***c-mos* and *cdc2* Cooperate in the Translational Activation of Fibroblast Growth Factor Receptor-1 during *Xenopus* Oocyte Maturation**

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During oocyte maturation in *Xenopus*, previously quiescent maternal mRNAs are translationally activated at specific times. We hypothesized that the translational recruitment of individual messages is triggered by particular cellular events and investigated the potential for known effectors of the meiotic cell cycle to activate the translation of the FGF receptor-1 (XFGFR) maternal mRNA. We found that both *c-mos* and *cdc2* activate the translation of XFGFR. However, although oocytes matured by injection of recombinant *cdc2*/cyclin B translate normal levels of XFGFR protein, *c-mos* depletion reduces the level of XFGFR protein induced by *cdc2*/cyclin B injection. In oocytes blocked for *cdc2* activity, injection of *mos* RNA induced low levels of XFGFR protein, independent of MAPK activity. Through the use of injected reporter RNAs, we show that the XFGFR 3' untranslated region inhibitory element is completely derepressed by *cdc2* alone. In addition, we identified a new inhibitory element through which both *mos* and *cdc2* activate translation. We found that *cdc2* derepresses translation in the absence of polyadenylation, whereas *mos* requires poly(A) extension to activate XFGFR translation. Our results demonstrate that *mos* and *cdc2*, in addition to functioning as key regulators of the meiotic cell cycle, cooperate in the translational activation of a specific maternal mRNA during oocyte maturation.

INTRODUCTION

During meiosis and the early stages of embryonic development in many metazoans, gene expression is regulated post-transcriptionally. Thus, during these important stages of development, mRNA localization, changes in mRNA stability, translational repression and regulated activation, and posttranslational modification of protein products are critical for driving the cell through the cell cycle, preparing the germ cells for fertilization, and establishing the embryonic body plan. During oogenesis in *Xenopus laevis*, maternal mRNAs accumulate in the growing oocyte, but a large number of these mRNAs are translationally repressed during this time. Such "masked" maternal messages are recruited for translation at various times after oocyte maturation is initiated, during which time the cell undergoes meiosis. How this complex regulation is achieved is not well understood.

Much of what is known about the specificity of translational activation has been found through analysis of the sequences within the mRNA responsible for translational recruitment. One conserved sequence near the 3' end of most mRNAs is the cytoplasmic polyadenylation element

(CPE), with the general sequence of U₄₋₆AU (Fox *et al.*, 1989; McGrew *et al.*, 1989). The CPE regulates elongation of the poly(A) tail in the cytoplasm, a process that has been shown to be critical for the translational activation of a number of mRNAs during *Xenopus* oocyte maturation (Richter, 1996; Wickens *et al.*, 1996). CPEs can be classified according to their exact sequence, and the type of CPE contained within an mRNA dictates not only the timing but also the cell cycle regulation of polyadenylation (Sheets *et al.*, 1994; Ballantyne *et al.*, 1997; de Moor and Richter, 1997). A protein that binds to the CPE, the cytoplasmic polyadenylation element-binding protein (CPEB), has been identified, and depletion of this protein ablates cytoplasmic polyadenylation both in vitro and in vivo (Paris *et al.*, 1991; Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). However, it is not yet clear how CPEB regulates adenylation through different CPEs with the required level of specificity.

Other sequences that regulate translation are inhibitory elements contained within 3' untranslated regions (3'UTRs). For a number of RNAs in a variety of species, specific interactions have been demonstrated between cellular proteins and 3'UTR regulatory sequences, including clam cyclin A and ribonucleotide reductase, rabbit erythroid 15-lipoxygenase, *Drosophila oskar* and *hunchback*, and *Caenorhabditis elegans fem-3* and *tra-2* mRNAs (Goodwin *et al.*, 1993;

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Ostareck-Lederer *et al.*, 1994; Kim-Ha *et al.*, 1995; Murata and Wharton, 1995; Walker *et al.*, 1996; Zhang *et al.*, 1997). Such RNA-protein interactions are postulated to repress translation, as has been shown for both lipoxygenase and *tra-2*. The 48-kDa cellular factor that binds the lipoxygenase 3'UTR has been shown to repress translation of this RNA in vitro (Ostareck-Lederer *et al.*, 1994). Additionally, the GLD-1 protein is a component of the direct repeat factor, which interacts with the *tra-2* 3'UTR element, and GLD-1 inhibits translation of this RNA both in vivo and in vitro (Jan *et al.*, 1999). Translational activation of mRNAs containing regulatory elements must accompany derepression of these sequences at the appropriate time and is thought to be achieved through modification, release, or degradation of the inhibitory proteins with which they interact. In support of this model, phosphorylation of the 82-kDa factor that binds the masking element in the clam ribonucleotide reductase and cyclin A RNAs is correlated with and may be important for translational activation of this RNA (Walker *et al.*, 1996; Minshall *et al.*, 1999). Because a number of different inhibitory elements thus far identified do not contain obvious homology to each other, these unique sequences can easily be regulated independently through the proteins that bind them and thus may serve as a source of specificity for translational regulation.

For an RNA element to dictate the timing of translation, however, these RNA sequences, or the proteins with which they interact, must be recognized by cellular factors that can either promote or derepress translation at the appropriate time. These cellular factors, then, must themselves be activated at particular times. One way that proper timing could be achieved is via an intrinsic clock that is set upon reentry into the cell cycle. In this situation, activation would proceed independently of cell cycle effectors. An alternative mechanism by which the cell could achieve the proper timing of translational recruitment is through its assessment of the extent of cell cycle progression. In this case, the activation of a specific cell cycle effector would serve to signal that a particular milestone had been met. Cell cycle regulators then would not only drive the cell through meiosis but would also serve as cellular signals to initiate specific molecular events, such as the translational activation of a subset of mRNAs. Thus, the cellular factors that recruit specific mRNAs for translation during meiosis may be activated by, or may even be composed of, key components of the cell cycle machinery.

Early in progesterone-stimulated meiosis of *Xenopus* oocytes, *c-mos*, which is a quiescent mRNA in the immature oocyte, is translated (Sagata *et al.*, 1988). After the translation of low levels of mos protein, a serine/threonine kinase, a protein kinase cascade is activated, all members of which are present in the immature oocyte in an inactive form. Mos activates the MAPK pathway by directly phosphorylating mek, a MAPK kinase, which then phosphorylates and activates MAPK (Nebreda *et al.*, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993). MAPK, then, is thought to activate cdc2/cyclin B indirectly by down-regulating its inhibitory kinase Myt1 (Haccard *et al.*, 1995; Huang *et al.*, 1995; Huang and Ferrell, 1996; Palmer *et al.*, 1998). cdc2 is a serine/threonine kinase that requires interaction with cyclin B1 or B2 for its activity. Through a positive feedback loop involving mos, cdc2/cyclin B, and members of the MAPK

pathway, the translation, stability, and activity of mos protein increases, as shown by the fact that oocytes that lack functional cdc2 accumulate very little mos protein (Nebreda *et al.*, 1995). This positive feedback loop also contributes to higher levels of activated cdc2/cyclin B, which ultimately drives the cell into meiosis and germinal vesicle breakdown (GVBD). Oocytes exit from meiosis I after the degradation of cyclin B protein, with a corresponding decrease in cdc2 activity (Huchon *et al.*, 1993). Entry into meiosis II accompanies the reactivation of cdc2/cyclin B and requires new translation of mos (Daar *et al.*, 1991; Kanki and Donoghue, 1991). Oocytes ultimately arrest in metaphase of meiosis II, at which time the mature oocyte is ready for fertilization.

