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NOTES

Meiotic Induction by *Xenopus* Cyclin B Is Accelerated by Coexpression with mos^{Xe}

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We have investigated the relationship between *Xenopus laevis* c-*mos* (mos^{Xe}) and the cyclin B component of maturation-promoting factor. Microinjection of *Xenopus* oocytes with in vitro-synthesized RNAs encoding *Xenopus* cyclin B1 or cyclin B2 induces the progression of meiosis, characterized by germinal vesicle breakdown (GVBD). By preinjecting oocytes with a mos^{Xe}-specific antisense oligonucleotide, we show that GVBD induced by cyclin B does not require expression of the mos^{Xe} protein. GVBD induced by cyclin B proceeds significantly faster than GVBD induced by progesterone or Mos^{Xe}. However, coinjection of RNAs encoding cyclin B1 or cyclin B2 with mos^{Xe} RNA results in a 2.5- to 3-fold acceleration in GVBD relative to that induced by cyclin B alone. This acceleration of GVBD does not correlate with changes in the level of cyclin B1 and cyclin B2 phosphorylation.

Numerous studies have demonstrated the apparently universal function of maturation (M-phase)-promoting factor (MPF) as the agent responsible for the progression of meiosis in maturing oocytes and as the inducer of the G2 to M phase transition in eukaryotic cells (22). MPF consists of the $p34^{cdc2}$ protein kinase and one or more of the cyclin B proteins (2, 4, 10, 11). MPF in oocytes exists in an inactive state known as pre-MPF (1), which can be activated in vivo by a variety of agents, including progesterone, small amounts of active MPF (12), okadaic acid (13), and overexpressed cyclin proteins (23, 30, 32), and by overexpression of the *Xenopus laevis* c-*mos* gene (mos^{Xe}) (8, 27) or the v-*mos* oncogene (7). Little is known about the mechanism of activation of MPF, but it likely involves dephosphorylation of $p34^{cdc2}$ and phosphorylation of cyclin B (5, 9, 10, 14, 15, 20, 25).

The expression of mos^{Xe} is required for progesterone-induced maturation of *Xenopus* oocytes (28). mos^{Xe} mRNA is present during oocyte growth and maturation and persists in the developing embryo through blastulation; however, the mos^{Xe} protein is only detected during hormone-induced oocyte maturation and is rapidly degraded shortly after fertilization (31). The inhibition of mos^{Xe} translation in oocytes by microinjection with mos^{Xe}-specific antisense oligonucleotides prevents hormone-induced germinal vesicle breakdown (GVBD) (28). Prophase-arrested oocytes can be induced to undergo GVBD by microinjection of in vitro-transcribed mos^{Xe} RNA (8, 27), demonstrating that expression of mos^{Xe} is sufficient to initiate oocyte maturation. Finally, immunoprecipitates containing the mos^{Xe} protein were recently shown to phosphorylate the *Xenopus* cyclin B2 protein in vitro (26).

When expressed in cleaving *Xenopus* embryos, the mos^{Xe} protein can induce mitotic arrest in a manner analogous to cytostatic factor (CSF) (29). CSF is an activity present in extracts from unfertilized eggs that maintains the egg in a state of meiotic arrest, possibly by stabilizing MPF (17, 21). Injection of mos^{Xe} RNA into one blastomere of a two-cell embryo induces mitotic cleavage arrest of the injected blastomere. In addition, neutralization or immunodepletion of the mos^{Xe} protein in unfertilized egg extracts with mos^{Xe}-specific antibodies abolishes CSF activity (29). These results demonstrate that Mos^{Xe} can act to arrest mitotic cleavage, thereby preventing the decrease in MPF activity that accompanies the normal cell cycle.

