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 (GVBD) and the completion of first meiosis. Prosthetic poly(A) contains 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 (Figure 1B, bar 4) and c-mos (Figure 1C, lane 4), showing that rescue by prosthetic poly(A) requires that it be targeted to c-mos mRNA.

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.
requirements 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
reporter mRNA in the absence of progesterone. It ends at the same position as 3′ frame followed by 238 nucleotides of sequence from the reporter mRNA is composed of the luciferase open reading

The number of hours after GVBD. (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 was 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
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 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′,5′-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-
...tion 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 (Galli 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 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 SpeI 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 [γ-32P]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 SpeI and HindIII-digested pGEM –83/HindIII, followed by ligation into pGEM7Z/EcoRI (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 SpeI and SpeI sites on one end and a HindIII site on the other end.

Non-specific 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 EcoRI 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. Polyadenylation in meiotic maturation...
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 DraI (Sheets et al., 1994). A typical transcription cell reaction contained 2 µg of plasmid, 1 mM each NTP (except GTP), 5 mM MgCl2, 2.5 mM MnCl2, 250 mM NaCl, 50 µg/ml bovine serum albumin (BSA), 1 U/µl RNAse (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 poly(A) synthesis, 2 mM 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 10X 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.

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 MgCl2, 2.5 mM MnCl2, 250 mM NaCl, 50 µg/ml bovine serum albumin (BSA), 1 U/µl RNAse (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 poly(A) synthesis, 2 mM 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 10X 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. Progestosterone (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 on the same day. Progestosterone. Oocyte maturation was determined by analyzing the oocytes for the presence of a white spot at two to three times GVBD (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). Progestosterone 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 were then 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 MgCl2, 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 Gotot 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 2X 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 AutoPhos alkaline phosphatase substrate (Boehringer Mannheim) and a FluorImager 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 (Verrott 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-32P]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 2×106 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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References


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