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# Evidence that Mos protein may not act directly on cyclin

(*Xenopus*/meiosis/oocyte maturation/germinal vesicle breakdown)

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**ABSTRACT** **Using affinity-purified antiserum we have examined cyclin B2 levels in *Xenopus* oocytes at various stages of oogenesis. We found that cyclin B2 is detected from stage 2 to stage 6 as two bands, one of which is phosphorylated, and that cyclin B2 mass increases about 28-fold between stage 2 and stage 6. To examine the effect of Mos protein on cyclin phosphorylation, we microinjected synthetic *Xenopus* c-*mos* (c-*mos*$^{xe}$) RNA into stages 4, 5, and 6 *Xenopus* oocytes. In stage 6 oocytes, maturation was induced by c-*mos*$^{xe}$ RNA, and, as is the case with progesterone treatment, all cyclin B2 was shifted to the phosphorylated form. However, c-*mos*$^{xe}$ RNA injected into stage 4 or 5 oocytes did not induce maturation or cause a shift in the relative proportion of the two cyclin B2 bands. These data suggest that Mos does not act directly to phosphorylate cyclin B2, causing the band shift during maturation. Cyclin B2 synthesis increases about 2-fold during maturation, in concert with total protein synthesis. Data from experiments on cyclin B2 stability indicate that the half-life of cyclin B2 is about 85 hr in stage 6. This suggests that if Mos protein has a direct effect on cyclin stability, it does so only at a later stage in oocyte maturation, but not at the onset.**

The induction of maturation in amphibian oocytes by progesterone leads to the appearance of active maturation-promoting factor (MPF) via an event(s) that requires protein synthesis (1). The nature of this requirement is unknown. The cyclic appearance and disappearance of MPF in mitotically dividing cells is now known to result from the periodic synthesis and degradation of one component of MPF, a B-type cyclin protein (2–6). The other component of MPF, a serine/threonine protein kinase (p34$^{cdc2}$), is constitutively present in dividing cells and is somehow regulated by interaction with cyclin (3, 6–11).

Experiments conducted some time ago showed that amphibian oocytes already contain an inactive form of MPF (pre-MPF) that can be activated in the absence of protein synthesis (11–16). Gautier and Maller (17) have shown that full-grown (stage 6) *Xenopus* oocytes contain cyclin B2 already complexed with p34$^{cdc2}$. The additional reports that stage 6 oocytes also synthesize cyclin *de novo* (17, 18) suggest that the protein synthesis requirement for MPF activation does not involve the appearance of cyclin.

Several lines of evidence suggest that the synthesis of Mos protein in response to progesterone might account for the protein synthesis requirement. Thus, the injection of *Xenopus* c-*mos* (c-*mos*$^{xe}$) RNA into stage 6 oocytes induces maturation (19, 20), while destruction of endogenous c-*mos*$^{xe}$ mRNA blocks progesterone-induced maturation. Further, c-*mos*$^{xe}$ product is not present in stage 6 oocytes and its increase in synthesis during maturation precedes the activation of MPF (18, 19, 21).

The mechanism(s) by which c-*mos*$^{xe}$ product might lead to MPF activation is unclear. Since Mos protein is a serine/threonine protein kinase, one possibility is that it phosphorylates one of the components of MPF directly. In this regard, Roy *et al.* (22) have reported that cyclin B2 is a substrate for c-*mos*$^{xe}$ product *in vitro*. Hence, phosphorylation of cyclin could be involved in p34$^{cdc2}$ activation; cyclin phosphorylation during maturation is a prerequisite for MPF activation (17, 23). Alternatively, phosphorylation could prevent degradation of cyclin as implied from the demonstration that Mos protein maintains high MPF activity in a manner analogous to cytostatic factor (24).