Recently, there has been increased interest in discovering the roles that key components of the *Xenopus* meiotic cell cycle might play in initiating specific molecular events within the cell. Several reports have focused on the roles that two kinases, *c-mos* and cdc2, might play in activating the cytoplasmic polyadenylation of a number of maternal mRNAs (Ballantyne *et al.*, 1997; de Moor and Richter, 1997). The polyadenylation and translation of several mRNAs, including D7, cyclin B1, and histone B4, has been shown to be dependent on *c-mos* translation (Ballantyne *et al.*, 1997; de Moor and Richter, 1997), but for at least some of these RNAs, including cyclin B1 and histone B4, cdc2 may be the more proximal effector (de Moor and Richter, 1997).

Our laboratory has focused on the translational regulation of FGF receptor-1 mRNA (XFGFR), a maternal message translationally activated during oocyte maturation (Robbie *et al.*, 1995). Because FGF signaling is essential in the early embryo, the regulated expression of XFGFR protein during oocyte maturation is critical for normal development (Amaya *et al.*, 1991, 1993). Thus, XFGFR mRNA is one of many maternal messages that must be appropriately translated to prepare the cell for postfertilization events. We have previously identified a 180-nucleotide translation inhibitory element (TIE) contained within the XFGFR 3'UTR (Robbie *et al.*, 1995). This element, which interacts specifically with a 43-kDa cytoplasmic protein, represses translation in the immature oocyte and can direct the timing and regulation of translational activation of this mRNA during oocyte maturation (Robbie *et al.*, 1995; Culp and Musci, 1998).

In this report, we have explored the potential for known cell cycle effectors to function in the translational recruitment of XFGFR during *Xenopus* oocyte maturation. We found that both *c-mos* and cdc2 initiate events leading to the translation of XFGFR and that these two effectors cooperate in this process through distinct *cis* elements in the XFGFR mRNA by means of different mechanisms of activation.

MATERIALS AND METHODS

Isolation of XFGFR 5'UTR

The XFGFR 5'UTR was isolated by rapid amplification of cDNA ends from stage VI *Xenopus* oocyte total RNA with a XFGFR-specific primer, termed 6187, encoding bases 256–235 of the coding region (5'-GGATCTCCTCCCCTGTTATGCG-3'). A 0.4-kilobase (kb) fragment was amplified during the PCR step and blunt-end subcloned into the EcoRV site of Bluescript KS. Sequence analysis of this clone, R6, identified a 151-nucleotide region upstream of the XFGFR start of translation. We confirmed that this clone contains most if not all of the XFGFR 5'UTR by primer extension analysis (our unpublished results).

The nucleotide sequence of the XFGFR 5'UTR, with the initiating ATG underlined, is as follows: ACCCTACTAA TATAAGGAAT ATAAGTTGGA CGTGC GGAGA CAAAAAGAGA TCAA-GAAAAA AAAACCTGGA AACAGGAGCC GTGCAAAGCT TGTCTCGTGC CTGACACTGG AGGTCTCATG GATTCTGGGCA GTGTGCACTA GCCAACTTGG GATG.

Plasmid Construction

The construct β galUTR contains the XFGFR 3'UTR downstream of β -galactosidase, as described previously (Culp and Musci, 1998).

The XFGFR 5'UTR was placed upstream of the β -galactosidase reporter by PCR amplification of the 5'UTR from R6 with a primer containing a *Bam*HI site immediately adjacent to the start of the 5'UTR, xfr5utr_{bam} (5'-CAGTGGATCCACCCTACTAATATAAGGAAT-3'), and a downstream primer that places the translation-initiating ATG in the context of an *Nco*I site, xfr5utr_{nco} (5'-GAGACATGGCAAGTTGGCTAGTGC-3'). The amplification product was digested with *Bam*HI and *Nco*I and filled in with Klenow, and the fragment was subcloned into CScytobgal (Rupp *et al.*, 1994) that had been digested with *Hind*III/*Bam*HI and filled in with Klenow. This construct, CS5 β gal, places the XFGFR 5'UTR and β -galactosidase gene downstream of the cytomegalovirus (CMV) IE94 promoter and removes the SP6 promoter and β -globin 5'UTR. The 1.2-kb *Bgl*III/*Xba*I fragment of β galUTR, which contains the XFGFR 3'UTR, was then subcloned into *Bgl*III/*Xba*I-digested CS5 β gal to generate CS5 β galUTR. To enable the generation of RNA in vitro containing the XFGFR 5'UTR, we subcloned the SP6 promoter from SP64pA (Promega, Madison, WI) on a 0.2-kb *Nhe*I/*Hind*III fragment into the *Bam*HI site of CS5 β galUTR, creating 5 β galUTR. RNA generated from this plasmid contains 14 nucleotides of polylinker before the start of the XFGFR 5'UTR.

β galCPE and 5 β galCPE were generated by partially digesting β galUTR and 5 β galUTR, respectively, with *Bam*HI and *Afl*III, filling in the sites with Klenow, and reclosing the plasmid, thereby removing all but the last 193 nucleotides of the XFGFR 3'UTR, which contains the functional CPE of XFGFR.

The XFGFR 5'UTR was placed upstream of the XFGFR coding region by PCR amplification from R6 with the use of a T7 downstream primer located in the Bluescript vector and the xfr5utr_{bam} primer described above. The 0.45-kb amplified fragment was digested with *Bam*HI and *Xho*I and subcloned into CS2⁺, which contains the CMV IE94 promoter, followed by an SP6 promoter, a leader sequence derived from *Xenopus* β -globin 5'UTR, a polylinker, and SV40 polyadenylation sequences (Rupp *et al.*, 1994). The XFGFR coding region and 3'UTR were placed downstream of the 5'UTR by subcloning the *Xmn*I/*Xba*I fragment of XFR3'UTR (Robbie *et al.*, 1995) into the intermediate described above, which had been digested with *Xmn*I and *Xba*I. A polylinker containing an *Eco*RI restriction site, followed by 30 adenosine residues, an *Nsi*I restriction site, an *Xho*I site, and an *Xba*I site, was then subcloned into the *Eco*RI/*Xba*I sites of the cloning intermediate. We then replaced the 1.1-kb *Hind*III/*Sal*I fragment (containing the SP6 promoter and β -globin 5'UTR) of this construct with the 1.3-kb *Hind*III/*Sal*I fragment (containing the SP6 promoter 14 nucleotides upstream of the XFGFR 5'UTR start) from 5 β galUTR. This plasmid, 5XFRUTR, allows the generation of an RNA in vitro that does not contain β -globin 5'UTR sequences.