Prior to isolation of the *Xenopus* cyclin B genes (19), microinjection of RNAs encoding the clam or sea urchin cyclin protein was shown to induce GVBD in *Xenopus* oocytes (23, 30, 32). To determine the effects of mos^{Xe} expression on cyclin B-induced GVBD, *Xenopus* cyclin B1 and cyclin B2 cDNAs (19) were subcloned into pSP64(polyA), and cyclin B RNAs were synthesized in vitro as described previously (7). Microinjection of 50 ng of *Xenopus* cyclin B1 or cyclin B2 RNA into prophase-arrested oocytes consistently induced GVBD in nearly 100% of the recipient oocytes (Fig. 1), whereas microinjection of lower amounts of RNA (<5 ng) also induced GVBD but with reduced efficiency. Microinjection of more than 50 ng of the cyclin B RNAs has not been observed to affect the rate of GVBD. Although GVBD is induced in these oocytes, it is not known whether they complete meiotic maturation by reaching a metaphase II arrest or whether they abort the normal maturation process after GVBD.

Prophase-arrested oocytes do not contain detectable levels of mos^{Xe} protein (31). To test whether GVBD induced by microinjection of *Xenopus* cyclin B RNA requires translation of mos^{Xe}, we first injected oocytes with a mos^{Xe}-specific antisense oligonucleotide and then reinjected these oocytes with cyclin B1 or cyclin B2 RNA (Fig. 1). Microinjection of oocytes with this antisense oligonucleotide prevents translation of mos^{Xe} and blocks progesterone-induced oocyte maturation (7, 28). Stage VI oocytes were microinjected with either 50 nl of the mos^{Xe}-specific antisense oligonucleotide (2

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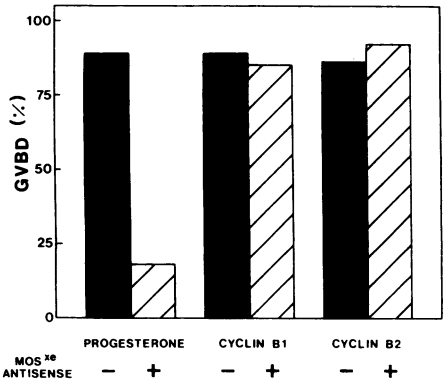

FIG. 1. Effect of blocking Mos^{Xe} translation on cyclin B-induced GVBD. Oocytes were manually dissected from the ovaries of *X. laevis* (obtained from Xenopus I). Stage VI oocytes (10 to 15 per sample) were microinjected with either a mos^{Xe}-specific antisense oligonucleotide (+) or MBS-H (−). Four hours later, the injected oocytes were incubated in 15 μM progesterone or reinjected with in vitro-synthesized cyclin B1 or cyclin B2 RNA, as indicated. Progesterone-treated and injected oocytes were incubated for ~12 h and then scored for GVBD by the appearance of a white spot in the pigmented animal pole (18). Oocytes were fixed in 5% trichloroacetic acid and manually dissected to confirm GVBD. The percent GVBD represents the mean from at least two experiments.

mg/ml) or 50 nl of modified Barth's solution (MBS-H) as described previously (7). Four hours later, the injected oocytes were incubated in 15 μM progesterone or reinjected with 50 nl of in vitro-synthesized cyclin B1 or cyclin B2 RNA (1 mg/ml). Whereas only 18% of the oocytes injected with the mos^{Xe} antisense oligonucleotide and then treated with progesterone underwent GVBD, 85 to 92% of the oocytes injected first with the mos^{Xe} antisense oligonucleotide and then with cyclin B1 or cyclin B2 RNA underwent GVBD. In control experiments, microinjection of the antisense oligonucleotide alone did not induce GVBD or have any other effect on the morphology of the oocytes. These results demonstrate that cyclin B overexpression can induce GVBD independent of mos^{Xe} translation and suggest that activation of MPF by this pathway can occur in the absence of mos^{Xe} protein.

