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Meiotic maturation in *Xenopus* requires polyadenylation of multiple mRNAs

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Cytoplasmic polyadenylation of specific mRNAs commonly is correlated with their translational activation during development. Here, we focus on links between cytoplasmic polyadenylation, translational activation and the control of meiotic maturation in *Xenopus* oocytes. We manipulate endogenous c-*mos* mRNA, which encodes a protein kinase that regulates meiotic maturation. We determined that translational activation of endogenous c-*mos* mRNA requires a long poly(A) tail *per se*, rather than the process of polyadenylation. For this, we injected 'prosthetic' poly(A)—synthetic poly(A) tails designed to attach by base pairing to endogenous c-*mos* mRNA that has had its own polyadenylation signals removed. This prosthetic poly(A) tail activates c-*mos* translation and restores meiotic maturation in response to progesterone. Thus the role of polyadenylation in activating c-*mos* mRNA differs from its role in activating certain other mRNAs, for which the act of polyadenylation is required. In the absence of progesterone, prosthetic poly(A) does not stimulate c-*mos* expression, implying that progesterone acts at additional steps to elevate c-Mos protein. By using a general inhibitor of polyadenylation together with prosthetic poly(A), we demonstrate that these additional steps include polyadenylation of at least one other mRNA, in addition to that of c-*mos* mRNA. These other mRNAs, encoding regulators of meiotic maturation, act upstream of c-Mos in the meiotic maturation pathway.

Keywords: c-*mos*/meiotic maturation/oocyte/polyadenylation/translational control

Introduction

Translational control is prominent in key decisions made during early development. Establishment of embryonic polarity and specification of cell fates exploit the regulation of mRNAs present in the egg before fertilization (reviewed in Curtis *et al.*, 1995; Wickens *et al.*, 1996). mRNAs encoding cyclins, cyclin-dependent kinases (CDKs) and their regulators are regulated extensively in time and space throughout early development. Their control is required to support and coordinate meiotic and embryonic cell cycles.

Progression of meiosis in vertebrates is linked intimately to the regulation of specific mRNAs. Vertebrate oocytes generally are arrested in prophase of meiosis I, and resume meiosis in response to external stimuli, such as progesterone. During meiotic maturation, they proceed to metaphase of meiosis II, where they arrest and await fertilization. Transcription is quiescent during this interval, making post-transcriptional controls critical. The c-*mos* proto-oncogene encodes a serine/threonine kinase required for control of meiotic maturation and for cell cycle arrest prior to fertilization (reviewed in Sagata, 1997). In *Xenopus*, translation of c-*mos* mRNA is required for meiotic maturation, and overexpression of c-*mos*, achieved by injecting either the mRNA or the protein, induces meiotic maturation in the absence of progesterone (Sagata *et al.*, 1988, 1989; Yew *et al.*, 1992). In mice, lack of a functional c-*mos* gene causes parthenogenetic activation of oocytes and reduced fertility, consistent with a role in oogenesis (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994).

Cytoplasmic polyadenylation is often correlated with, and can cause, translational activation of mRNAs during early development (reviewed in Richter, 1996; Wickens *et al.*, 1997). It requires two *cis*-acting sequence signals, AAUAAA and a U-rich element called a cytoplasmic polyadenylation element (CPE). The mechanisms and regulation of the process are highly conserved (Verrotti *et al.*, 1996). The developmental importance of regulated changes in poly(A) length has been demonstrated in two contexts. In the first, meiotic maturation of mouse and frog oocytes requires cytoplasmic polyadenylation of c-*mos* mRNA (Gebauer *et al.*, 1994; Sheets *et al.*, 1995). In *Xenopus*, maturation is prevented by targeted removal of the polyadenylation signals from endogenous c-*mos* mRNA; maturation is rescued by injection of a *trans*-acting prosthetic RNA that restores c-*mos* polyadenylation signals by base pairing to the endogenous, amputated, mRNA (Sheets *et al.*, 1995). In the second context, pattern formation in *Drosophila* requires both cytoplasmic polyadenylation of *bicoid* mRNA (Sallés *et al.*, 1994; Lieberfarb *et al.*, 1996) and regulated shortening of the poly(A) tail of *hunchback* mRNA (Wreden *et al.*, 1997).

The presence of a poly(A) tail is sufficient to enhance translation of certain synthetic mRNAs injected into oocytes (Galili *et al.*, 1988; Vassalli *et al.*, 1989; Paris and Richter, 1990; Sallés *et al.*, 1994). However, translational activation of other injected mRNAs requires the dynamic process of cytoplasmic polyadenylation, rather than the mere presence of poly(A) (McGrew *et al.*, 1989; Simon *et al.*, 1992). Similarly, ribose methylation of certain injected mRNAs requires the ongoing process of polyadenylation, consistent with a role for ribose methylation in their translational activation (Kuge and Richter, 1995). The ability of a prosthetic RNA containing c-*mos* polyadenylation signals to rescue translational activation of endogenous c-*mos* mRNA (Sheets *et al.*, 1995) does not distinguish whether activation requires polyadenylation *in vivo* or the mere presence of a long tail.

Endogenous mRNAs may be subject to mechanisms of regulation that are evaded by synthetic mRNAs injected into the cytoplasm (Bouvet and Wolffe, 1994; Braddock *et al.*, 1994). The nature of those mechanisms is not entirely clear, but may involve the 'nuclear experience' of natural mRNAs and translational repression by Y-box proteins (Bouvet and Wolffe, 1994; Meric *et al.*, 1997). Regardless, these findings caution against extrapolation solely from the behavior of injected mRNAs, and emphasize the importance of examining natural, endogenous mRNAs in studies of translational regulation. Very few such studies have been reported in the context of the linkage between cytoplasmic polyadenylation and translational control.