XFRCPE was generated by subcloning the 250-base pair (bp) *Bgl*III/*Xba*I fragment of 5 β galCPE containing the XFGFR CPE into *Xba*I-digested XFR plasmid. 5XFRCPE was generated by subcloning the 1-kb *Bgl*III/*Xho*I fragment of XFRCPE into *Bgl*III/*Xho*I-digested 5XFRUTR. XFR Δ UTR and 5XFR Δ UTR were generated by subcloning the 1-kb *Bam*HI/*Xho*I fragment of β gal Δ UTR (Culp and Musci, 1998) into the *Bam*HI/*Xho*I sites of XFRCPE and 5XFRCPE, respectively.

CS2 Δ 90 was generated by cloning the 965-bp *Hinc*II/*Spe*I fragment of cyc Δ 90 (provided by A. Murray, University of California, San Francisco, CA) into the *Xho*I/*Xba*I sites of CS2⁺6MT (Rupp *et al.*,

1994), placing the N-terminal truncated sea urchin cyclin B gene downstream of the CMV IE94 promoter and six myc tags (with which the cyclin gene is in frame).

RNA Synthesis

In vitro transcription was performed with the use of Message Machine kits (Ambion, Austin, TX). *mos* RNA was generated from the plasmid TZKA⁺ (a gift of M. Murakami and G. Vande Woude, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) by digesting the plasmid with *Hind*III and transcribing with T7 polymerase.

Wild-type (p34^{cdc2}) and dominant-negative (p34^{cdc2} K33R) *cdc2* RNAs were transcribed with T7 polymerase from the *Bam*HI-digested plasmids, pET8c-Xl_{cdc2} and pET8c-Xl_{cdc2} K33R (a kind gift of T. Hunt, Imperial Cancer Research Fund).

MAPK phosphatase RNA (MKP-1) was synthesized with T7 polymerase from *Hind*III-digested MKP-1 plasmid (provided by M. Whitman, Harvard Medical School, Boston, MA).

XFR RNA was transcribed with SP6 polymerase from the XFR plasmid (Amaya *et al.*, 1991) linearized at the *Sac*I site immediately downstream of the *Xenopus* β -globin 3'UTR sequences.

All templates for in vitro RNA synthesis containing the XFGFR 3'UTR or CPE are followed by 30 adenosine residues in the midst of several restriction sites. Digestion of these plasmids with *Nsi*I and transcription in vitro with SP6 yields RNAs that terminate with 30 adenosine residues. The poly(A) tail can be further elongated in vivo, as directed by the XFGFR cytoplasmic polyadenylation sequences. These synthetic RNAs are named for the plasmids from which they were generated. To test for the requirement for polyadenylation, some of these same constructs were digested with *Xba*I and transcribed in vitro, yielding an RNA that contains the 30 adenosines followed by 20 nonadenosine nucleotides. These additional 20 nucleotides prevent the RNA from serving as a substrate for polyadenylation in vivo (our unpublished results). The names for these RNAs are the same as the names for the templates from which they were derived, with a terminal X.

Oocyte Injections

Ovarian tissue was obtained from adult *X. laevis* that had been injected with 25 U of PMSG 24–48 h previously. Stage VI oocytes were manually defolliculated, and all experiments were performed in modified Barth's solution at 16°C.

To assay the ability of *cdc2* to activate endogenous XFGFR translation in intact versus *c-mos*-depleted or MAPK-blocked oocytes, oocytes were first injected with 60 ng of an oligonucleotide antisense to *c-mos*, as described previously (Culp and Musci, 1998), or with ~100 pg of in vitro transcribed RNA encoding MAPK phosphatase (MKP-1). In some experiments, ~50 pg of reporter RNA was then injected into the cytoplasm. The oocytes were incubated for at least 16 h. Injected and control intact oocytes were then induced to mature by injection of 50 nl of recombinant *cdc2*/cyclin B1 protein (kindly provided by J. Gautier, College of Physicians and Surgeons of Columbia University, New York, NY) into the cytoplasm. In metabolically labeled oocytes, egg-derived cytosolic factor-arrested extract was used as a source of activated *cdc2*/cyclin B. Cytosolic factor-arrested extract was prepared as described by Murray (1991). Oocyte maturation was induced by injecting 3 nl of cytosolic factor-arrested extract, which is approximately 0.3% of the oocyte volume. In all experiments in which we used the extract, the results were identical to those of experiments in which recombinant *cdc2*/cyclin B was used. For the nondestructible cyclin experiment, 1 ng (in 5 nl) of CS2 Δ 90 plasmid DNA was injected into the nucleus to induce maturation. Oocytes were collected for analysis 16–20 h after the induction of maturation.

To assay the translation of endogenous XFGFR in the absence of endogenous *cdc2* activity, oocytes were first injected with ~50 ng of synthetic RNA encoding wild-type (p34^{cdc2}) or dominant-negative

cdc2 (p34^{cdc2} K33R). For experiments in which the involvement of MAPK was assessed, ~100 pg of synthetic MKP-1 RNA was then injected. For experiments involving reporter RNAs, 50 pg of in vitro transcribed RNA was also injected. Oocytes were then incubated for at least 20 h. Injected and intact control oocytes were then incubated in 10 μ M progesterone and/or were injected with 100 pg of synthetic mos RNA. In experiments in which the involvement of mek was assessed, 50 nl of recombinant activated mek (approximate activity of 9 pmol $\cdot$ min⁻¹ $\cdot$ μ l⁻¹) was injected (a kind gift of J. Maller, University of Colorado Health Sciences Center, Denver, CO). Oocytes were then incubated for 16–20 h before collecting for analysis.

Metabolic labeling of endogenous XFGFR was performed by injecting 50 nl of 5 $\times$ concentrated [³⁵S]methionine/cysteine (3.5 mCi, >1000 Ci/mmol; Promix, Amersham, Arlington Heights, IL) into groups of 60 intact, *c-mos*-depleted, or cdc2-blocked oocytes at various times after injection of cdc2/cyclin B or synthetic mos RNA. Oocytes were incubated for 1 h after injection and then collected for analysis.

XFGFR protein derived from synthetic XFR RNA was metabolically labeled by injecting XFR RNA into intact, *c-mos*-depleted, or cdc2-blocked oocytes. After incubation overnight in Promix at 0.5 mCi/ml, oocytes were washed five times and incubated in media containing 1 mM each cold L-methionine and L-cysteine for 3 h. Oocytes were then injected with cdc2/cyclin B or synthetic mos RNA and harvested at various times thereafter.

Protein Analyses

Western analysis of endogenous and injected XFGFR was performed as described previously (Robbie *et al.*, 1995). Briefly, a pool of 20 oocytes was partially purified on wheat germ lectin Sepharose (Pharmacia, Piscataway, NJ). Bound proteins were electrophoresed on a 7.5% polyacrylamide gel and transferred to Immobilon P (Bio-Rad, Richmond, CA), and XFGFR protein was detected with a polyclonal (R#1) or a monoclonal (Ab50) antibody followed by a secondary antibody conjugated to HRP and detection with the use of Renaissance chemiluminescence reagents (New England Nuclear, Boston, MA).