GVBD induced by overexpression of mos^{Xe} proceeds more slowly than GVBD induced by progesterone treatment (8, 27) (Fig. 2). Thus, Mos^{Xe} probably functions at an early point in the maturation process. To test whether expression of mos^{Xe} would affect the rate of cyclin B-induced GVBD, we injected oocytes with a mixture of mos^{Xe} RNA and cyclin B1 or cyclin B2 RNA (Fig. 2). For comparison, other oocytes were injected with a mixture of cyclin B1 or cyclin B2 RNA along with RNA encoding an inactive mos^{Xe} protein containing a lysine-to-arginine mutation in the canonical ATP-binding domain. We have previously shown that this altered protein, Mos^{XeR90}, fails to induce GVBD when expressed in oocytes (8). Oocytes were microinjected with 50 nl of the following: cyclin B1 or cyclin B2 RNA (1 mg/ml), a mixture of mos^{Xe} RNA and cyclin B1 or cyclin B2 RNA, or a mixture of mos^{XeR90} RNA and cyclin B1 or cyclin B2 RNA. (In the RNA mixtures, each RNA had a final concentration of 1 mg/ml.) Additional oocytes were incubated in 15 μM progesterone or injected with 50 nl of mos^{Xe} RNA (1 mg/ml).

In these experiments, oocytes injected with only cyclin B1 or cyclin B2 RNA reached $GVBD_{50}$ (the point at which 50% of the injected oocytes have undergone GVBD) in about 70% of the time required for progesterone-treated oocytes to reach $GVBD_{50}$. Similar results were obtained from oocytes coinjected with mos^{XeR90} RNA and cyclin B1 or cyclin B2 RNA. However, oocytes coinjected with cyclin B1 or cyclin B2 RNA together with wild-type mos^{Xe} RNA reached $GVBD_{50}$ in only about 25% of the time needed for progesterone-stimulated oocytes. This acceleration of cyclin B-induced GVBD by Mos^{Xe} is apparently not due to an increase in cyclin B translation as a result of mos^{Xe} expression, since we did not detect an increase in cyclin B synthesis as a result of coexpression of cyclin B with mos^{Xe} compared with coexpression with mos^{XeR90} (data not shown). These results clearly demonstrate that coexpression of mos^{Xe} with cyclin B1 or cyclin B2 dramatically accelerates GVBD.

Both cyclin B proteins can be phosphorylated by $p34^{cdc2}$ in vitro (3, 9, 10, 24), and an activity that can phosphorylate cyclin B appears in maturing oocytes coincident with the activation of MPF (10). Whether $p34^{cdc2}$ phosphorylates cyclin B in vivo or some other protein kinase(s) phosphorylates cyclin B during maturation, however, is not known. Since the mos^{Xe} gene encodes a protein kinase that is required for progesterone-induced maturation and can accel-

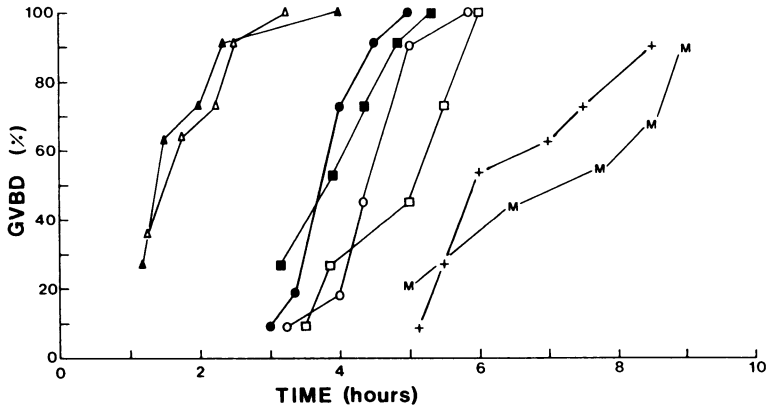

FIG. 2. Effect of coexpression of cyclin B and mos^{Xe} on GVBD. Stage VI oocytes (10 to 15 per sample) were microinjected with cyclin B1 (○) or cyclin B2 (●) RNA; a mixture of mos^{Xe} RNA and cyclin B1 (△) or cyclin B2 (▲) RNA; or a mixture of mos^{XeR90} RNA and cyclin B1 (□) or cyclin B2 (■) RNA. Additional oocytes were incubated in 15 μM progesterone (+) or injected with mos^{Xe} RNA alone (M). Oocytes were scored for GVBD as described in the legend to Fig. 1.