In this report, we examine translational activation of endogenous c-*mos* mRNA, focusing on the role of poly(A) in its translational activation and in the regulation of meiotic maturation. We exploit a prosthetic RNA strategy to provide a poly(A) tail to an endogenous mRNA that has had its own polyadenylation signals removed. We find that a long poly(A) tail *per se* is sufficient to support translational activation of c-*mos* mRNA and hence meiotic maturation in response to progesterone. In the absence of progesterone, meiotic maturation is not induced, implying additional targets of progesterone action. We demonstrate that polyadenylation of other mRNAs, in addition to c-*mos*, is required to induce meiotic maturation. These other progesterone targets lie upstream of the accumulation of c-Mos protein.

Results

A long poly(A) tail is sufficient to stimulate expression of c-mos

To test the role of poly(A) in stimulating c-*mos* expression, we used the strategy depicted in Figure 1A. Injection of an antisense oligonucleotide directed to the c-*mos* 3′-untranslated region (UTR), followed by cleavage of the mRNA by endogenous RNase H results in an 'amputated' c-*mos* mRNA that lacks polyadenylation signals. As shown previously, injection of the antisense oligonucleotide prevents oocyte maturation in response to progesterone (Figure 1B, bar 2; Sheets *et al.*, 1995); it also prevents accumulation of c-Mos, as expected (Figure 1C, lane 2). Injection of recombinant c-Mos protein into these cells induces oocyte maturation, confirming that the antisense oligonucleotide specifically prevents accumulation of c-Mos (data not shown).

To determine whether a long poly(A) tail is sufficient to elevate c-Mos levels in response to progesterone, we injected a prosthetic RNA capable of annealing to amputated c-*mos* mRNA, and carrying only a poly(A) tail. We refer to this RNA as 'prosthetic poly(A)'. Oocyte maturation was assayed by the appearance of a white spot at the animal pole, indicative of nuclear breakdown and completion of first meiosis. Prosthetic poly(A) that contains 130 adenosine residues rescues progesterone-induced oocyte maturation (Figure 1B, bar 3) and c-Mos levels (Figure 1C, lane 3). Prosthetic poly(A) typically rescued c-Mos levels to 40% of the amount in uninjected, progesterone-matured cells. Injection of an RNA that carries a poly(A) tail but is incapable of annealing to amputated c-*mos* mRNA ['nonspecific poly(A)'] does not rescue maturation (Figure 1B, bar 4) or c-Mos (Figure 1C, lane 4), showing that rescue by prosthetic poly(A) requires that it be targeted to c-*mos* mRNA.

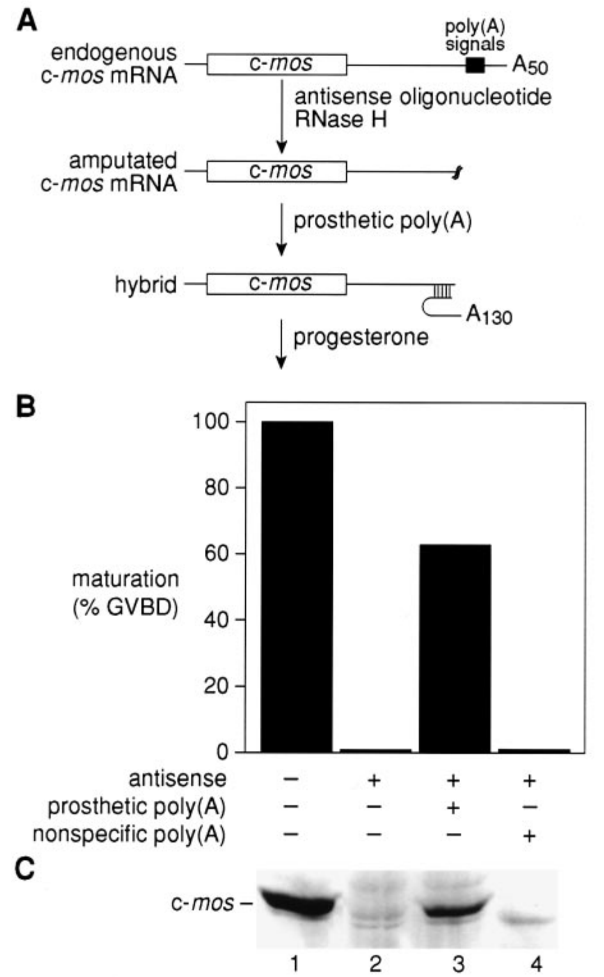

Fig. 1. Poly(A) rescues c-Mos levels and meiotic maturation in progesterone-treated oocytes. Oocytes were injected with antisense oligonucleotide, then with either prosthetic poly(A) containing 130 adenosines or non-specific poly(A) containing 130 adenosines, and finally treated with progesterone. Non-specific poly(A) contains polylinker sequence instead of complementarity to the c-*mos* 3′-UTR. The experiment was repeated eight times. Results of a representative experiment are shown. In this and all subsequent experiments, oocyte maturation was assayed by monitoring the appearance of a white spot at the animal pole of the oocyte, indicative of germinal vesicle (nuclear) breakdown (GVBD) and the completion of first meiosis. (**A**) Experimental design. The polyadenylation signals (filled box) include both AAUAAA and a CPE. Prosthetic poly(A) contains only a region of complementarity and a stretch of poly(A). (**B**) Histogram of maturation results. (**C**) Immunoblot of c-*mos*. For lanes 1 and 3, lysates were prepared only from cells which had undergone GVBD. A protein which migrates slightly faster than c-Mos is recognized non-specifically by the anti-c-*mos* antibody, as determined by peptide neutralization of the antibody prior to use (data not shown).

