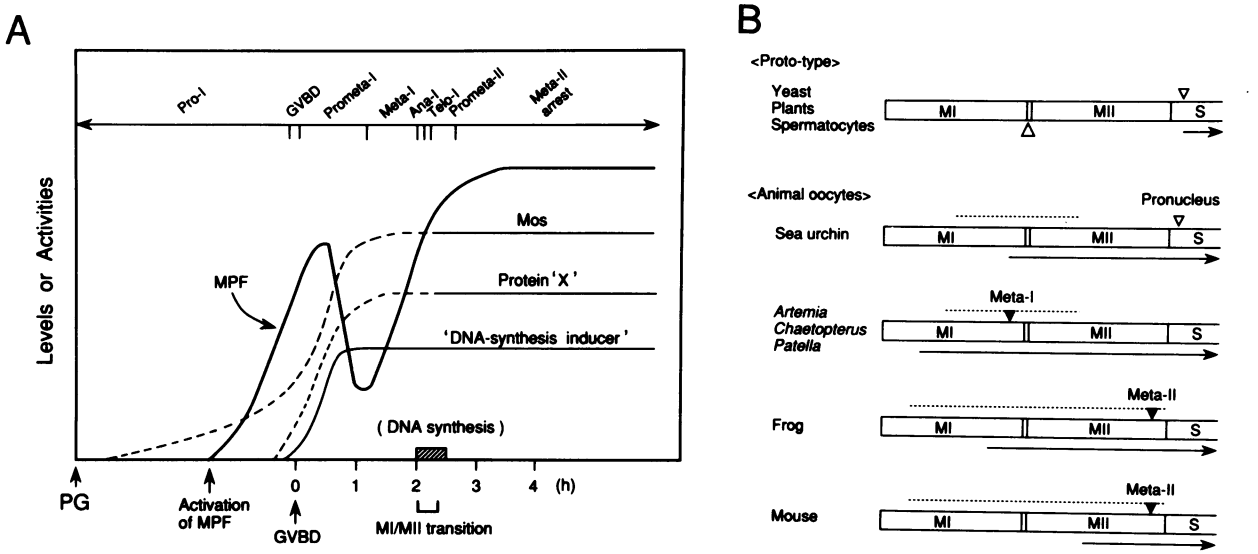

Fig. 5. Expression patterns of factors involved in DNA replication-inducing ability and its suppression during normal *Xenopus* oocyte maturation (**A**) and possible mechanisms of suppression of DNA replication in various types of meiosis (**B**). (A) Results obtained in this work are summarized. Development of the DNA replication-inducing ability depends on a new protein(s) ('DNA-synthesis inducer' in the figure), which is synthesized and accumulated around GVBD. This ability is normally suppressed during maturation and until fertilization, apparently via functions of Mos and protein 'X', which are also newly synthesized during oocyte maturation and somehow act to prematurely reactivate MPF (or cdc2 kinase) well before MII. This premature MPF reactivation is almost certainly the direct cause of the suppression of DNA replication after MI, as demonstrated by using a dominant-negative cdc2 mutant. The expression pattern of Mos has been described (Sagata *et al.*, 1988; Watanabe *et al.*, 1989). [During MI, Mos is very unstable (Nishizawa *et al.*, 1992), as indicated by the dashed line.] Protein 'X' is assumed to be expressed after GVBD and to be unstable at least during MI (to account for the results in Figure 2C). The biological function of Mos (as CSF) in metaphase II arrest (Sagata *et al.*, 1989b) may also be understood to be the prevention of DNA replication prior to fertilization, as discussed in the text. The time of DNA replication in oocytes treated with CHX (after GVBD) or injected with anti-Mos antibody (at GVBD) is indicated by the shaded box. Pro-I, prophase I; Prometa-I, prometaphase I; Meta-I, metaphase I; Ana-I, anaphase I; Telo-I, telophase-I; Prometa-II, prometaphase-II; Meta-II, metaphase II; GVBD, germinal vesicle breakdown; MI, meiosis I; MII, meiosis II; PG, progesterone. (**B**) Possible models for omitting DNA replication in various types of meiosis. Meioses in yeast, many plants and animal spermatocytes are postulated to be the prototype and to lack components required for, and thus simply to 'omit', entry into S phase or DNA replication during their MI/MII transitions (denoted by △). (Relevant to this postulation may be the fact that MI/MII transitions in these meioses commonly proceed up to nuclear reformation and partial chromosome decondensation; Hotta, 1988; John, 1990). In contrast, animal eggs of many species develop DNA-replicating ability during oocyte maturation for immediate use after fertilization (Masui and Clarke, 1979; Benbow, 1985). In these cases, therefore, DNA replication must be actively 'suppressed' during maturation for meiosis to occur successfully. Based on the present study, we postulate that development of the DNA-replicating ability depends on new protein synthesis, and that suppression of this ability is accomplished at least partly by the function of Mos (and protein 'X') or its functional equivalent, which appears only during maturation. However, the time of expression of the new protein needed for DNA replication (solid arrow) and that needed for its suppression (dashed line) seem to differ depending on the species. In vertebrates (frog and mouse), the protein needed for DNA replication probably appears shortly before [in frogs (Gurdon, 1967; Benbow and Ford, 1975; and this study)] or during [in mice (Clarke *et al.