TABLE 1. Comparison of cyclin B1 and B2 phosphorylation when coexpressed with mos^{Xe} versus mos^{XeR90}[a]

Expt no.	Cyclin B phosphorylation			
	At GVBD		After 2-h labeling	
	B1	B2	B1	B2
1	2.1	0.3	8.4	0.4
2	1.2	0.4	7.6	0.6
3	0.4	0.2	8.6	1.0
Avg ± SD	1.2 ± 0.7	0.3 ± 0.1	8.2 ± 0.4	0.7 ± 0.2

[a] Results from three identical experiments are presented which were obtained by scanning the autoradiograms. The autoradiograms for one experiment are presented in Fig. 3. Cyclin B phosphorylation is presented as the ratio of the amount of ^{32}P-labeled cyclin B measured when coexpressed with wild-type mos^{Xe} compared with the amount of ^{32}P-labeled cyclin B measured when coexpressed with mos^{XeR90}. The amount of ^{32}P-labeled cyclin B was first normalized to the amount of ^{35}S-labeled cyclin B protein.

erate cyclin-induced GVBD, it is possible that Mos^{Xe} activates MPF via the phosphorylation of cyclin B. In a recent study, immunoprecipitates of Mos^{Xe} were shown to phosphorylate cyclin B2 in vitro (26). However, this might not be the result of an intrinsic activity of the mos^{Xe}-encoded protein kinase, since in the same report an intracellular complex containing Mos and $p34^{cdc2}$ was noted to exist in *mos*-transformed cells.

An alternative approach is to look for a change in the level of cyclin B phosphorylation in oocytes overexpressing mos^{Xe}. If the cyclin B proteins are substrates for the mos^{Xe} kinase and if the amount of Mos^{Xe} is a limiting factor, then an increase in the level of cyclin B1 and cyclin B2 phosphorylation should occur in oocytes overexpressing cyclin B and mos^{Xe}. We compared the level of cyclin B phosphorylation in oocytes that had been injected with a mixture of cyclin B and mos^{XeR90} RNAs or with a mixture of cyclin B and wild-type mos^{Xe} RNAs (Fig. 3). Antisera raised against synthetic peptides corresponding to the C termini of cyclin B1 and cyclin B2 recognized the proteins in oocytes, and immunoprecipitation of the cyclin B proteins was specifically blocked by preincubation of the sera with the antigenic peptides (data not shown). Oocytes were microinjected with 50 nl of a mixture containing cyclin B1 or cyclin B2 RNA and either mos^{XeR90} or wild-type mos^{Xe} RNA (each RNA at 1 mg/ml). For ^{35}S labeling, injected oocytes were immediately incubated in MBS-H containing [^{35}S]cysteine and [^{35}S]methionine. For ^{32}P labeling, injected oocytes were immediately incubated in MBS-H containing 5 mCi of $^{32}P_i$ per ml. Cell lysates were prepared after the labeled oocytes had undergone GVBD and then immunoprecipitated with anti-cyclin B1 or anti-cyclin B2 serum (Fig. 3, lanes 2, 3, 5, and 6). Both cyclin B1 and cyclin B2 were phosphorylated in the injected oocytes. After adjustment for the amount of ^{35}S-labeled cyclin B (shown in Fig. 3A), the phosphorylation of cyclin B1 at GVBD increased only slightly, if at all, when coexpressed with wild-type mos^{Xe} versus mos^{XeR90} (Table 1). However, the level of phosphorylation at GVBD of cyclin B2 when coexpressed with mos^{Xe} was approximately one-third the level obtained when coexpressed with mos^{XeR90}. Normalizing the amount of ^{32}P-labeled cyclin B protein (which represents both preexisting and newly translated cyclin) to the amount of ^{35}S-labeled cyclin is possible because the pool of preexisting cyclin B protein is the same for oocytes coinjected with either mos^{Xe} or mos^{XeR90} RNA.