The current study was undertaken to evaluate the above possibilities. Regarding phosphorylation, we took advantage of the fact that stage 4 oocytes contain pre-MPF, which can be activated by injection of cyclin or active MPF (11–15, 25) but not by exposure of the oocytes to progesterone (26). Thus, we reasoned that if Mos protein interacts directly with pre-MPF, injection of c-*mos*$^{xe}$ RNA into stage 4 oocytes should lead to cyclin phosphorylation and MPF activation. This was not the case. To evaluate the possible effect of Mos protein on cyclin stability, we first measured cyclin stability in the absence of Mos protein. Our results suggest that cyclin is synthesized continuously, as a constant percentage of total protein synthesis, and that cyclin is very stable, even before the induction of maturation. Thus, it is unlikely that Mos protein has a direct effect on cyclin stability.

## MATERIALS AND METHODS

**Animals and Preparation of Oocytes.** Female *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI) and maintained as described (27). Ovaries were surgically removed from hypothermically anesthetized females. Oocytes were staged (28) based on the measurement of oocyte diameter with an ocular micrometer. Oocytes at all stages of oogenesis used in these studies were manually defolliculated and cultured in OR2 medium (29).

**Purification of *Escherichia coli* Cyclin B2 Protein.** An *E. coli* strain containing the *Xenopus* cyclin B2 expression plasmid was generously provided by M. Kirschner (University of California, San Francisco). Purification of cyclin B2 was carried out according to the method of Gautier *et al.* (6). For further purification, isolated cyclin B2 was electrophoresed in an SDS/10% polyacrylamide gel (30). Regions of the gel corresponding to cyclin B2 bands were cut out and homogenized with a Brinkmann homogenizer in a siliconized glass tube. Protein was extracted from the homogenized material with 5 ml of 1% SDS at 37°C for 24 hr. The acrylamide was removed by centrifugation and cyclin B2 was precipitated with trichloroacetic acid at a final concentration of 30% (wt/vol). The protein pellet was dissolved in 80 mM Tris, pH 6.8/10% glycerol/70 mM 2-mercaptoethanol/2% SDS.

**Preparation of c-*mos*$^{xe}$ RNA.** The plasmid pTZXA+, containing the clone with the entire c-*mos*$^{xe}$ coding region, was

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Abbreviations: GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor.

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a generous gift of G. Vande Woude (National Cancer Institute, Frederick, MD). After the plasmid was linearized with *Hin*dIII, c-*mos*$^{xe}$ RNA was transcribed *in vitro* by using T7 polymerase and a 5′ cap (Pharmacia). The concentration of synthetic RNA was determined with an LKB spectrophotometer (31).

**Western Blotting.** Oocytes were homogenized in 100 μl of buffer A (32). Protein samples were precipitated with 4 vol of acetone and electrophoresed in an SDS/10% polyacrylamide gel. The gel was transferred to an Immobilon-P transfer membrane (Millipore), and the blot was probed with an affinity-purified antibody against *Xenopus* cyclin B2, which has no crossreactivity with cyclin B1 (6), in a hybridization incubator (Robbins Scientific, Sunnyvale, CA) at 37°C for 1 hr. After the blots were washed with 0.5% Nonidet P-40 in Tris-buffered saline for 30 min, they were incubated for 60 min, with $^{125}$I-labeled protein A (NEN; 1 μCi/ml; 1 μCi = 37 kBq) in Tris-buffered saline containing 5% bovine serum albumin. The membranes were then washed, dried, and exposed to XAR film (Kodak).

**Labeling of Cyclin B2 and Total Proteins.** For protein synthesis studies, oocytes cultured in OR2 with or without progesterone (10 μg/ml, Sigma) were injected at different time points with [$^{35}$S]methionine/[$^{35}$S]cysteine (Tran$^{35}$S-label, ICN) as described (33). For protein phosphorylation analysis, 20 nl of [γ-$^{32}$P]ATP (NEN; specific activity, 5 mCi/ml) was injected, and the oocytes were incubated for 6–7 hr. At the appropriate times, oocytes were homogenized in 100 or 200 μl of buffer A for total protein analysis or immunoprecipitation, respectively. Protein was then precipitated with acetone and either directly electrophoresed or first immunoprecipitated. For immunoprecipitation, protein was resuspended in 0.5% SDS buffer, boiled for 5 min, and then incubated with 1:250 diluted affinity-purified antiserum followed by protein A-agarose beads (6).