*, 1988; Kubiak, 1989)] MII, while the protein (i.e. Mos) needed for suppressing DNA replication is expressed almost continuously throughout maturation (up to metaphase II arrest also caused by this protein; Paules *et al.*, 1989; Sagata *et al.*, 1989a; Watanabe *et al.*, 1989). In invertebrate oocytes arrested at metaphase I (*Artemia*, *Chaetopterus* and *Patella*), parthenogenetic activation (and hence DNA replication) can be induced by a protein synthesis inhibition (Fautrez and Fautrez-Firlefyn, 1961; Zampetti-Bosseler *et al.*, 1973; Néant and Guerrier, 1988). In these species, therefore, the protein needed for DNA replication must have already appeared before metaphase I arrest, and the functional equivalent of Mos or protein 'X' may appear spanning the MI/MII transition (to suppress DNA replication during the transition, which is triggered by fertilization). Even in exceptional invertebrate oocytes that complete meiosis before fertilization (sea urchin), the Mos- or protein 'X'-equivalent protein might appear spanning the MI/MII transition, since the protein needed for DNA replication is already present in the mature egg (Young *et al.*, 1969) and so must have appeared during maturation. Mos (and protein 'X') or its functional equivalent probably suppresses DNA replication by prematurely reactivating MPF to directly induce MII; this premature MPF reactivation may be responsible for condensed chromosomes and the absence of nuclear reformation that are commonly seen during the MI/MII transitions in oocytes of many species (Masui, 1985; John, 1990). Finally, metaphase arrest, whether in MI or MII, may also be understood as a way for preventing DNA replication before fertilization, as discussed in the text, and may require another new or labile protein(s), besides Mos or its functional equivalent, as suggested in *Xenopus* oocyte maturation (Yew *et al.*, 1991; Gabrielli *et al.*, 1993). In some species, this new protein may be cyclin (van Loon *et al.*, 1991; Kubiak *et al.*, 1993). MI, meiosis I; MII, meiosis II; S, S phase; Meta-I, metaphase I; Meta-II, metaphase II; ▼, metaphase arrest; ▽, post-meiotic arrest.

around GVBD (Figure 5A). This protein, which may well correspond to the 'I-factor', seems to be required for the G_1/S transition, because oocytes treated with CHX at or before GVBD also reformed an interphase nucleus (but did not replicate DNA and were thus arrested presumably in G_1) (Figure 1C). Cdk2 kinase and cyclin A (Pines, 1993), which are both newly synthesized and accumulated during maturation (Kobayashi *et al.*, 1991; Gabrielli *et al.*, 1993), are good candidates for such a protein (though our preliminary results showed that this is not the case). Our results and experimental systems may help to identify this important protein.

Involvement of Mos (and protein 'X') in the suppression of DNA replication

The DNA replication-inducing ability is normally suppressed, not to be realized during maturation (until fertilization) (Masui and Clarke, 1979; Benbow, 1985). This suppression must also depend on a new or labile protein(s), because inhibition of protein synthesis in maturing oocytes permitted DNA replication as described above. Using c-*mos* antisense oligonucleotides and neutralizing anti-Mos antibodies, we showed clearly that one of the new proteins needed for suppressing DNA replication is Mos; mere inhibition of Mos function around GVBD allowed oocytes