Endogenous XFGFR protein was detected in metabolically labeled oocytes as described previously (Culp and Musci, 1998). Briefly, groups of 60 oocytes per time point were harvested, and XFGFR protein was partially purified on wheat germ lectin Sepharose. After the bound proteins were boiled off the Sepharose beads, XFGFR was immunoprecipitated with R#1 antiserum, separated by PAGE, and visualized by autoradiography. Radiolabeled XFGFR protein derived from injected synthetic RNA was immunoprecipitated directly with R#1 antiserum from extracts of 20 pooled oocytes and detected by PAGE and autoradiography.

Mos protein levels were assessed by immunoprecipitation followed by Western analysis. In most cases, the same oocyte extracts from which XFGFR protein had been isolated were then precleared with 1 μ g of rabbit immunoglobulin G, and one-fourth volume of 5 $\times$ immunoprecipitation buffer (250 mM Tris, pH 8.0, 50 mM EDTA, 5 mM EGTA, 5% Triton X-100, 2.5% deoxycholic acid, 0.5% SDS, 500 mM NaCl) was added. Mos protein was immunoprecipitated with 2 μ l of a polyclonal antibody (*c-mos*^{xc}; Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-Sepharose (Pharmacia). The bound material was separated on a 10% polyacrylamide gel, and Western analysis was performed with a mAb (5S; courtesy of G. Vande Woude) at a 1:500 dilution.

β -Galactosidase protein levels were assessed by immunoprecipitation followed by Western analysis, similar to that for mos. Pools of 20 oocytes were immunoprecipitated with 0.3 μ l of a polyclonal antibody (Cappel, Organon Teknika, Durham, NC), and Western analysis was performed with a mAb (Promega) at a 1:5000 dilution.

For MAPK protein analysis, pools of 10 oocytes were lysed in 100 μ l of oocyte lysis buffer (Amaya *et al.*, 1991), and 3 μ l (0.3 oocyte equivalent) was electrophoresed on a 12.5% polyacrylamide gel (100:1 acrylamide/bisacrylamide). MAPK was detected with a poly-

clonal antibody (DC3; kindly provided by J. Ferrell, Stanford University School of Medicine, Stanford, CA).

H1 Kinase Assays

cdc2 activity was assayed by homogenizing groups of five oocytes in 50 μ l of 100 mM KCl, 15 mM MgCl₂, 5 mM EGTA, 80 mM β -glycerophosphate, pH 7.4. Five microliters of the lysate was incubated in a 20- μ l reaction containing 20 mM HEPES, pH 7.8, 30 mM β -mercaptoethanol, 10 mM MgCl₂, 100 μ g/ml BSA, 125 μ g/ml histone H1, 0.25 μ Ci of [γ -³²P]ATP, 100 μ M ATP for 15 min at 30°C, after which the reaction was electrophoresed on a 12.5% polyacrylamide gel and subjected to autoradiography.

RNA Analysis

Total RNA was isolated with the use of Trizol reagent (Life Technologies, Grand Island, NY). For RNase protection analysis, 5 μ g of total RNA was hybridized to a ³²P-labeled XFGFR coding region antisense probe, JM450, as described previously (Culp and Musci, 1998).

High-resolution Northern analysis was performed as described previously (Culp and Musci, 1998). Briefly, 40 μ g (for analysis of endogenous XFGFR) or 20 μ g (for analysis of injected RNA) of total RNA was hybridized to an oligonucleotide antisense to the XFGFR 3'UTR ~500 nucleotides from the 3' end. After digestion with RNase H, the RNA was electrophoresed on a 5% denaturing polyacrylamide gel and subjected to Northern analysis. The membrane was hybridized in Northern Max solution (Ambion) at 37°C for 16 h to a ³²P-labeled 414-bp *Rsa*I fragment derived from the 3' end of the XFGFR 3'UTR.

RESULTS

c-mos Is Required for Normal Levels of XFGFR Protein

In previous work, we found that XFGFR protein first appears during oocyte maturation, just before GVBD, and is dependent on *c-mos* translation, which occurs early in progesterone-mediated oocyte maturation (Culp and Musci, 1998). This inability of progesterone alone to trigger the increase in XFGFR protein levels suggested that XFGFR protein appearance is likely not timed intrinsically but is dependent on cell cycle progression. However, this result did not necessarily imply that *c-mos* leads directly to the appearance of XFGFR protein, because *c-mos* is required for the activation of other known cell cycle regulators, including cdc2, mek, and MAPK, which may themselves regulate XFGFR protein levels. In fact, oocytes matured by injection of recombinant cdc2/cyclin B translate normal levels of XFGFR protein (Culp and Musci, 1998). However, in these oocytes, *c-mos* is translated to normal levels and mek and MAPK are also activated. Thus, we wondered whether the appearance of XFGFR protein after cdc2 injection was the result of a cdc2-activated pathway or whether XFGFR might be dependent on one of these other known kinases. To address this question, we tested whether cdc2, in the absence of *c-mos*, could induce the appearance of XFGFR protein. Oocytes were depleted of *c-mos* by antisense oligo injection and then injected with recombinant cdc2/cyclin B. Western analysis revealed that XFGFR protein accumulates in response to cdc2 in the absence of *c-mos*. However, the level of XFGFR protein produced is reduced in *c-mos*-depleted oocytes compared with progesterone-matured oocytes or intact cdc2-matured oocytes (Figure 1). These *c-mos*-

Figure 1. Oocytes induced to mature with *cdc2* exhibit reduced levels of XFGFR protein when depleted of *c-mos*. Intact or *c-mos*-depleted oocytes were induced to mature by injection of recombinant *cdc2*/cyclin B (*cdc2*/B1), and some oocytes were also injected with in vitro synthesized *mos* RNA. Immature and progesterone-matured oocytes are included for comparison. (Top panel) Extracts of oocytes were assessed for XFGFR translation by Western analysis with the Ab50 antibody. (Second panel) *mos* protein levels were determined in the same oocyte extracts as in the top panel by immunoprecipitation with a polyclonal anti-*mos*^{xc} antibody (Santa Cruz Biotechnology), followed by Western analysis with a monoclonal anti-*mos* antibody (5S). The upper band in all lanes is the immunoglobulin G pulled down in the immunoprecipitation step. (Third panel) The stage of oocyte arrest was inferred from the level of *cdc2* activity in oocyte extracts, as assayed in vitro with exogenously provided histone H1 as a substrate for *cdc2*. (Bottom panel) XFGFR mRNA levels were assessed by RNase protection analysis.

depleted oocytes do not translate detectable levels of *mos* protein, and the reduction in XFGFR protein levels in *c-mos*-depleted oocytes is not due to reduced XFGFR mRNA levels in these oocytes (Figure 1). Normal XFGFR protein levels can be rescued by injection of in vitro synthesized *mos* RNA into *c-mos*-depleted oocytes just before *cdc2* injection. These oocytes translate *mos* protein levels at least as high as that in intact mature oocytes. (The synthetic *mos* RNA was not cleaved in these oocytes because the RNA was injected >20 h after oligo injection, by which time the oligonucleotide is no longer intact.)