These results show that although GVBD induced by both cyclin B1 and cyclin B2 is similarly accelerated by Mos^{Xe},

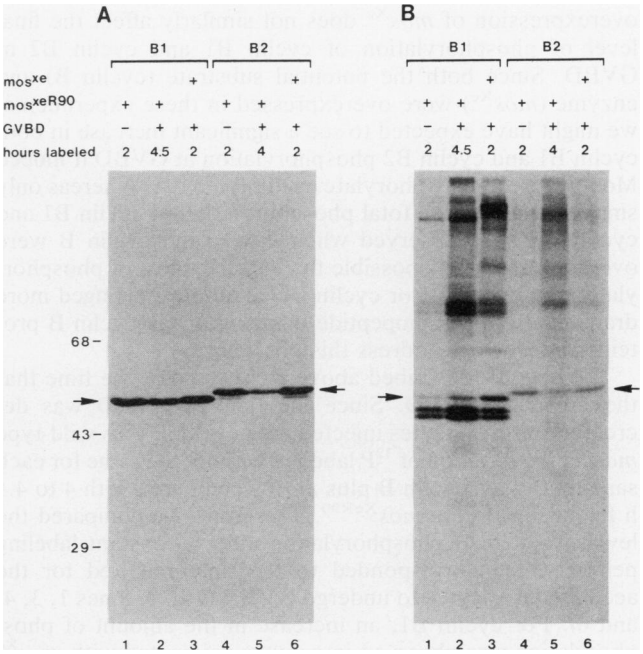

FIG. 3. Immunoprecipitation of cyclin B proteins from (A) ^{35}S-labeled or (B) ^{32}P-labeled oocytes coinjected with cyclin B RNA and either mos^{Xe} or mos^{XeR90} RNA. Stage VI oocytes were microinjected with a mixture of in vitro-synthesized cyclin B1 or cyclin B2 RNA together with either mos^{Xe} RNA (16 oocytes) or mos^{XeR90} RNA (32 oocytes) as indicated. For ^{35}S labeling, eight oocytes from each set injected with a mos^{Xe} RNA mixture and 16 oocytes from each set injected with a mos^{XeR90} RNA mixture were incubated in MBS-H containing 0.5 mCi each of [^{35}S]cysteine and [^{35}S]methionine per ml. For ^{32}P labeling, the oocytes remaining from each set were incubated in MBS-H containing 5 mCi of $^{32}P_i$ per ml. Immediately after the oocytes that were coinjected with cyclin B and mos^{Xe} RNAs underwent GVBD (approximately 2 h after injection), they were lysed in 100 μl of ice-cold lysis buffer (8.5 mM Tris-HCl [pH 6.8], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, 2 mM ATP, 2 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM EGTA [ethylene glycol tetraacetic acid], 0.5 μg of leupeptin per ml, 10 μg of aprotonin per ml, 10 μM pepstatin A). In addition, half of the oocytes injected with cyclin B and mos^{XeR90} RNAs were lysed after an equivalent labeling period (2 h after injection). The remaining cyclin B- and mos^{XeR90}-injected oocytes were incubated until they had undergone GVBD (approximately 4 to 4.5 h after injection) and then were lysed as above. To ensure that the amount of each cyclin B antiserum used was sufficient to quantitatively recover the ^{35}S- and ^{32}P-labeled cyclin B proteins, 5 μl of each antiserum was used to immunoprecipitate the labeled cyclin B proteins from various numbers of injected oocytes. The amount of cyclin B protein recovered was plotted versus the number of oocytes. The number of oocytes used for the subsequent experiments was in the middle of the linear range of recovery of cyclin B with 5 μl of antiserum. Lysates from six oocytes for each sample were preadsorbed with 25 μl of a 50% (vol/vol) suspension of protein A-Sepharose in lysis buffer for 25 min at 4°C. After centrifugation at 10,000 × *g*, the supernatants were incubated with excess anti-cyclin B1 or anti-cyclin B2 serum for 1.5 h at 4°C. The immune complexes were collected with protein A-Sepharose as described above, layered onto 1 ml of lysis buffer containing 10% sucrose, and pelleted for 10 min at 2,500 × *g*. The beads were washed twice with lysis buffer, pelletting as above after each wash. The immunoprecipitated proteins were analyzed by 12.5% SDS-PAGE and fluorography (^{35}S-labeled samples) for 3 h or autoradiography with an intensifying screen (^{32}P-labeled samples) for 23 h. The arrows indicate the positions of the cyclin B1 and B2 proteins. The incorporation of radioactivity into specific proteins was quantitated by scanning laser densitometry and is presented in Table 1. Sizes are shown in kilodaltons.