to enter interphase and replicate DNA after MI (Figure 2). (Thus, interphase nucleus reformation in oocytes treated with CHX can be attributed, at least in part, to inhibition of Mos synthesis.) Besides Mos, however, another new or labile protein(s) (referred to hereafter as 'X') also appeared to be necessary for suppressing DNA replication, since maturing oocytes injected with stable GST−Mos protein and then treated with CHX still showed DNA replication (Figure 2C). This protein may be related to the protein postulated to be necessary for entry into MII (Yew *et al.*, 1991; see also the legend to Table I), but cannot be cyclin A or B whose new synthesis is not required for normal maturation (Minshull *et al.*, 1991). Indeed, protein 'X' might be maturation-specific as Mos is (Sagata *et al.*, 1988, 1989b), because injection of Mos alone into fertilized eggs did not appear to suppress DNA replication (not shown). Thus, Mos and protein 'X' may function cooperatively to suppress DNA replication in a maturation-specific manner (Figure 5A).

Mechanisms of suppression of DNA replication

To explore how Mos (and protein 'X') suppresses the DNA replication-inducing ability that develops soon after GVBD, we monitored the cytology and the activity and molecular state of MPF (or its catalytic subunit cdc2 kinase) during maturation. To our surprise (cf. Gerhart *et al.*, 1984; Daar *et al.*, 1991; Kanki and Donoghue, 1991; Yew *et al.*, 1991; Gabrielli *et al.*, 1993), we found that MPF is precociously inactivated after GVBD (during prometaphase I) and is prematurely reactivated well before MII (during metaphase I to anaphase I) (Figures 3B and 5A). [This finding suggests that anaphase is not necessarily triggered directly by MPF inactivation, as shown recently in other systems (Holloway *et al.*, 1993; Surana *et al.*, 1993). The precocious inactivation of MPF before metaphase I may also occur during starfish oocyte maturation—see Figure 2 in Galas *et al.* (1993)—and may thus be universal in the animal kingdom.] Moreover, even during the transient MPF inactivation, cdc2 kinase remained dephosphorylated on Tyr15, whereas it was transiently dephosphorylated on Thr161, as expected. Importantly, however, ablation of Mos around GVBD inhibited the premature reactivation of MPF, reversed the phosphorylation state of cdc2 Tyr15 (at least partially) and even allowed nuclear reformation and DNA replication just after MI (Figure 3B and C). These results suggest strongly that Mos (presumably with protein 'X') functions to prematurely reactivate cdc2 kinase to induce MII directly, thereby preventing entry into interphase and DNA replication that would otherwise occur immediately after MI. [The MPF activity already high in telophase I (see Figure 5A) will surely not allow these events to occur and is probably responsible for maintenance of the condensed chromosomes during the MI/MII transition (see Figure 3A).] Indeed, using a dominant-negative cdc2 mutant, we demonstrated clearly that mere and direct inhibition of the premature MPF reactivation allows maturing oocytes to enter interphase and replicate DNA (Figure 4). Thus, it seems almost certain that the premature MPF reactivation mediated by Mos function is the direct cause of suppression of DNA replication after MI. This premature MPF reactivation (or direct entry into MII) is very similar to 'premature mitosis', which omits S phase and occurs in yeast overproducing cdc25 (Tyr15 phosphatase; Kumagai and Dunphy, 1991) (Enoch and Nurse, 1990) or in mammalian cells expressing Tyr15-mutated cdc2 (Krek and Nigg, 1991b), and might thus be regarded as a sort of 'premature mitosis'.

The molecular mechanism(s) by which Mos (and protein 'X') acts to prematurely reactivate MPF is largely unknown. Formally, Mos and/or protein 'X' could function in the pathway for sustaining cdc25 activity to preserve cdc2 Tyr15 dephosphorylation (cdc25 is only partially inactivated during the period of transient MPF inactivation after GVBD; Izumi *et al.*, 1992; our unpublished data). Equally, either or both of them could function in the pathway leading to permanent inactivation of wee1 kinase, which antagonizes cdc25 by phosphorylating cdc2 Tyr15 (Nurse, 1990; Heald *et al.*, 1993). However, it is also possible that they function in a quite different pathway, such as that for incomplete cyclin degradation during maturation (Kobayashi *et al.*, 1991) or cdc2 Thr161 phosphorylation (Solomon *et al.*, 1992; Poon *et al.*, 1993). The problems of whether the Mos function in these or other possible pathways is also involved in meiotic initiation (Freeman *et al.*, 1989; Sagata *et al.*, 1989a) or metaphase II arrest (Sagata *et al.*, 1989b) and whether it is mediated by mitogen-activated protein kinase (MAPK) function (Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993) remain to be elucidated. [Tyrosine dephosphorylation of MAPK (see the last lane of the lower panel in Figure 3B) and failure in spindle formation (Figure 3C) in Mos-ablated maturing oocytes are not inconsistent with the involvement of MAPK in the Mos pathway; Posada *et al.*, 1993; Haccard *et al.*, 1993.]