Translation of *c-mos* has been shown to be required for oocytes to enter meiosis II, whereupon the mature oocyte arrests at metaphase, when *cdc2* kinase activity is high. In contrast, *c-mos*-depleted oocytes arrest in meiosis I, with reduced *cdc2* activity levels (Figure 1) (Daar *et al.*, 1991; Kanki and Donoghue, 1991). To determine whether a process activated during meiosis II or a continued high level of *cdc2* activity might be more directly related to the *c-mos* dependence for normal levels of XFGFR protein, we investigated an alternative method to arrest the oocytes in meiosis I. Maturing oocytes exit from metaphase of meiosis I as a result of cyclin B protein degradation. Oocytes expressing a form of cyclin B protein ($\Delta 90$) that is unable to be degraded

Figure 2. *c-mos* depletion, and not meiosis I arrest or decreased *cdc2* levels, is responsible for the reduced XFGFR protein levels in *c-mos*-depleted oocytes. Intact or *c-mos*-depleted oocytes were induced to mature and arrest in meiosis I by injection of an expression construct containing nondestructible cyclin B ($\Delta 90$). Immature and progesterone-matured oocytes are also shown. (Top panel) XFGFR protein levels, assayed by Western analysis with the Ab50 antibody. (Middle panel) *mos* protein levels were assessed in the same extracts by immunoprecipitation followed by Western analysis. (Bottom panel) *cdc2* activity was assessed in vitro with histone H1 as substrate.

are unable to exit from meiosis I because of the continued presence of cyclin B protein complexed with *cdc2*, and thus they maintain a high level of *cdc2* activity (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Huchon *et al.*, 1993). Consequently, oocytes expressing $\Delta 90$ undergo GVBD and arrest in metaphase of meiosis I. However, these oocytes translate levels of XFGFR protein equivalent to those of progesterone-matured oocytes that arrest in meiosis II (Figure 2). Thus, entry into meiosis II is not required for normal levels of XFGFR protein. Oocytes expressing $\Delta 90$ contain high levels of *cdc2* activity as a result of the stable $\Delta 90$ -cyclin B/*cdc2* complex (Figure 2). To determine whether this high *cdc2* activity is sufficient to achieve maximal XFGFR protein in the absence of *c-mos*, we expressed $\Delta 90$ in intact oocytes and in oocytes that were depleted of *c-mos*. Although *cdc2* activity was equally high in both *c-mos*-depleted and intact oocytes, the amount of XFGFR protein was greatly reduced in the absence of *c-mos*. Thus, high *cdc2* activity alone is insufficient to achieve maximal XFGFR protein levels, and although *cdc2* can activate a pathway leading to an increase in XFGFR, *c-mos* is required for normal levels of XFGFR protein.

mos Effects an Increase in XFGFR Protein in the Absence of *cdc2* Activity

Because the results described above suggested that *c-mos* appears to be important for normal levels of XFGFR protein, we wished to determine whether *c-mos* alone can induce the appearance of XFGFR protein in the absence of *cdc2* activity or whether *c-mos* simply enhances the effect of the *cdc2*-initiated pathway. To test this, we blocked *cdc2* activity with a dominant-negative form of *cdc2* (p34^{cdc2} K33R) (Nebreda

Figure 3. Oocytes blocked for *cdc2* accumulate XFGFR protein in response to *mos*. (A) XFGFR Western analysis with Ab50 antibody on progesterone-matured intact oocytes and oocytes expressing p34^{cdc2} or p34^{cdc2} K33R treated with progesterone (Prog). Immature oocytes are also shown. (B) Intact or p34^{cdc2} K33R-expressing oocytes were treated with progesterone (Prog) or injected with synthetic *mos* RNA. (Top panel) XFGFR protein levels, as assayed by Western analysis with R#1 antibody. The lower band in all lanes is a nonspecific cross-reacting protein. (Second panel) *mos* protein levels were assayed in the same extracts that had been assayed for XFGFR by immunoprecipitation, followed by Western analysis for *mos* protein. The upper band in all lanes is the immunoglobulin G protein pulled down in the immunoprecipitation step. (Third panel) Histone H1 was used as an *in vitro* substrate to assess *cdc2* activity levels. (Bottom panel) MAPK activation was assessed by the differential migration on Western blots of inactive and activated forms of MAPK (lower and upper arrow, respectively).

et al., 1995). Although wild-type p34^{cdc2} has no effect on the ability of progesterone to induce oocyte maturation or XFGFR translation, oocytes expressing p34^{cdc2} K33R do not mature in response to progesterone, and very little XFGFR protein is detected (Figure 3). However, these oocytes also accumulate very little *mos* protein, as a result of the absence of the positive feedback loop with *cdc2* (Figure 3B) (Nebreda *et al.*, 1995). Thus, to test the ability of *mos* to activate XFGFR in the absence of *cdc2*, we increased the amount of *mos* protein in *cdc2*-blocked oocytes by injecting synthetic *mos* RNA. Although p34^{cdc2} K33R-expressing oocytes injected with *mos* RNA did not undergo GVBD or activate *cdc2*, they did accumulate *mos* protein to levels at least as high as those in progesterone-matured oocytes (Figure 3B). This increase in *mos* protein results in an increase in XFGFR protein as well; however, XFGFR levels are still reduced compared with the levels in intact oocytes matured by either proges-

terone or *mos* RNA injection. Thus, *mos* increases XFGFR protein levels in the absence of *cdc2* activity and in the absence of cell cycle progression, but the amount of XFGFR protein that accumulates in response to *mos* is greatly reduced in the absence of functional *cdc2*.

Data from other authors have suggested that functional *cdc2* not only increases *mos* protein levels but that phosphorylated *mos* protein, the more stable and active form of the protein, accumulates only after GVBD and *cdc2* activation (Watanabe *et al.*, 1989; Nishizawa *et al.*, 1992). Therefore, we wished to determine whether the lower XFGFR protein levels in the absence of *cdc2* activity might be explained solely by a lower activity of *mos* protein. We assessed the activity of *mos* protein in *cdc2*-blocked oocytes by assaying MAPK activation because *mos* is an activator of mek, which in turn phosphorylates and activates MAPK (Nebreda *et al.*, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993). Although activation of MAPK within a single oocyte is an all-or-none event (Ferrell and Machleder, 1998), we used the proportion of oocytes with activated MAPK as an indicator of *mos* activity. In immature oocytes, inactive MAPK is hypophosphorylated and migrates at 42 kDa. Oocytes matured by progesterone or *mos* RNA injection contain activated MAPK, which is completely phosphorylated, and therefore exhibit a reduced mobility of ~44 kDa (Figure 3B). Progesterone treatment of oocytes blocked for *cdc2* activity revealed no detectable activated MAPK, reflecting the low *mos* activity in these oocytes. After *mos* RNA injection, however, MAPK was activated. Between different experiments, we observed variations in the proportion of oocytes containing activated MAPK in *cdc2*-blocked, *mos*-injected oocytes, ranging from 50 to 100% (our unpublished results). The levels of XFGFR protein in these oocytes also vary and correlate with MAPK activation, but they never equal the levels seen in intact mature oocytes, despite complete MAPK activation and high *mos* activity levels. We conclude, therefore, that although *mos* can effect an increase in XFGFR protein levels in the absence of functional *cdc2*, *cdc2* is necessary for the normal complement of XFGFR protein.

mos Increases XFGFR Protein Levels Independently of the MAPK Pathway

The experiments described above indicate that *mos* can direct an increase in XFGFR protein in the absence of *cdc2* activity. However, because we observed that an increase in *mos* protein resulted in both a greater proportion of activated MAPK and increased levels of XFGFR protein, we wished to determine whether members of the MAPK pathway might be more proximal to XFGFR or whether the level of MAPK activation is simply an index of *mos* activity. We addressed this question in two ways, in both cases with the use of a MAPK phosphatase (MKP-1) (Sun *et al.*, 1993), which dephosphorylates and inactivates MAPK. In the first approach, we expressed MKP-1 in oocytes lacking functional *cdc2* (p34^{cdc2} K33R) and expressing exogenous *mos* RNA. Although a marked reduction in activated MAPK was observed in oocytes expressing MKP-1, the level of XFGFR protein was equivalent to that in *cdc2*-blocked oocytes injected with *mos* RNA alone (Figure 4A).