The prosthetic poly(A) RNAs lack the signals required for cytoplasmic polyadenylation, AAUAAA and a CPE, and so should not receive poly(A) during maturation. Nevertheless, to test this possibility rigorously, we performed an experiment in which we injected prosthetic poly(A) terminating in a 3′ deoxyadenosine, to block any potential elongation of the tail. A prosthetic RNA containing 130 adenosine residues and ending in a 3′ deoxyadenosine rescued maturation in 100% of the oocytes

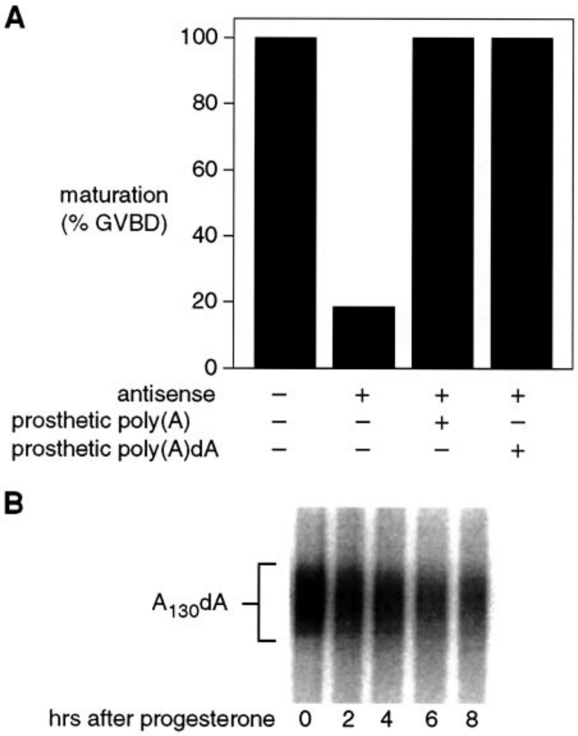

Fig. 2. Prosthetic poly(A) does not receive additional poly(A) during maturation. Oocytes were injected with antisense oligonucleotide, then with either prosthetic poly(A) or prosthetic poly(A) terminating in 3′ deoxyadenosine (‘prosthetic poly(A) dA’), and finally were treated with progesterone. The experiment was repeated twice. Results of a representative experiment are shown. (**A**) Histogram of maturation results. (**B**) Lengths of injected RNA during oocyte maturation. Prosthetic A_{130}dA RNAs used in (A) were recovered at various times after progesterone addition, as indicated below each lane, and were analyzed by denaturing gel electrophoresis. GVBD began 6 h after progesterone addition; by 8 h, the prosthetic RNAs had rescued GVBD in 45% of the cells. RNAs shown were isolated from cells that had not yet undergone GVBD. As expected, following GVBD, the prosthetic poly(A) RNAs were fully deadenylated due to the default deadenylation pathway, and persisted in deadenylated form (Fox and Wickens, 1990; Varnum and Wormington, 1990).

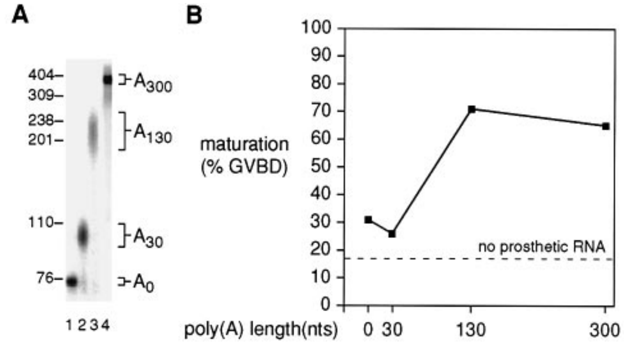

Fig. 3. Rescue of maturation depends on a minimum length of poly(A). Oocytes were injected with antisense oligonucleotide, then with prosthetic RNA carrying a poly(A) tail of 0, 30, 130 or 300 adenosines [mean length of poly(A)], and finally treated with progesterone. The experiment was repeated five times. In each experiment, all four prosthetic RNAs were injected, and oocytes from a single frog were used. Results of a representative experiment are shown. (**A**) PhosphorImager scan of a denaturing polyacrylamide gel, showing prosthetic RNAs before injection. The migration of single-stranded DNA markers is indicated on the left. (**B**) Plot of maturation results. In this experiment, 100% of uninjected, progesterone-treated cells matured. The dotted line indicates maturation of cells injected only with antisense oligonucleotide.

tested (Figure 2A). Moreover, as shown in Figure 2B, the injected RNA did not significantly change in length prior to nuclear breakdown. We conclude that the poly(A) tail itself is necessary and sufficient for progesterone-induced accumulation of c-Mos, and hence for oocyte maturation.

Rescue of oocyte maturation depends on a minimum length of poly(A)

The poly(A) tail on c-*mos* mRNA increases from 40 and 75 adenosines in a resting stage VI oocyte to a heterogeneous length with a mean of 125 adenosines during oocyte maturation (Sheets *et al.*, 1994). To determine whether rescue of maturation by prosthetic poly(A) requires a minimum length of poly(A), we injected prosthetic RNAs with four different lengths of poly(A) into oocytes that previously had been injected with the antisense oligonucleotide. The RNAs injected are shown in Figure 3A. Efficient rescue of progesterone-induced maturation requires that the prosthetic poly(A) carries at least 130 adenosines (Figure 3B). Prosthetic RNAs carrying either 0 or 30 adenosines do not rescue maturation significantly. The stabilities of the four prosthetic RNAs in oocytes are comparable (data not shown). Thus, the length of poly(A) required for rescue of maturation correlates with the length of poly(A) present on endogenous c-*mos* mRNA during maturation. A tail of 30 nucleotides, which should be sufficient to bind poly(A)-binding protein (Sachs and Kornberg, 1990), does not support maturation.