Prevention of parthenogenetic activation: a probable ultimate biological function of Mos

After the initiation of maturation, oocytes of many species are arrested again either at metaphase I (many invertebrates) or metaphase II (vertebrates) until fertilization (Masui, 1985) (Figure 5B). However, the reason why they are arrested prematurely at metaphase I or II, not after the completion of meiosis (this occurs, albeit exceptionally, in some invertebrates such as sea urchins; Figure 5B), has long remained a mystery (Masui, 1985). As known (Masui and Clarke, 1979; Masui, 1985; John, 1990), oocytes arrested at metaphase I or II are easily parthenogenetically activated either spontaneously or experimentally, implying that they have already acquired DNA-replicating ability before their arrest (Figure 5B). Although not noted previously, this fact suggests strongly that meiotic arrest at metaphase, whether in MI or MII, is a way of preventing the already acquired DNA-replicating ability from being realized before fertilization. (If not arrested at 'metaphase' these oocytes would undergo parthenogenetic activation, entering 'interphase' and replicating DNA before fertilization.) In vertebrates, meiotic arrest at metaphase II is caused by the cytostatic factor (CSF) which appears only during maturation and stabilizes MPF (Masui and Markert, 1971; Newport and Kirschner, 1984), and an essential component of this factor is now known to be Mos (Sagata *et al.*, 1989b). Thus, based on the above considerations, the biological function of Mos (as CSF) in metaphase II arrest may now be understood to be the prevention of undesirable DNA replication or parthenogenetic activation before fertilization. Evidently, this function is analogous to, and an extension of, that of Mos during the MI/MII transition, which could also be interpreted as prevention of parthenogenetic activation. It may be, therefore, that the ultimate biological function of Mos during

oocyte maturation is the prevention of parthenogenetic activation, probably to guarantee reduction of the chromosome number, a central objective of meiosis, in eggs. This view is very intriguing because Mos is expressed only during oocyte maturation (Sagata *et al.*, 1988; Paules *et al.*, 1989) and disappears on fertilization, or soon after the completion of meiosis (Watanabe *et al.*, 1989; Lorca *et al.*, 1991; Weber *et al.*, 1991; Nishizawa *et al.*, 1993).

Possible models for omitting DNA replication in various types of meiosis

In this work we showed that even maturing *Xenopus* oocytes have the capacity to enter interphase after MI and replicate DNA. This suggests that meiosis evolved by acquiring a specific mechanism(s) to omit or suppress DNA replication between the two successive divisions. The simplest way to acquire such a mechanism would then be the loss of some component(s) (e.g. G_1 cyclins, DNA polymerase α, etc.) needed for entry into S phase or DNA replication. This type of meiosis might be assumed to be the prototype and could occur in sporulation or gametogenesis in many organisms such as yeast and plants (Hotta, 1988; John, 1990) (see the legend to Figure 5B for details). However, animal eggs as female gametes are unique in that they prepare in advance almost all components that are necessary for rapid cell divisions immediately after fertilization (Davidson, 1986). Thus, as mentioned, the eggs of many species do develop the ability to induce DNA replication some time during oocyte maturation (Benbow, 1985; Masui, 1985) (Figure 5B). Logically, this would in turn indicate that the maturing oocytes must also develop some specific mechanism(s) to suppress the DNA replication-inducing ability until fertilization. As described here in *Xenopus*, this mechanism almost certainly involves the function of Mos (and protein 'X'), which appears only during maturation and acts to maintain and (re)activate MPF (Sagata *et al.*, 1988, 1989a,b; Freeman *et al.*, 1989; O'Keefe *et al.*, 1989; Paules *et al.*, 1989; Daar *et al.*, 1991; Kanki and Donoghue, 1991; Yew *et al.*, 1991; this study). This function of Mos may be well conserved in other vertebrates too, because ablation of c-*mos* mRNA, like protein synthesis inhibition (Clarke *et al.*, 1988), appears to allow maturing mouse oocytes to enter interphase after MI and often to divide (O'Keefe *et al.*, 1989). (In mice, however, the DNA replication-inducing ability seems to develop only after entry into MII; see Figure 5B and its legend.) Mos has not been detected in invertebrate oocytes, but its homolog or a functionally equivalent protein may exist and act similarly, because inhibition of protein synthesis causes parthenogenetic activation (and thus DNA replication) in metaphase I-arrested oocytes of some invertebrates such as *Chaetopterus* and *Patella* (Zampetti-Bosseler *et al.*, 1973; Néant and Guerrier, 1988) (see the legend to Figure 5B for details). Thus, it seems very likely that the mechanism for suppressing DNA replication via the function of Mos or its functional equivalent has evolved only for meiosis in animal oocytes, in which DNA replication-inducing ability specifically develops for immediate use after fertilization. In this context, Mos, though reportedly expressed in rodent testes (Herzog *et al.*, 1988; van der Hoorn *et al.*, 1991), may not be essential for meiosis in spermatocytes, which neither need to develop the DNA replication-inducing ability nor undergo meiotic arrest at metaphase, situations perhaps similar to those in meioses in yeast and plants (Figure 5B).