We then tested whether MAPK might be involved in the *cdc2*-directed increase in XFGFR protein. As other authors have shown, MAPK is not activated in *c-mos*-depleted oo-

Figure 4. Members of the MAPK pathway do not contribute to the accumulation of XFGFR protein. (A) XFGFR (top panel) and MAPK (bottom panel) Western analysis on oocytes injected with p34^{cdc2} K33R RNA, plus or minus MKP-1 RNA, followed by *mos* RNA injection. Immature and progesterone-matured oocytes are also shown. (B) Recombinant *cdc2*/cyclin B was injected into intact, *c-mos*-depleted, or MKP-1-expressing oocytes. Immature oocytes are also included. Extracts of oocytes were assayed for XFGFR translation (top panel), MAPK activation (second panel), *mos* protein levels (third panel), and *cdc2* activity (bottom panel). (C) XFGFR Western analysis on extracts of oocytes injected with activated mek protein (act mek) in the presence or absence of p34^{cdc2} K33R. Immature and progesterone-matured oocytes are included for comparison. (D) *mos* protein levels assayed in immature oocytes, progesterone-matured oocytes, and oocytes injected with p34^{cdc2} K33R RNA and either stimulated with progesterone or injected with activated mek protein. XFGFR Western analysis was performed with R#1 antibody (A and C) or Ab50 antibody (B). MAPK Western blots show the locations of hyperphosphorylated, activated MAPK (top arrow) and the hypophosphorylated, inactive form (bottom arrow).

ocytes induced to mature with *cdc2* (Figure 4B) (Gotoh *et al.*, 1995). Thus, we tested whether the reduced XFGFR protein levels in *c-mos*-depleted, *cdc2*/cyclin B-injected oocytes is a result of *c-mos* depletion itself or the inability to activate the MAPK pathway. Oocytes expressing MKP-1 were induced to mature by *cdc2*/cyclin B injection. We confirmed that although MKP-1-expressing oocytes contain no detectable activated MAPK and these oocytes arrest in meiosis I, as shown by the low histone H1 kinase activity, XFGFR protein levels are not reduced in these oocytes (Figure 4B). In fact, a slight increase in XFGFR protein levels was observed in the absence of active MAPK, suggesting a possible inhibitory function of MAPK on XFGFR protein levels. In these oocytes, the amount of *mos* protein produced is somewhat reduced, consistent with a break in the positive feedback loop between *mos* and the MAPK pathway. However, this level of *mos* protein produced contributes sufficiently to XFGFR protein levels, because oocytes depleted of both MAPK and *c-mos* activities have levels of XFGFR protein equivalent to those of oocytes depleted of just *c-mos* (our unpublished results). These data demonstrate that oocytes that have intact *cdc2* and *mos* activities but no active MAPK exhibit no reduction in XFGFR protein.

To further explore the possibility that the MAPK pathway has a negative effect on XFGFR protein levels, we activated the MAPK pathway directly with a recombinant activated mek protein. We injected activated mek into intact oocytes or oocytes blocked for *cdc2* activity. In intact oocytes, activated mek induced oocyte maturation and the appearance of XFGFR protein (Figure 4C). However, the level of XFGFR protein in mek-matured oocytes was reduced compared

with that in progesterone-matured oocytes. In contrast, we detected no protein in *cdc2*-blocked oocytes injected with activated mek. We confirmed the findings of other authors that activation of the MAPK pathway induces *mos* translation (Figure 4D) (Matten *et al.*, 1996; Roy *et al.*, 1996). However, because of the lack of *cdc2* activity, the level of *mos* protein in these oocytes was quite low and was similar to that in progesterone-stimulated, *cdc2*-blocked oocytes (Figure 4D). That some *mos* protein was present but no XFGFR protein was detected in mek-injected, *cdc2*-blocked oocytes suggests that the MAPK pathway does negatively regulate the appearance of XFGFR protein during oocyte maturation. Taken together, these data demonstrate that *c-mos* and *cdc2* both contribute to the increase in XFGFR protein during meiosis independently of the MAPK pathway.

XFGFR Protein Accumulation Occurs with Neither a Delay Nor a Major Decrease in Protein Stability in Oocytes Depleted of c-mos or cdc2

Our results demonstrate that *c-mos* and *cdc2* each directs an increase in XFGFR protein levels, although both effectors are required for the full complement of XFGFR protein attained in intact mature oocytes. However, the submaximal protein levels in the absence of one of the two effectors might have several possible explanations: a reduced rate of translation, a decreased stability of the protein produced, or a delay in the normal increase in translation or protein stability. We first addressed whether the timing of translation is intact in the absence of either *c-mos* or *cdc2*. We examined XFGFR translation by pulse-label analysis after *cdc2* injection into intact

Figure 5. The timing of protein accumulation is intact and XFGFR protein is stable in the absence of either *c-mos* or *cdc2* activities. (A) Immunoprecipitation of endogenous XFGFR protein from intact or *c-mos*-depleted oocytes metabolically labeled during the course of *cdc2*-induced meiosis. (B) Endogenous XFGFR protein immunoprecipitated from intact or p34^{cdc2} K33R-expressing (*cdc2*-blocked) oocytes metabolically labeled at various times after *mos* RNA injection. Oocytes were labeled by injecting [³⁵S]methionine/cysteine at various times after *cdc2*/cyclin B or *mos* RNA injection (the times in hours are indicated above the lanes), incubating for 1 h, and then harvesting for protein analysis. The ratio of the intensities of the

and *c-mos*-depleted oocytes. At various times after *cdc2*/cyclin B injection, oocytes were metabolically labeled for 1 h, the oocytes were collected, and XFGFR protein levels were assessed by immunoprecipitation. Although no labeled XFGFR protein was detected in immature oocytes, in both intact and *c-mos*-depleted oocytes injected with *cdc2*, labeled XFGFR protein was detected by the first time point after *cdc2*/cyclin B injection, and translation continued throughout the course of the experiment (Figure 5A). However, the amount of labeled protein in *c-mos*-depleted oocytes was reduced compared with that in intact oocytes.