Progesterone is required for rescue of c-Mos protein accumulation and oocyte maturation

Overexpression of c-*mos*, achieved by injection of active c-Mos protein, is sufficient to induce oocyte maturation in the absence of protein synthesis (Yew *et al.*, 1992). If the sole target of progesterone in inducing meiotic maturation were activation of c-*mos* polyadenylation, then addition of a prosthetic poly(A) tail to c-*mos* mRNA should induce c-*mos* expression on its own, and thereby cause maturation in the absence of progesterone. To test this hypothesis, endogenous c-*mos* mRNA was ablated by injection of the antisense oligonucleotide, then supplied with a poly(A) tail by injection of prosthetic poly(A). In the absence of progesterone, this induced neither germinal vesicle breakdown (GVBD) (Figure 4A, bar 2) nor c-Mos accumulation (Figure 4B, lane 2). We conclude that progesterone-induced maturation requires additional steps, besides polyadenylation of c-*mos* mRNA.

Two distinct levels of c-Mos have been observed during progesterone-induced oocyte maturation (Gotoh *et al.*, 1995). Shortly after progesterone addition, there is a slight increase in accumulated c-Mos; at nuclear breakdown, c-Mos levels increase dramatically and a high level persists until metaphase arrest. We verified this pattern in a time course of c-*mos* induction (Figure 4C). c-Mos levels increased slightly ~1 h following progesterone addition (Figure 4C, lane 3), then increased dramatically at GVBD (Figure 4C, lane 6).

Polyadenylation of c-*mos* mRNA occurs before GVBD (Ballantyne *et al.*, 1997), suggesting that it might have a role in the initial increase. To determine whether polyadenylation is necessary and sufficient for the initial increase in c-Mos levels, the levels of c-Mos seen in

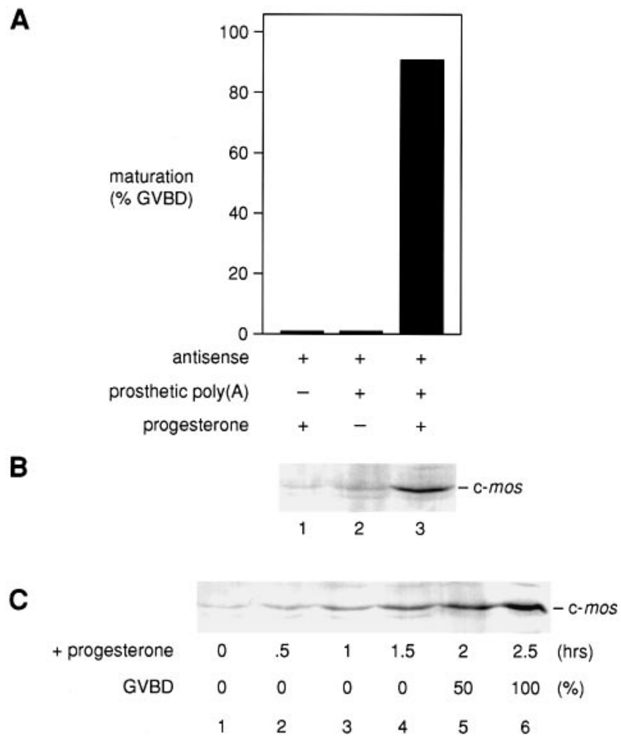

Fig. 4. Progesterone is required for rescue of c-Mos accumulation and oocyte maturation. Oocytes were injected with antisense oligonucleotide, then with prosthetic poly(A) containing 130 adenosines, and finally some oocytes were treated with progesterone. Uninjected oocytes, taken from the same frog as the injected oocytes, were frozen at various times after progesterone application for comparison with injected cells. The experiment was repeated five times. Results of a representative experiment are shown. (**A**) Histogram of maturation results. (**B**) Immunoblot of c-*mos*. For lane 3, lysate was prepared only from cells which had undergone GVBD. (**C**) Immunoblot of c-*mos*, showing the time course of c-Mos accumulation during oocyte maturation. The number of hours after progesterone application, and the percentage GVBD of the total pool of cells at each time point, are shown below. For each sample, 10 oocytes were frozen, with the percentage GVBD in each sample representative of the percentage GVBD in the total pool of oocytes.

Figure 4B were compared with those seen during the time course of maturation. To make such comparisons possible, oocytes used for the experiments in Figure 4A–C were all taken from the same frog. Injection of the antisense oligonucleotide prevents accumulation of the low level of c-Mos (compare lane 1 in Figure 4B with lane 3 in Figure 4C). Injection of prosthetic poly(A) does not elevate c-Mos levels significantly (Figure 4B, lane 2 versus Figure 4C, lane 1). We conclude that, in the absence of progesterone, the presence of a long poly(A) tail is insufficient to reach the low level of c-Mos observed during oocyte maturation.

Prosthetic poly(A) stimulates translation of a reporter mRNA in the absence of progesterone

To determine whether progesterone treatment is a general requirement for prosthetic poly(A)-stimulated translation, we used a chimeric luciferase–c-*mos* reporter mRNA. The reporter mRNA is composed of the luciferase open reading frame followed by 238 nucleotides of sequence from the 3′ end of the c-*mos* 3′-UTR, but not including the polyadenylation signals. It ends at the same position as endogenous c-*mos* mRNA following amputation with the antisense oligonucleotide. Oocytes were injected with 0.5 fmol of reporter mRNA, which approximates the amount of endogenous c-*mos* mRNA in an oocyte, as determined by a nuclease protection assay (data not shown). Translation of this reporter mRNA does not increase during oocyte maturation; however, its translation is stimulated by subsequent injection of prosthetic poly(A) carrying 130 adenosines, with or without progesterone treatment (Figure 5). We conclude from this experiment that prosthetic poly(A) can stimulate translation of an mRNA in the absence of progesterone, and thus that endogenous c-Mos accumulation is regulated by a mechanism to which the reporter mRNA is not subject.