Materials and methods

Preparation and microinjection of Xenopus oocytes

Fully grown stage VI oocytes were obtained, treated with collagenase to remove follicle cells and cultured at 19–21°C as described previously (Sagata *et al.*, 1988). Oocyte maturation was induced by the addition of progesterone (5 μg/ml). In some cases, maturing oocytes at various times before or after GVBD, which usually occurred 3–4 h after progesterone addition, were treated with CHX (100 μg/ml) or APD (20 μg/ml). Oocytes were microinjected, usually at the time of GVBD, with 40 nl of a solution containing each of the various reagents (see below), as described previously (Sagata *et al.*, 1988).

For kinetic analyses of both cytological and biochemical events throughout post-GVBD maturation (see Figure 3), ~800 fully grown oocytes from a single female were treated with progesterone. Approximately 3 h later, 150 oocytes just undergoing GVBD were collected within a window of 10 min and these 'synchronized' oocytes were pooled. At 15 min intervals, eight oocytes were removed from the pooled oocytes and four of them were fixed for cytological examination (see below), while the other four oocytes were quickly frozen in dry ice/ethanol for biochemical analyses (i.e. histone H1 kinase assay and Western blot analysis; see below).

Oligonucleotides

Oligonucleotides used for microinjection were a mixture (40 ng total per oocyte; each 0.5 mg/ml in water) of two c-*mos*-specific sense (A^+ and G^+; Sagata *et al.*, 1988) or antisense (A^- and G^-) oligonucleotides.

Antibodies

Anti-Mos antibody was purified from rabbit polyclonal anti-Mos antiserum (termed K2; Nishizawa *et al.*, 1992) by affinity chromatography on agarose beads (Affigel 15, Bio-Rad) coupled with bacterially produced Mos protein, as described previously (Harlow and Lane, 1988). 140 ng of anti-Mos antibody (3.5 mg/ml in 100 mM NaCl, 10 mM sodium citrate) or control preimmune rabbit IgG were injected into individual oocytes. The anti-Mos antibody inhibited Mos kinase activity *in vitro* and blocked maturation of injected immature oocytes (data not shown), indicating that it had a neutralizing activity against Mos (initiation of maturation requires Mos; Sagata *et al.*, 1988).

GST–Mos fusion protein

For construction of the chimeric gene encoding GST–Mos fusion protein, a pair of synthetic oligonucleotides and a 1.3 kb *Sst*II–*Eco*RI fragment, which respectively encoded the third to 12th and 13th to the last amino acids of *Xenopus* Mos (Sagata *et al.*, 1988), were inserted into the *Bam*HI (blunt-ended)/*Eco*RI sites of the plasmid pGEX-3X (Smith and Johnson, 1988). The GST–Mos protein produced in *Escherichia coli* was purified by glutathione–Sepharose chromatography (Pharmacia) as described (Smith and Johnson, 1988). The purified GST–Mos protein (20 ng; 0.5 mg/ml in 4 mM potassium citrate, 10 mM potassium glutamate, pH 7.0) was injected into individual oocytes; this fusion protein (65 kDa in size) was capable of initiating maturation (and hence was active) when injected into immature oocytes, and was stable in maturing oocytes at least during the experiment (our unpublished data). Moreover, the GST–Mos protein showed the normal CSF activity (Sagata *et al.*, 1989b) when injected into two-cell embryos (our unpublished data), indicating that the GST moiety did not affect the Mos activity.