We also compared the timing of *mos*-induced XFGFR protein appearance in the presence and absence of *cdc2* activity. We injected synthetic *mos* RNA into intact oocytes or oocytes blocked for *cdc2* activity and metabolically labeled the oocytes for 1 h at various times after *mos* RNA injection. We detected labeled XFGFR protein in both intact and *cdc2*-blocked oocytes at the first time point after *mos* RNA injection (Figure 5B). Although the rate of XFGFR protein accumulation in intact oocytes initially increased and then subsequently decreased during *mos*-stimulated oocyte maturation, XFGFR protein accumulated in *cdc2*-blocked oocytes at a constant low level throughout the duration of the experiment. From this analysis, we conclude that although either *c-mos* or *cdc2* alone is capable of effecting an increase in XFGFR protein levels with no discernible delay, the rate of protein accumulation is compromised in the absence of the other effector.

We then addressed by pulse-chase analysis whether the reduced levels of XFGFR protein in the absence of either *c-mos* or *cdc2* might be the result of a decreased stability of the protein in the absence of one of the effectors. For this analysis, we used a synthetic RNA encoding the XFGFR coding region alone (XFR) (Robbie *et al.*, 1995). This RNA is translated in immature oocytes, allowing us to compare directly the stability of XFGFR protein in immature oocytes with that in oocytes with intact *mos* and/or *cdc2* activities. Immature intact oocytes, *c-mos*-depleted oocytes, or *cdc2*-blocked oocytes that had been injected with synthetic XFR RNA were metabolically labeled overnight. After extensive washing and incubation in cold amino acids, oocytes were injected with either *cdc2*/cyclin B or *mos* RNA and collected at various times for later immunoprecipitation. We found that XFGFR protein was quite stable in all oocytes (Figure 5B). Quantitation of several such experiments revealed that although XFGFR protein had a half-life of more than 5 h in immature oocytes, XFGFR protein appeared to be slightly more stable in oocytes that had intact *cdc2* or *c-mos* activities (7 and 9 h, respectively; our unpublished results). However, this difference is not sufficient to account for the disparity between intact and *c-mos*-depleted oocytes in a 1-h pulse

Figure 5 (cont.) signals in *c-mos*- or *cdc2*-depleted oocytes was compared with that of the cognate intact oocyte sample and is reported below each lane as the expression ratio. Imm, immature oocytes labeled similarly for 1 h. (C) Pulse-chase analysis of metabolically labeled intact, *c-mos*-depleted, or *cdc2*-blocked oocytes expressing synthetic XFR RNA. After incubation in [³⁵S]methionine/cysteine and extensive washing in cold amino acids, immature oocytes were injected with either *cdc2*/cyclin B or synthetic *mos* RNA at time 0 and were collected at the indicated times thereafter for analysis by immunoprecipitation.

Figure 6. *c-mos* and *cdc2* function through different *cis* elements on the XFGFR RNA. (A) Scheme of synthetic RNAs tested. RNAs contain either the *Xenopus* β -globin (white) or XFGFR (gray) 5'UTR sequence and the β -galactosidase (white) or XFGFR (gray) coding region. 3' untranslated sequences used include the 3'UTR from *Xenopus* β -globin (white), the entire XFGFR 3'UTR (gray), with or without the 180-nucleotide TIE (black), or just the last 193 nucleotides of the XFGFR 3'UTR containing the CPE. All of the RNAs featured a poly(A) tail containing 30 adenosine residues. The RNAs terminating with the poly(A) tail can be further adenylated in vivo (arrows). RNAs containing additional nonadenosine residues beyond the poly(A) tail are not substrates of adenylation in vivo (X).

(B) Scheme of results of Western analyses. Immature oocytes were injected with the synthetic RNAs diagrammed in A, and protein levels were assayed under the following conditions: *cdc2*⁻/*mos*⁻, immature oocytes; *cdc2*⁺/*mos*⁺, intact oocytes matured by *cdc2*/cyclin B injection; *cdc2*⁺/*mos*⁻, *c-mos*-depleted oocytes injected with *cdc2*/cyclin B; *cdc2*⁻/*mos*⁺, p34^{cdc2} K33R-expressing oocytes injected with *mos* RNA. Oocytes injected with RNAs containing the XFGFR coding region were analyzed for XFGFR protein by Western analysis. Reporter protein levels in oocytes injected with RNAs containing the β -galactosidase coding region were analyzed by immunoprecipitation followed by Western analysis. Autoradiographs were subjected to scan analysis, and the levels of protein relative to those in intact mature oocytes are presented as follows: + + + +, 80–100%; + + +, 60–80%; + +, 40–60%; +, 20–40%; -, <20% of the amount in intact mature oocytes. Within a given experiment, translation in mature oocytes was similar in oocytes injected with different RNAs containing the same coding region.

label of endogenous XFGFR protein (Figure 5C). To generate such a difference in a 1-h pulse, the half-life of the protein in the absence of one of the effectors would need to be <15 min. These results suggest that both *mos* and *cdc2* contribute to an increase in the level of XFGFR protein, primarily by effecting an increase in translation, but that they also function to stabilize XFGFR protein.

cis Elements Involved in Activating XFGFR Translation

With our finding that both *cdc2* and *c-mos* activate XFGFR translation, we then wished to distinguish the functions of these two effectors in this process. To identify potential regulatory elements within the XFGFR RNA and to determine whether they might respond differently to the two effectors, we generated synthetic RNAs that contained various portions of the XFGFR RNA. We especially wished to examine the activation of the 3'UTR, because our previous work had identified within this region a 180-nucleotide element, the TIE, which inhibits the translation of a heterologous reporter (Robbie *et al.*, 1995). The TIE also mimics the timing of activation of endogenous XFGFR translation and thus appears to serve as a key element for the translational activation of this mRNA (Culp and Musci, 1998). However, to ensure that an intact synthetic RNA could recapitulate the translation profile of endogenous XFGFR mRNA, we first generated RNA in vitro that contained all of the elements of

endogenous XFGFR: the 5'UTR, the coding region, and the 3'UTR. This RNA, 5XFRUTR, was injected into intact oocytes, *c-mos*-depleted oocytes, and oocytes blocked for *cdc2* function. We then injected the oocytes with *cdc2*/cyclin B or *mos* RNA and assessed XFGFR protein levels by Western analysis. The results are shown schematically in Figure 6. As expected, 5XFRUTR RNA mimicked endogenous XFGFR mRNA, because this synthetic RNA was repressed in the immature oocyte, was activated during *cdc2*-induced oocyte maturation, and could be activated by *mos* in the absence of *cdc2*. Also, like endogenous XFGFR, 5XFRUTR was not completely activated by either *mos* or *cdc2* in the absence of the other effector.