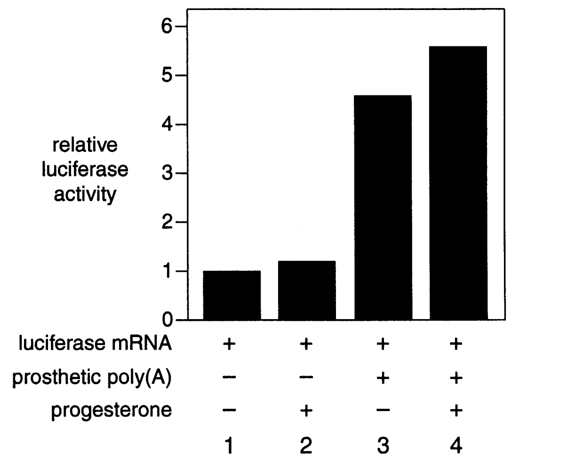

Fig. 5. Prosthetic poly(A) stimulates translation of a reporter mRNA in the absence of progesterone. Oocytes were injected with luciferase–c-*mos* reporter mRNA, then with prosthetic poly(A) containing 130 adenosines, and finally some oocytes were treated with progesterone. Following meiotic maturation of progesterone-treated cells, oocyte lysates were prepared and luciferase activity was measured. The experiment was repeated four times. Results of a representative experiment are shown. Luciferase activity is shown relative to sample 1.

Cordycepin prevents rescue of meiotic maturation by prosthetic poly(A)

3′ deoxyadenosine (cordycepin) inhibits polyadenylation by preventing the formation of additional phosphodiester bonds after its incorporation into an RNA chain *in vivo* and *in vitro* (e.g. Darnell *et al.*, 1971; Maale *et al.*, 1975; Moore *et al.*, 1986; Sheets *et al.*, 1987). Incubation of *Xenopus* oocytes in media containing cordycepin prevents oocyte maturation in response to progesterone (Kuge and Inoue, 1992). This is to be expected, since polyadenylation of c-*mos* mRNA is required for meiotic maturation. To determine whether additional mRNA(s) besides c-*mos* must be polyadenylated in order for oocyte maturation to occur, we used cordycepin to inhibit polyadenylation in the same cells into which the antisense oligonucleotide and prosthetic poly(A) had been injected. If c-*mos* is the only mRNA whose polyadenylation is required for oocyte maturation, then prosthetic poly(A) directed to amputated c-*mos* mRNA should rescue maturation in oocytes that have been treated with cordycepin.

Incubation of oocytes in culture media containing cordycepin inhibited oocyte maturation (Figure 6A, compare bars 2 and 3). Following cordycepin treatment, oocytes were injected with the antisense oligonucleotide, followed

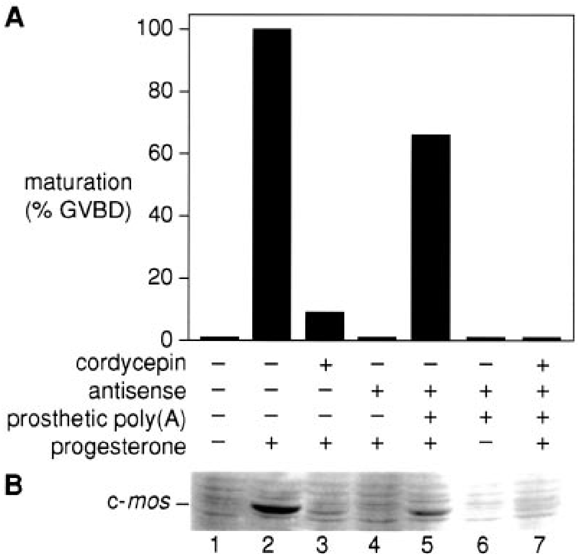

Fig. 6. Cordycepin prevents rescue of maturation and c-Mos accumulation by prosthetic poly(A). Oocytes were first incubated in media containing or not containing cordycepin for 12 h. Oocytes were then injected with antisense oligonucleotide, followed by injection of prosthetic poly(A) containing 130 adenosines, and finally treated with progesterone. The experiment was repeated three times. Results of a representative experiment are shown. (**A**) Histogram of maturation results. (**B**) Immunoblot of c-*mos*. For lanes 1, 3, 4, 6 and 7, lysates were prepared only from cells which had not undergone GVBD. For lanes 2 and 5, lysates were prepared only from cells which had undergone GVBD.

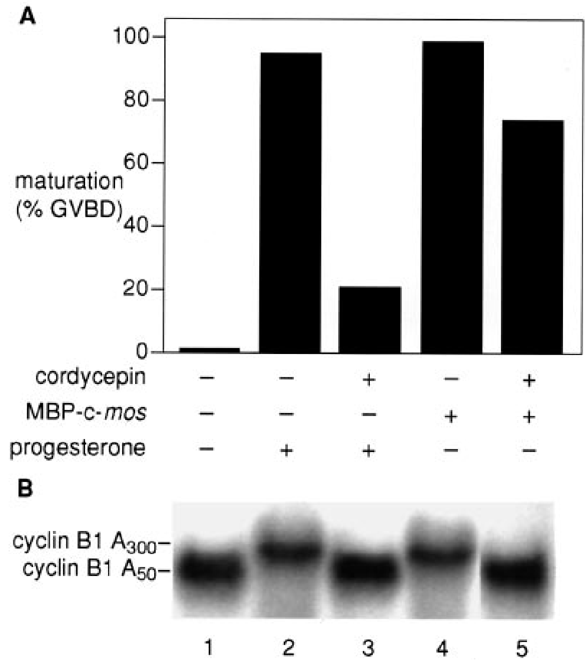

Fig. 7. Cordycepin does not block c-Mos-induced oocyte maturation, and does prevent polyadenylation of endogenous mRNA. Oocytes were first incubated in media containing or not containing cordycepin for 12 h. MBP–c-*mos* was then injected into some oocytes, and progesterone was applied to others. (**A**) Histogram of maturation results. Data shown are the averages of four independent experiments. (**B**) Northern blot of cyclin B1 mRNA. For lanes 1 and 3, oocyte RNA was isolated only from cells which had not undergone GVBD. For lanes 2, 4 and 5, oocyte RNA was isolated only from cells which had undergone GVBD.

by prosthetic poly(A), and treated with progesterone. Cordycepin prevents rescue of both maturation (Figure 6A, compare bar 7 with bar 5) and c-Mos accumulation (Figure 6B, compare lane 7 with lane 5) by prosthetic poly(A). Cordycepin does not affect total protein synthesis, translation of an injected reporter mRNA or the stimulation of that mRNA's translation by prosthetic poly(A) (data not shown). These data raise the possibility that polyadenylation of one or more mRNAs in addition to c-*mos* is required for c-*mos* translation and oocyte maturation.