A dominant-negative cdc2 mutant

A cDNA clone of a *Xenopus* homolog of the *cdc2* gene was obtained by PCR of the phage DNA from a *Xenopus* ovarian cDNA library (Rebagliati *et al.*, 1985); the 5′ and 3′ primers used for PCR spanned an initiator ATG codon and a terminator TAA codon, respectively, of the *Xenopus cdc2* gene (Pickham *et al.*, 1992) and had an artificial *Xba*I site (upstream of the ATG codon) and *Bam*HI site (downstream of the TAA codon), respectively. The cloned 0.92 kb *Xba*I–*Bam*HI fragment, spanning the whole coding sequence, was inserted into the *Xba*I/*Bam*HI sites of a pIBI31-based plasmid vector (pIBIDSph); pIBIDSph was constructed by *Sph*I digestion of pIBI31, followed by T4 DNA polymerase treatment (to remove an ATG codon in the *Sph*I site), blunt-end ligation, *Mlu*I digestion and then ligation to an *Xba*I linker. The K33R cdc2 mutant was prepared by replacing the wild-type Lys33 codon (AAG) with an Arg codon (CGC) by oligonucleotide-directed

mutagenesis, and *in vitro* transcription of the plasmids was performed using T7 RNA polymerase, as described (Nishizawa *et al.*, 1992). Oocytes were injected at GVBD with 60 ng of the *in vitro* transcribed mRNA (1.0 mg/ml in water). Injection of K33R mRNA but not wild-type mRNA prevented activation of the endogenous cdc2 kinase in progesterone-treated immature oocytes or fertilized eggs (our unpublished data), indicating that the K33R cdc2 mutant acted in a dominant-negative manner.

Analysis of DNA synthesis

Oocytes were injected with 40 nl of water containing [α-^{32}P]dCTP (100 mCi/ml; 3000 Ci/mmol) and treated subsequently with progesterone to induce maturation. Maturing oocytes at various times or after various treatments were individually subjected to DNA extraction, and samples with equal numbers of total counts (5×10^6 c.p.m., roughly equivalent to 0.5 oocytes) were analyzed by 1% agarose gel electrophoresis and autoradiography, as described previously (Newport and Kirschner, 1984). Under the present conditions, the sheared nuclear DNA fragments migrated faster than the closed and open circular mitochondrial DNAs.

Histone H1 kinase assay

Frozen oocytes (usually four in number) were homogenized on ice in 40 μl of extraction buffer (EB; Gerhart *et al.*, 1984) and centrifuged briefly at 2°C. 4 μl of the supernatant were subjected to histone H1 kinase assay (in the presence of [γ-^{32}P]ATP) followed by SDS–PAGE, essentially as described previously (Dabauvalle *et al.*, 1988).

Western blot analysis

Frozen oocytes (usually four in number) were homogenized on ice in 40 μl EB and centrifuged briefly at 2°C. The supernatant was diluted with an equal volume of 2 × Laemmli's sample buffer and a portion (equivalent to two oocytes) was subjected to SDS–PAGE to make a Western blot. The blot was probed with monoclonal anti-PSTAIR antibody (Yamashita *et al.*, 1991) (diluted 1:2000); in some cases the blot was deprobed and then reprobed with monoclonal anti-phosphotyrosine antibody (UBI; diluted 1:1000), essentially as described previously (Harlow and Lane, 1988; Ferrell *et al.*, 1991). Antibodies were detected using enhanced chemiluminescence reagents (Amersham).

Cytological examination

Oocytes were fixed in Smith's solution, dehydrated and embedded in Paraplast Plus (Sherwood Medical). Sections were routinely prepared at 8 μm thickness, stained with Feulgen's stain and counter-stained with fast green, as described previously (Nishizawa *et al.*, 1992).

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Very recently, K.Ohsumi, W.Sawada and T.Kishimoto (submitted) have also shown similar regulation of the MI/MII transition during *Xenopus* oocyte maturation. In addition, in collaboration with S.Aizawa and his colleagues (submitted), we have recently found that oocytes from c-*mos*-deficient mice undergo parthenogenetic activation and DNA replication after MII; this time of DNA replication (i.e. after MII) is apparently different from that in *Xenopus* oocytes (this study) and from that in mouse oocytes treated with puromycin (Clarke *et al.*, 1988) or c-*mos* antisense oligonucleotides (O'Keefe *et al.*, 1989), and might be relevant to the late and gradual occurrence (in MII) of the DNA replicating ability in maturing mouse oocytes (see the text and the legend to Figure 5).