**Cytosol Preparation for Cytostatic Factor Assay.** Oocyte extract was prepared according to Daar *et al.* (34). Fifteen to twenty stage 4 oocytes were injected with c-*mos*$^{xe}$ RNA, incubated 6–7 hr, and then rinsed with extraction buffer and homogenized. After centrifugation, 40 nl of the supernatant was injected into either a fertilized egg or one cell of a two-cell embryo. Development of the embryos was monitored under the dissecting microscope.

## RESULTS

**Effects of c-*mos*$^{xe}$ Product on Germinal Vesicle Breakdown (GVBD).** In stage 6 oocytes, injection of 2.5 ng of c-*mos*$^{xe}$ RNA induced 3 of 15 oocytes to mature, as evidenced by GVBD 12–24 hr after injection; 15 of 18 and 18 of 18 matured with 5 ng and 10–40 ng, respectively. In most cases, $GVBD_{50}$ in c-*mos*$^{xe}$ RNA-injected oocytes occurred about 30–60 min faster than with progesterone. Furthermore, when c-*mos*$^{xe}$ RNA-injected stage 6 oocytes were treated with progesterone immediately after injection, the time of $GVBD_{50}$ was about 2–3 hr earlier than with progesterone alone. Both stage 4 and stage 6 oocytes translate c-*mos*$^{xe}$ RNA equally well (data not shown). Thus, we assumed that 10–40 ng of c-*mos*$^{xe}$ RNA should have been more than sufficient to cause an effect in stage 4 oocytes. However, GVBD never occurred in stage 4 oocytes after injection of 10, 20, or 40 ng of c-*mos*$^{xe}$ RNA, even after >24 hr of incubation.

One explanation for the absence of GVBD in stage 4 oocytes is that the Mos protein synthesized is inactive. To test this, a biological assay was used. A major characteristic of Mos protein is its cytostatic factor activity (24), defined as an activity that arrests dividing cells in metaphase (35). Accordingly, a cytoplasmic extract, prepared from stage 4 oocytes injected with c-*mos*$^{xe}$ RNA, was injected into either fertilized eggs or one cell of a two-cell embryo. In two experiments, arrested cleavage was observed in 86% and 100% of the recipients (data not shown). While cytological analysis was not undertaken, these are the results expected from cytostatic factor activity, suggestive of active Mos protein.

**Effects of c-*mos*$^{xe}$ Product on Phosphorylation.** Using antibody against *Xenopus* cyclin B2, Gautier and Maller (17) and Izumi and Maller (36) identified two immunoreactive proteins in Western blots of stage 6 oocytes; phosphorylation of the slower-migrating protein was demonstrated by immunoprecipitation of $^{32}$P-labeled oocytes. In maturing oocytes, all of the cyclin became phosphorylated, as evidenced by the conversion of two bands to one slower band at a time approximately coincident with MPF activation (0.6 $GVBD_{50}$). Our data, obtained by using the same antibody, confirm and extend the results mentioned above and those reported by Gautier *et al.* (6). As shown in Fig. 1, in oocytes induced to mature either with progesterone or by injection of synthetic c-*mos*$^{xe}$ RNA, there is a shift from two immunoreactive cyclin proteins to a single cyclin protein at 0.6 $GVBD_{50}$. That the single band is phosphorylated is confirmed in Fig. 1*C*, which shows immunoprecipitation of cyclin from $^{32}$P-labeled oocytes.