Polyadenylation is not required downstream of c-Mos protein

To determine whether meiotic maturation requires additional polyadenylation events downstream of c-Mos, we injected recombinant c-Mos protein into cordycepin-treated oocytes. The recombinant c-Mos protein is a bacterially expressed fusion protein between maltose-binding protein (MBP) and *Xenopus* c-*mos* (Yew *et al.*, 1992). Overexpression of c-*mos* by injection of MBP–c-Mos efficiently induces meiotic maturation in the absence of progesterone (Figure 7A, bar 4), as observed previously (Yew *et al.*, 1992). Cordycepin treatment does not significantly block c-*mos*-induced oocyte maturation (Figure 7A, bar 5), suggesting that meiotic maturation does not require polyadenylation after c-Mos accumulation.

Following uptake by the cell, cordycepin is converted to the triphosphate, 3′ deoxyadenosine triphosphate (Kuge and Inoue, 1992). This molecule is an ATP analog, and in principle could affect other ATP-dependent processes besides polyadenylation, including action as a competitive inhibitor of CDK1 kinase activity. In this regard, the rescue of maturation by injected c-Mos protein provides an important control, as it demonstrates that cordycepin does not inhibit any cell cycle kinases downstream of c-Mos, including CDK1.

To ensure that cordycepin had indeed prevented cytoplasmic polyadenylation, we examined endogenous cyclin B1 mRNA by Northern blotting. Polyadenylation of cyclin B1 mRNA occurs late during maturation, and can be detected by a change in electrophoretic mobility on a Northern blot (Ballantyne *et al.*, 1997). As shown in Figure 7B, cyclin B1 mRNA receives poly(A) in response to progesterone (lane 2) or MBP–c-Mos (lane 4). As expected, cordycepin prevents its polyadenylation in response to either inducer (Figure 7B, lanes 3 and 5).

Discussion

Our results lead to the following main conclusions. The presence of a long poly(A) tail on endogenous c-*mos* mRNA is sufficient to stimulate c-Mos accumulation and hence maturation in response to progesterone; the act of polyadenylation *in vivo* is not essential. An additional progesterone-sensitive event is required to elevate c-Mos levels and induce maturation. One such event is likely to be polyadenylation of another mRNA, in addition to c-*mos* mRNA.

Previous results from injection of certain synthetic mRNAs suggest that the dynamic process of poly(A) addition, not the long poly(A) tail itself, is required for translational activation in response to progesterone, and have linked the polyadenylation process to 2′-*O*-methyl-

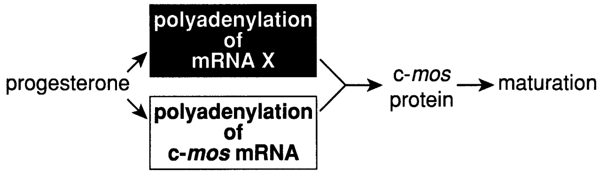

Fig. 8. Model for role of cytoplasmic polyadenylation in oocyte maturation. Progesterone activates cytoplasmic polyadenylation of c-*mos* and at least one other mRNA (X). These events together lead to c-*mos* accumulation, which in turn propels meiotic maturation.

ation of the cap structure (Kuge and Richter, 1995). The experiments reported here exploit and manipulate endogenous c-*mos* mRNA, and demonstrate that the presence of a long poly(A) tail is sufficient. The different conclusions drawn from these two groups of experiments could be due to a difference between endogenous and injected mRNAs, or among the particular mRNAs used. The mere presence of poly(A) stimulates translation of various injected mRNAs (Galili *et al.*, 1988; Vassalli *et al.*, 1989; Paris and Richter, 1990; Sallés *et al.*, 1994), consistent with our findings using endogenous c-*mos* mRNA.

In the absence of progesterone, a prosthetic poly(A) tail stimulates translation of a reporter mRNA (Figure 5), but does not cause increased accumulation of c-Mos (Figure 4). This could reflect the difference between the UTRs of the endogenous and synthetic mRNAs, or the repressive effect of 'nuclear experience' on endogenous mRNAs (Bouvet and Wolffe, 1994; Braddock *et al.*, 1994). Alternatively, effects on c-Mos turnover could account for the difference (Nishizawa *et al.*, 1992). These possibilities do not compromise our interpretation that a long poly(A) tail is sufficient to rescue maturation induced by progesterone.

We propose that polyadenylation of another mRNA, in addition to c-*mos* mRNA, is required to cause elevation of c-Mos and induce maturation (Figure 8). In this model, progesterone activates cytoplasmic polyadenylation of c-*mos* mRNA, and that of an unidentified mRNA, X. This model is consistent with the data presented here. Injection of prosthetic poly(A) targeted to amputated c-*mos* mRNA does not induce c-Mos accumulation or oocyte maturation on its own, because mRNA X requires progesterone in order to undergo polyadenylation. However, upon progesterone treatment, mRNA X is polyadenylated, enabling prosthetic poly(A) to rescue c-Mos accumulation and meiotic maturation. Cordycepin blocks rescue by prosthetic poly(A) in the presence of progesterone because it prevents polyadenylation of mRNA X. Cordycepin does not affect meiotic maturation induced by injection of c-Mos because no polyadenylation events are required downstream of c-Mos protein. This model is consistent with the finding that recombinant c-Mos protein induces maturation in the absence of protein synthesis (Yew *et al.*, 1992), since polyadenylation and translation of mRNA X precede c-Mos accumulation: if a high level of c-Mos is provided by injection, additional protein synthesis is dispensable.