overexpression of mos^{Xe} does not similarly affect the final level of phosphorylation of cyclin B1 and cyclin B2 at GVBD. Since both the potential substrate (cyclin B) and enzyme (mos^{Xe}) were overexpressed in these experiments, we might have expected to see a significant increase in both cyclin B1 and cyclin B2 phosphorylation at GVBD if indeed Mos^{Xe} were to phosphorylate cyclin B in vivo. Whereas only small changes in the total phosphorylation of cyclin B1 and cyclin B2 were observed when mos^{Xe} and cyclin B were overexpressed, it is possible that specific sites of phosphorylation on cyclin B1 or cyclin B2 could have changed more dramatically. Phosphopeptide mapping of the cyclin B proteins is needed to address this possibility.

The oocytes described above were lysed at the time that they reached GVBD. Since the time to GVBD was decreased for the oocytes injected with cyclin B plus wild-type mos^{Xe}, the duration of ^{32}P labeling was not the same for each sample: 2 h for cyclin B plus mos^{Xe} compared with 4 to 4.5 h for cyclin B plus mos^{XeR90}. Therefore, we compared the level of cyclin B phosphorylation after a constant labeling period which corresponded to the time required for the accelerated oocytes to undergo GVBD (Fig. 3, lanes 1, 3, 4, and 6). For cyclin B1, an increase in the amount of phosphorylation was observed in oocytes coinjected with mos^{Xe} RNA compared with oocytes coinjected with mos^{XeR90} RNA (Table 1, 2-h labeling). This enhanced rate of cyclin B1 phosphorylation in oocytes undergoing accelerated GVBD could be a consequence of the overall increase in protein phosphorylation that is known to accompany GVBD (16). For cyclin B2, a slight decrease in phosphorylation was detected in oocytes coinjected with mos^{Xe} RNA and labeled for 2 h compared with oocytes coinjected with mos^{XeR90} RNA (Table 1). These results, obtained after a 2-h labeling period, are consistent with those found from labeling through GVBD. Thus, we did not observe any qualitative fluctuations in cyclin B phosphorylation that correlate with the acceleration of GVBD induced by coexpression with mos^{Xe}.

During this work, we also examined the potential interaction between mos^{Xe} and another component of MPF, $p34^{cdc2}$. Microinjection of in vitro-synthesized *Xenopus* $p34^{cdc2}$ RNA alone into oocytes did not induce GVBD. Moreover, microinjection of RNA encoding $p34^{cdc2}$ did not accelerate GVBD induced by either mos^{Xe} or cyclin B RNA (data not shown). Thus, overexpression of $p34^{cdc2}$ is insufficient to activate the existing pool of pre-MPF in prophase-arrested oocytes. In addition, since $p34^{cdc2}$ is dephosphorylated during activation (5, 9, 14, 15, 20, 25), it is unlikely to be a direct substrate for the mos^{Xe} protein kinase. It is possible that Mos^{Xe} may phosphorylate an as yet unidentified protein that regulates MPF activity without directly phosphorylating cyclin B or $p34^{cdc2}$. One group of potential regulators of MPF activation are the protein phosphatases and their inhibitors, which have been shown to play a role in regulating MPF activity (6, 13).

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