Surprisingly, stage 4 oocytes also contained two immunoreactive proteins (Fig. 2), which, by analogy to stage 6 oocytes, we assume represent phosphorylated and unphosphorylated forms of cyclin B2. In two separate experiments we attempted to confirm this by injecting [γ-$^{32}$P]ATP, as described above with stage 6 oocytes. It should be pointed out that the specific activity of the precursor pool of the stage 4 oocytes was 10 times greater than that of stage 6. In spite of this, phosphorylation of cyclin was barely detectable in control oocytes, with no significant difference in c-*mos*$^{xe}$ RNA-injected oocytes. The difficulty in detecting phosphorylated cyclin could be due to a cyclin kinase(s) that is less active in stage 4 than in stage 6; alternatively, one cannot rule out that cyclin could be inaccessible to the kinase(s). Yet, since Mos protein has no effect on phosphorylation, although

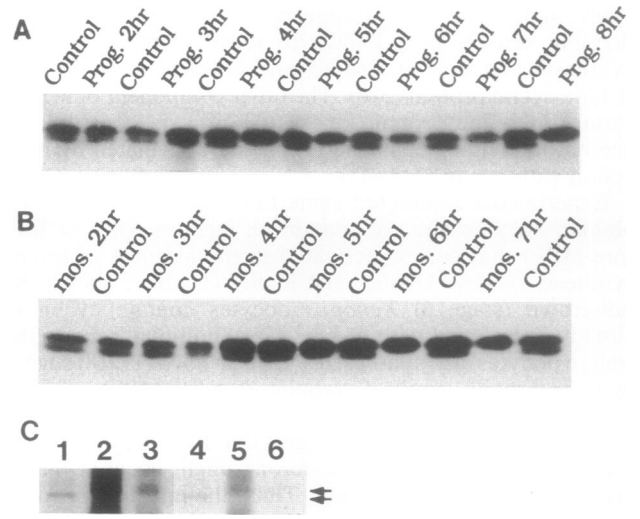


FIG. 1. Western blot analysis (*A* and *B*) and immunoprecipitation (*C*) of cyclin B2 from stage 6 oocytes. The bands in the upper position are phosphorylated and the bands in the lower position are unphosphorylated. Five oocytes were used for each lane. (*A*) Progesterone (Prog.)-treated oocytes and controls at various time points after treatment as indicated above each lane ($GVBD_{50}$ at 6 hr). (*B*) c-*mos*$^{xe}$ RNA-injected oocytes (20 ng per oocyte) and controls ($GVBD_{50}$ at 8 hr). Data are from two independent experiments. (*C*) Lane 1, [$^{35}$S]methionine-labeled oocytes; lanes 2 and 3, [γ-$^{32}$P]ATP-labeled oocytes treated with or without progesterone, respectively; lanes 4–6, same as lanes 1–3 but with a shorter exposure time. Arrows at right indicate the two cyclin B2 bands.

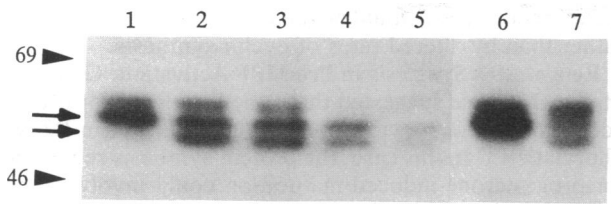


FIG. 2. Western blot analysis of cyclin B2 protein 10 hr after c-*mos*$^{xe}$ RNA injection or progesterone treatment. Five oocytes were used for each lane. Arrows designate the two bands of cyclin B2. The identity of the third band is unknown and was not seen in all blots. Lanes 1–5, c-*mos*$^{xe}$ RNA-injected oocytes in stage 6, late stage 5 (1150 μm in diameter), early stage 5 (1000 μm), late stage 4 (850 μm), and early stage 4 (600 μm), respectively; lane 6, progesterone-treated stage 6 oocytes; lane 7, control stage 6 oocytes.

Mos extracted from stage 4 oocytes is biologically active, we conclude either that Mos does not function *in vivo* as a cyclin kinase or that it does not phosphorylate cyclin to a significant degree. This view is supported further by the observation that in stage 4 oocytes, injection of c-*mos*$^{xe}$ RNA did not result in a change in the relative ratio between the two protein bands seen in Western blots.