An important result supporting the model is that cordycepin prevents c-Mos accumulation in the presence of a prosthetic poly(A) tail directed to amputated c-*mos* mRNA and progesterone. Since cordycepin is an ATP analog, the possibility exists that it has effects on other processes, and that these underlie the results. However, several experiments indicate that the inhibitory effect of cordycepin is likely to be exerted through its effects on polyadenylation. First, cordycepin does indeed prevent poly(A) addition to endogenous mRNAs, even late in maturation. Second, cordycepin treatment does not inhibit translation in the oocyte, or its stimulation by poly(A). Finally, and perhaps most importantly, cordycepin has no effect on the ability of c-Mos to induce maturation. This result demonstrates that cordycepin does not inhibit factors in the maturation pathway downstream of c-Mos, such as CDK1. Previous experiments from others affirm that cordycepin specifically inhibits polyadenylation (Kuge and Inoue, 1992).

A simple prediction of the model depicted in Figure 8 is that an unknown protein, X, is required for c-Mos accumulation and meiotic maturation. The protein might be involved in either c-*mos* translational activation or c-Mos stabilization. Using a dominant-negative form of CDK1 that blocks progesterone-induced maturation, Nebreda *et al.* (1995) deduced that c-Mos accumulation requires a newly synthesized protein, most likely a cyclin. Protein X may be this inferred molecule. Several restrictive criteria must be satisfied in identifying mRNA X. Upon progesterone treatment, its translation must be enhanced in a polyadenylation-dependent fashion. Further, its translational activation must be required for meiotic maturation, since its polyadenylation is required for the accumulation of c-Mos. It follows that polyadenylation of mRNA X should be independent of c-*mos* polyadenylation; thus mRNA X should be a class I mRNA as defined by Ballantyne *et al.* (1997). Identification of mRNA X now is an important objective in unraveling the connections between polyadenylation, translational control and regulation of the cell cycle.

Materials and methods

Synthetic RNAs and oligonucleotides

Prosthetic poly(A) is composed of pAB6/SpeI RNA followed by a poly(A) tail. pAB6/SpeI RNA contains 73 nucleotides: 14 nucleotides of vector sequence at its 5′ end, followed by 54 nucleotides of sequence complementary to positions –196 to –143 in the c-*mos* 3′-UTR, followed by five nucleotides of polylinker. To prepare pAB6/SpeI RNA, plasmid pAB6 was cut with *Spe*I and transcribed *in vitro* using an Ampliscribe SP6 RNA polymerase transcription kit (Epicentre). A typical transcription reaction contained 2 μg of plasmid, 5 mM each NTP (except GTP), 4 mM ApppG cap analog (NEB), 1 mM GTP and 20 μCi of [α-^{32}P]UTP. pAB6/SpeI RNA was purified as described (Fox *et al.*, 1989), prior to *in vitro* polyadenylation.

Plasmid pAB6 was constructed by annealing two DNA oligonucleotides, then ligating the annealed oligonucleotides with *Sph*I- and *Hin*dIII-digested pGEM –83/+2 c-*mos* (Sheets *et al.*, 1994). The annealed oligonucleotides contain 54 nucleotides of sequence complementary to positions –196 to –143 [relative to the poly(A) site] of the c-*mos* 3′-UTR, plus *Spe*I and *Sph*I sites on one end and a *Hin*dIII site on the other end.

Nonspecific poly(A) is composed of pGEM7Z/EcoRI RNA followed by a poly(A) tail. pGEM7Z/EcoRI RNA contains 80 nucleotides transcribed from the polylinker of plasmid pGEM7Z (Promega). To prepare pGEM7Z/EcoRI RNA, pGEM7Z was cut with *Eco*RI and transcribed *in vitro* using an Ampliscribe SP6 transcription kit, under the same conditions as used above for pAB6/SpeI RNA. pGEM7Z/EcoRI RNA was purified as described (Fox *et al.*, 1989), prior to *in vitro* polyadenylation.

Luciferase mRNA contains 1680 nucleotides of the luciferase gene followed by 238 nucleotides of sequence corresponding to positions –321 to –83 of the c-*mos* 3′-UTR. Luciferase mRNA was prepared by transcription of the pLuc/c-*mos* plasmid using T7 RNA polymerase, after cleavage with *Dra*I (Sheets *et al.*, 1994). A typical transcription reaction contained 2 μg of plasmid, 1 mM each NTP (except GTP), 5 mM m^7GpppG cap analog (NEB), 0.5 mM GTP, 20 μCi of [α-^{32}P]UTP and 80 U of T7 RNA polymerase (Promega). The transcription reaction was terminated by addition of 1 U of RNase-free DNase I; then free nucleotides were removed by passing the reaction over a Sephadex G50 QuickSpin column (Pharmacia). The mRNA was purified as described (Fox *et al.*, 1989), and resuspended at a final concentration of 10 fmol/μl in 88 mM NaCl. The integrity of the mRNA was verified by agarose gel electrophoresis prior to injection into oocytes.

The anti-c-*mos* antisense oligonucleotide used in this study is identical to oligonucleotide –126A in Sheets *et al.* (1995). It was purified by denaturing polyacrylamide gel electrophoresis followed by HPLC, by the manufacturer (NEB), and was resuspended at a final concentration of 2 mg/ml in 88 mM NaCl prior to injection into oocytes.