**c-*mos*$^{xe}$ Product and Cyclin B2 Stability.** To determine whether c-*mos*$^{xe}$ product might alter cyclin stability, we first needed to estimate the stability of the endogenous protein in the absence of Mos protein. We used two approaches in this regard. First, using the polyclonal antibody we estimated cyclin mass as well as the rate of cyclin synthesis. From these two values it is possible to calculate the half-life of the steady-state pool of cyclin as described (37). Alternatively, we performed experiments in which oocytes were treated with cycloheximide to measure the loss of radioactivity in prelabeled cyclin B2 as a function of time. The data from both kinds of experiments are summarized below.

By comparing data of the type shown in Fig. 1 with that obtained from Western blots prepared with known amounts of purified cyclin, the mass of cyclin B2 was estimated, not only in stage 6 oocytes but throughout the preceding stages of oogenesis. As shown in Fig. 3, we detected some cyclin B2 already by stage 2 of oogenesis, and by the time stage 6 was reached, the amount of cyclin B2 was estimated to have increased 28-fold to about 418 pg per oocyte. This latter value is about twice that reported by Kobayashi *et al.* (23) for stage 6 oocytes. The pattern of increase in cyclin B2 follows closely

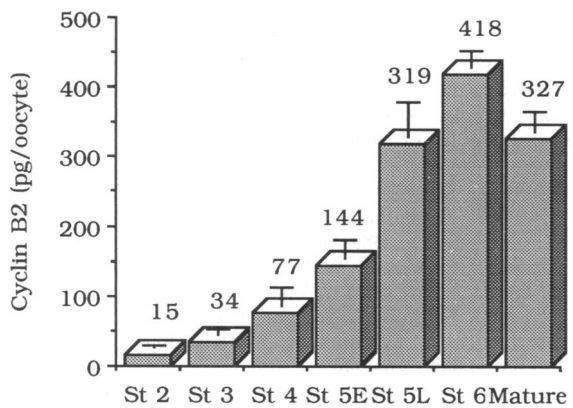


FIG. 3. Cyclin B2 concentration in various stages (St.) of oocytes from two different experiments showing standard error. The original values were obtained by measuring radioactivity (cpm) in protein bands cut out of blotted Immobilon membrane. The concentration is calculated based on cpm from samples and cpm from *E. coli* cyclin B2 marker with known concentrations. St. 5E, early stage 5 oocytes; St. 5L, late stage 5 oocytes (see Fig. 2 legend); Mature, oocytes processed at $GVBD_{50}$.

the pattern of increase in the total protein synthetic rate, which is about 50-fold from stage 2 to stage 6 (38). This relationship could be interpreted to mean that cyclin synthesis occurs throughout oogenesis as a constant percentage of total protein synthesis. To explore this possibility further, we measured the proportion of total protein synthetic activity devoted to newly synthesized cyclin in stage 6 oocytes. This also allowed an estimate of the rate of cyclin B2 synthesis in stage 6 oocytes.

Based on data from several experiments, we estimate that radioactive cyclin B2 represents 0.013–0.021% of total newly synthesized protein, not only in control stage 6 oocytes but also in oocytes induced to mature with progesterone (Table 1). The actual rate of total protein synthesis in stage 6 oocytes, as well as during oocyte maturation, has been measured repeatedly in numerous experiments over the years (39). Consistent with previous studies, our data show about a 2-fold increase in total protein synthesis by the time of $GVBD_{50}$. Based on previous data of total protein synthesis (about 20 ng/hr per oocyte) and a mean percentage of cyclin B2 synthesis from Table 1 of 0.017, we estimate that the rate of cyclin B2 synthesis in stage 6 oocytes is about 3.4 pg/hr per oocyte, increasing about 2-fold after GVBD to 6.8 pg/hr per oocyte (Table 1; refs. 26, 33, 38, and 40). Cyclin B2 would continue to be synthesized at the higher rate throughout maturation. It is interesting that the radioactive cyclin B2 is detected as two bands, which, based on relative mobility, represent unphosphorylated and phosphorylated cyclin. As was the case with total cyclin described earlier, newly synthesized cyclin B2 becomes completely phosphorylated during maturation and at about 0.6 $GVBD_{50}$ (data not shown).

Assuming a cyclin B2 mass in stage 6 oocytes of 418 pg and a rate of synthesis of 3.4 pg/hr, we estimate that the half-life of cyclin B2 is 85 hr (37). In an attempt to verify this number, stage 6 oocytes were labeled with [$^{35}$S]methionine for 1 hr and incubated with cycloheximide (50 μg/ml). Radioactivity in cyclin was subsequently measured at various times. As

Table 1. Incorporation of $^{35}$S into cyclin B2 and total protein in control and progesterone-treated oocytes

| Time after treatment | cpm per oocyte | | (B2/total) × 100 |
|---|---|---|---|
| | Total* | B2* | |
| Control | 402,677 | 52 | 0.013† |
| | 158,415 | 16 | 0.010‡ |
| | 209,205 | 32 | 0.015§ |
| 1 hr | 381,573 | 73 | 0.019† |
| | 178,863 | 29 | 0.016‡ |
| | 243,885 | 51 | 0.021§ |
| 3 hr | 426,796 | 81 | 0.019† |
| | 238,181 | 40 | 0.016‡ |
| | 397,485 | 79 | 0.020§ |
| 6 hr (GVBD) | 585,938 | 75 | 0.013† |
| | 360,762 | 49 | 0.014‡ |
| | 507,468 | 107 | 0.021§ |
| 9 hr | 523,008 | 124 | 0.024† |
| | 392,333 | 74 | 0.019‡ |
| | 515,643 | 100 | 0.019§ |

*Actual radioactivity in total protein and cyclin B2 was measured in groups of 5, 10, or 15 oocytes in the respective experiments. The values shown were obtained by dividing total radioactivity by the appropriate number of oocytes in each sample.

†‡Oocytes with the same symbols are from the same female and total protein was based on measurement of radioactivity in perchloric acid-fixed oocytes. The values for cyclin B2 were obtained from the cyclin bands cut from an SDS/polyacrylamide gel.

§Oocytes from the same female, but the total protein represents radioactivity in acetone-precipitated pellets; the values for cyclin B2 were obtained from immunoprecipitated protein after acetone precipitation of the total protein, but before electrophoresis.

shown in Fig. 4, there is minimal loss of label even after 46 hr of continuous culture in cycloheximide. This result supports the view that newly synthesized cyclin is very stable, at least in $G_2$ arrested oocytes. We conclude from these observations that it is highly unlikely that any agent which induces oocyte maturation, including c-*mos*$^{xe}$ product, would do so by exerting an effect at the level of cyclin stability.

## DISCUSSION

The demonstration of a protein synthesis requirement in the induction of oocyte maturation was based on experiments showing that cycloheximide inhibits GVBD when added to oocytes prior to steroid exposure, but not when added an hour or so after exposure to progesterone (13). The additional observations that oocytes contain pre-MPF and that MPF can induce GVBD in the presence of cycloheximide (12, 15, 41) suggest that protein synthesis is required to produce a protein(s) that, directly or indirectly, activates pre-MPF. The identity of this protein remains unknown.

**Role of Cyclin Synthesis in Pre-MPF Activation.** Considerable evidence now exists that the periodic synthesis and degradation of cyclin proteins drives the periodic appearance and disappearance of MPF activity in mitotically dividing cells (1, 42, 43). The induction of meiosis in *Xenopus* oocytes does not appear to proceed in the same manner. Thus, stage 6 oocytes already contain cyclin B2 protein (Fig. 1; refs. 17 and 23). Furthermore, the cyclin B2 present in stage 6 oocytes already is complexed with p34$^{cdc2}$, which nevertheless does not exhibit MPF activity (17, 23). Finally, Minshull *et al.* (18) have shown that destruction of all cyclin mRNA by injection of antisense oligodeoxynucleotides into oocytes does not prevent the induction of maturation by progesterone. Nevertheless, stage 6 oocytes are synthesizing cyclin B2 even before progesterone exposure, and cyclin synthesis continues during progesterone-induced maturation (Fig. 3; refs. 17 and 23). What role, if any, does cyclin synthesis play in maturation?