In vitro polyadenylation

The protocol for *in vitro* polyadenylation is based on previously determined optimum conditions for *Escherichia coli* poly(A) polymerase (Sippel, 1973). Each reaction contained 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 50 μg/ml bovine serum albumin (BSA), 1 U/μl RNasin (Promega) and 1 mM dithiothreitol (DTT). Typical reactions also contained 1 μM RNA, 130 μM ATP and 0.027 U/μl of *E.coli* poly(A) polymerase (Pharmacia). To terminate prosthetic poly(A) with 3′ deoxyadenosine, cordycepin was substituted for ATP in the reaction. A typical reaction was 300 μl in volume and was incubated for 1 h at 37°C. Polyadenylation reactions were stopped by addition of EDTA to 0.1 M. Then 1 μl of 1 mg/ml proteinase K/ 100 μl reaction was added, along with one-tenth volume of 10× proteinase K reaction buffer [0.5 M Tris–HCl (pH 7.9), 0.1 M EDTA, 0.1 M NaCl, 2% SDS], and incubated for 15 min at 37°C. Polyadenylated RNA was purified essentially as described (Fox *et al.*, 1989), and resuspended at a final concentration of 8 pmol/μl in 88 mM NaCl. The length and integrity of the polyadenylated RNA were verified by polyacrylamide gel electrophoresis prior to injection into oocytes.

Oocyte injection

Xenopus laevis oocytes were isolated, injected and incubated as described (Ballantyne *et al.*, 1997). In all injection experiments, 50 nl of the appropriate solution was injected. Progesterone (Sigma) was added to the media to achieve a final concentration of 10 μg/ml, as appropriate. In experiments involving cordycepin, oocytes were incubated at 18°C for 12 h in media containing 10 mM cordycepin (Sigma) prior to injection or treatment with progesterone. Oocyte maturation was determined by analyzing the oocytes for the presence of a white spot at two to three times $GVBD_{50}$ (the time after progesterone addition when half of the uninjected oocytes display a white spot). Cells were frozen at this time for RNA, protein or luciferase activity analysis. Oocytes from different frogs were used in all experiments, and at least 20 oocytes were used for each data point.

In experiments involving antisense oligonucleotide, 100 ng of antisense oligonucleotide was injected into each oocyte. Oocytes were incubated at 24°C for 1–2 h to allow cleavage of c-*mos* mRNA before subsequent treatment, either with progesterone or by injection or prosthetic poly(A). Where prosthetic poly(A) was used, oocytes were injected with 400 pmol of prosthetic poly(A). Progesterone was applied to the oocytes within 5 min of prosthetic RNA injection. Where luciferase mRNA was injected, 0.5 fmol of luciferase mRNA was injected into each oocyte. Oocytes then were incubated at 24°C for 1 h, followed by injection of prosthetic poly(A).

Immunoblots

Groups of 10 oocytes were homogenized in 100 μl of ice-cold oocyte homogenization buffer [20 mM Tris–HCl (pH 7.5), 12.5 mM β-glycerophosphate, 15 mM NaF, 10 mM EGTA, 2 mM $MgCl_2$, 50 mM NaCl, supplemented with 6 mM DTT, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 15 μg/ml benzamidine], based on Gotoh *et al.* (1995). Lysates were centrifuged at 14 000 *g* for 10 min at 4°C to remove cell debris, and the clear cytosol was collected. Two oocyte equivalents (10 μl) were mixed with 2 μl of β-mercaptoethanol and 10 μl of 2× Laemmli sample buffer, and samples were loaded onto a 12% SDS–PAGE gel (Harlow and Lane, 1988). Proteins were transferred to Immobilon P (Millipore) as described (Ballantyne *et al.*, 1995).

Blocking, incubation with antibodies and washing of blots were performed according to standard protocols (Sambrook *et al.*, 1989). To detect c-Mos, membranes were incubated with anti-c-Mos antibody (Santa Cruz Biotechnology) and subsequently with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Kirkegaard & Perry). Incubation with antibodies was done in TBST [20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.05% Tween-20] containing 5% non-fat dry milk. Immunoreactive bands were detected using AttoPhos alkaline phosphatase substrate (Boehringer Mannheim) and a FlourImager SI (Molecular Dynamics), and quantitated using ImageQuant software (Molecular Dynamics).

Analysis of injected and endogenous RNAs

Isolation and purification of RNA was done essentially as described (Verrotti *et al.*, 1996), except that 2–10 cells were homogenized together, using 100 μl of homogenization solution per cell. For analysis of injected RNA, one oocyte equivalent was loaded on a 6% polyacrylamide gel containing 8.3 M urea, and bands were detected using a PhosphorImager (Molecular Dynamics).

For Northern analysis of endogenous cyclin B1 mRNA, three oocyte equivalents of RNA were loaded on a 0.8% formaldehyde agarose gel. Electrophoresis and transfer of RNA by capillary action to Biotrans nylon membrane (ICN) were performed as described (Sambrook *et al.*, 1989). To detect the RNA, a double-stranded DNA probe complementary to the entire open reading frame of cyclin B1 was used. The probe was made in a standard PCR including 100 ng of plasmid, 50 pmol of each primer, 100 μM each dCTP, dGTP and dTTP, and 50 μCi of [α-^{32}P]dATP (6000 Ci/mmol). Free nucleotides were removed by passing the reaction over a Sephadex G50 QuickSpin column (Pharmacia). Membranes were pre-hybridized for 1 h at 65°C in hybridization solution [1 mM EDTA, 0.25 M Na phosphate (pH 7.2), 7% SDS, 1% BSA]. Hybridization was for 16 h at 65°C, using 2×10^6 c.p.m./ml of probe in hybridization solution. Membranes were washed twice for 20 min at 65°C in wash solution [1 mM EDTA, 40 mM Na phosphate (pH 7.2), 5% SDS]. Bands were detected using a PhosphorImager.

Luciferase assay

Oocytes were pooled into groups of five, and homogenized in 200 μl of 1× cell lysis buffer (Promega). Then 5–10 μl of each homogenate were assayed in duplicate. The reaction was initiated by adding 100 μl of luciferase assay reagent (Promega). Photons were counted with a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Average luciferase activity values were calculated for each type of sample, from at least four pools of five cells.

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