Fig. 3 shows that cyclin B2 mass increases about 28-fold between stage 2 and stage 6 of oogenesis, a result that would occur if cyclin B2 synthesis were maintained as a constant percentage of total protein synthesis throughout oogenesis (26). Interestingly, if we assume that the percentage equates with that actually measured in stage 6 oocytes (about 0.017%) and that newly synthesized cyclin B2 has a half-life of about 85 hr, the steady-state levels of cyclin B2 in different stages of oogenesis would be essentially those measured (Fig. 3). While total protein synthesis increases about 2-fold during maturation (1), cyclin synthesis also has increased about 2-fold by the time of GVBD (Table 1). One interpretation of the data presented here is that cyclin B2 synthesis, like p34$^{cdc2}$ synthesis, is constitutive throughout oogenesis and oocyte maturation. Thus, cyclin levels, at least through the meiotic cell cycles, would be regulated solely by degradation, rather than by altered rates of cyclin synthesis.

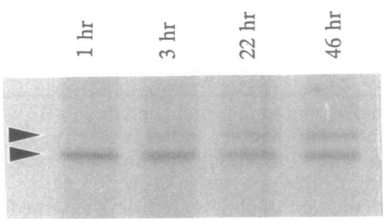


FIG. 4. Immunoprecipitated cyclin B2 protein from stage 6 oocytes (indicated by arrowheads). Oocytes were labeled with [$^{35}$S]methionine for 1 hr and then incubated in OR2 medium (29) containing cycloheximide (50 μg/ml) until processing. The time points at which oocytes were processed are indicated above the lanes.

**Role of Mos Synthesis in Pre-MPF Activation.** Gautier and Maller (17) have suggested that "the key event for pre-MPF activation in the oocyte is the regulation of cyclin B2 phosphorylation." In this case, the protein synthesis requirement for progesterone-induced maturation could involve appearance of cyclin kinase. In turn, Mos protein fits many of the expectations for a cyclin kinase whose synthesis is induced by progesterone. First, cyclin B2 is a substrate for Mos protein kinase *in vitro* (22). Second, injection of c-*mos*$^{xe}$ mRNA into stage 6 oocytes induces maturation (GVBD) and the resulting complete phosphorylation of endogenous cyclin B2 (21, 22). Finally, c-*mos*$^{xe}$ product is not detectable prior to the induction of oocyte maturation but appears approximately coincident with the activation of pre-MPF (21). However, neither the current data nor experiments reported by Freeman *et al.* (44) support the hypothesis suggested by Roy *et al.* (22) that Mos protein kinase phosphorylates cyclin B2 to any significant extent *in vivo*. In particular, the data in Fig. 2 show clearly that phosphorylated cyclin B2 already exists *in vivo* as early in oogenesis as stage 4, and neither stage 4 nor stage 6 oocytes contain endogenous Mos protein. Further, injection of c-*mos*$^{xe}$ RNA, at least in stage 4 oocytes, causes no change in the ratio of phosphorylated to nonphosphorylated cyclin B2. We conclude from these observations that Mos protein kinase has no direct effect on cyclin B2.

Considering the above, if we continue to assume that synthesis of Mos accounts for the protein synthesis requirement during the cycloheximide-sensitive period of progesterone-induced maturation, how might Mos protein kinase function in leading to activation of pre-MPF? At this point, we can only speculate. However, any hypothesis that includes the need to synthesize one or more new proteins in response to progesterone must of necessity consider mechanisms that involve the recruitment of mRNA onto polysomes (39). Thus, one possible mechanism by which Mos induces maturation in stage 6 oocytes could be that Mos kinase plays some role in message recruitment, which leads to the synthesis of a protein required for activation of pre-MPF. Further work is necessary to identify the real substrate of Mos protein kinase *in vivo* to better understand its role in oocyte maturation.

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