We then tested whether the 3'UTR was sufficient to mimic the translational regulation by *mos* and *cdc2* that we observed for full-length XFGFR RNA. We injected synthetic RNA, β galUTR, which contains the β -galactosidase coding region and the entire XFGFR 3'UTR, into intact oocytes, *c-mos*-depleted oocytes, or *cdc2*-blocked oocytes, and β -galactosidase translation was assessed after *cdc2* or *mos* RNA injection (Figure 6). As expected, translation of this RNA was repressed in immature oocytes as a result of the presence of the TIE within the XFGFR 3'UTR, and translation was activated during *cdc2*-induced oocyte maturation. However, we found that oocytes blocked for *cdc2* activity and injected with synthetic *mos* RNA did not activate translation of the β galUTR reporter RNA above that of the re-

pressed state in the immature oocyte. In addition, oocytes induced to mature by *cdc2* but lacking *c-mos* translated at least as much β -galactosidase protein as intact *cdc2*-matured oocytes (Figure 6). Thus, *mos* is neither capable of nor required for derepression of the XFGFR 3'UTR, and *cdc2* alone is capable of fully activating RNA that contains the TIE. These observations are in contrast with the results observed for endogenous XFGFR, in which complete translational activation by *cdc2* required *c-mos*, and *mos* was able to partially activate translation in the absence of *cdc2* activity. This result demonstrates that *cdc2* completely derepresses XFGFR translation through the 3'UTR but that *mos* cannot.

We then tested whether the XFGFR 5'UTR might contain an element through which *mos* might function. Translation of RNA containing the XFGFR 5'UTR and 3'UTR (5 β galUTR) was assayed as described above. Like β galUTR, 5 β galUTR was repressed in the immature oocyte and was activated by *cdc2*-induced maturation, but it was neither activated by nor dependent on *c-mos* for complete activation by *cdc2* (Figure 6). Thus, *mos* does not activate XFGFR translation through either the 5'UTR or the 3'UTR.

Because *mos* does not activate translation through the XFGFR untranslated regions, we hypothesized that the coding region might be involved in *mos*-dependent translation. However, in confirmation of previous results, XFR RNA, which contains the XFGFR coding region alone (with 5' and 3' untranslated region sequences derived from *Xenopus* β -globin), was not repressed in immature oocytes (Figure 6) (Robbie *et al.*, 1995). The amount of XFGFR protein was not significantly altered by *mos* alone, which is further evidence that *mos* activity does not greatly affect the stability of the XFGFR protein. A slight increase in protein levels was observed during *cdc2*-induced oocyte maturation; however, this modest change was consistent with the global increase in translation during meiosis and likely was not due to cell cycle-regulated activation of this RNA specifically (Wasserman *et al.*, 1982). We conclude from this result that although the coding region might contribute to the *mos* dependence of XFGFR RNA, on its own the coding region is not regulated by *mos*.

Because neither the coding region nor the untranslated regions in isolation exhibited the *mos* dependence observed for endogenous RNA, we considered the possibility that multiple regions of the XFGFR RNA in combination, as in the intact endogenous mRNA, form a regulatory element. Thus, we constructed four additional RNAs containing the XFGFR coding region and various portions of the untranslated regions, including the 5'UTR, the TIE-deleted 3'UTR, and the terminal 193 nucleotides of the 3'UTR (5XFR Δ UTR, XFR Δ UTR, 5XFR CPE, and XFR CPE). This latter region of the XFGFR 3'UTR, which we call the XFGFR CPE, contains U-rich sequences and directs cytoplasmic polyadenylation in vivo (our unpublished results), despite a lack of identity with the consensus CPE (Fox *et al.*, 1989; McGrew *et al.*, 1989). Unexpectedly, these four RNAs had the same translation profile (Figure 6). These RNAs were translationally repressed in the immature oocyte, despite the absence of the TIE; they were activated during *cdc2*-induced oocyte maturation; and either *mos* or *cdc2* could partially activate them in the absence of the other effector (Figure 6). Because the presence or absence of either the 5'UTR or the majority of the 3'UTR had no effect on translation in this assay, we

conclude that *mos* activates translation through sequences contained within the coding region and the CPE.

Our observation that the translation of XFR CPE RNA was inhibited in the immature oocyte, whereas the XFGFR coding region alone was not repressed, suggested that at least a portion of an inhibitory element is contained within the XFGFR CPE. However, RNA containing the β -galactosidase coding region and the XFGFR CPE (β galCPE) was not repressed in immature oocytes, and translation was not appreciably altered by the addition of either *mos* or *cdc2* activities (Figure 6). Thus, the XFGFR CPE alone cannot regulate the translation of a heterologous gene.

Finally, we wished to determine whether the 5'UTR sequences might contain any regulatory function. We constructed 5 β galCPE RNA, which contains the XFGFR 5'UTR, the β -galactosidase coding region, and the XFGFR CPE (to allow polyadenylation in vivo). Like β galCPE, 5 β galCPE exhibited no translational repression in immature oocytes, and neither *cdc2* nor *mos* greatly affected translation of this RNA (Figure 6). We conclude that the XFGFR 5'UTR does not contribute to either translational repression or activation of XFGFR during oocyte maturation.

These experiments demonstrate that two inhibitory elements are contained within the XFGFR mRNA. The first is the TIE, which resides within the 3'UTR and is completely derepressed by *cdc2* during oocyte maturation; *mos* does not contribute to this process. The second is formed by a combination of sequences in the coding region and the CPE and is activated during meiosis by *mos* and *cdc2* in a cooperative manner.

Requirement for Polyadenylation in Activating *mos*-dependent Translation

In the experiments described above, all RNAs containing XFGFR 3'UTR sequences terminated with short poly(A) tails (A_{30}), which may be elongated in vivo as directed by the XFGFR CPE. We next examined whether *mos* and *cdc2* require the process of polyadenylation to derepress XFGFR by assessing the translational activation of reporter RNAs that cannot be adenylated. These RNAs contain a poly(A) region (A_{30}) that is followed by and terminates with 20 nonadenosine nucleotides, which prevents these RNAs from serving as substrates for adenylation in vivo (our unpublished results). Our previous work had shown that the poly(A) tail of XFGFR mRNA, which is normally extensively elongated during progesterone-mediated oocyte maturation, is not appreciably elongated when oocytes are induced to mature by *cdc2* injection (Culp and Musci, 1998), suggesting little, if any, requirement for adenylation in *cdc2*-induced translational activation. We first tested whether *cdc2* requires any poly(A) tail elongation to derepress the TIE. We injected nonadenylatable RNA containing the XFGFR 3'UTR (β galUTRx) into intact, *c-mos*-depleted, or *cdc2*-blocked oocytes; subsequently, we injected either *cdc2*/cyclin B or *mos* RNA and assayed the amount of reporter protein produced. As expected, *mos* alone did not derepress this RNA. In contrast, the translational activation of β galUTRx by *cdc2* was equivalent to that of β galUTR, despite the inability of β galUTRx to be further adenylated (Figure 6). Thus, derepression of the TIE by *cdc2* does not require polyadenylation.

Figure 7. *mos* induces XFGFR polyadenylation independently of coding region sequences. Synthetic *mos* RNA was injected into intact oocytes or oocytes that had been injected with 5XFRUTR or 5βgalUTR RNA. Sixteen hours later, total RNA was extracted and subjected to high-resolution Northern analysis, with a probe derived from the 3' terminus of the XFGFR 3'UTR. The extent of poly(A) elongation is indicated by the bracket.

We then tested whether poly(A) tail elongation is required for derepression of the XFGFR coding region/CPE by *mos* or *cdc2*. We found that, like XFRCPe, the nonadenylatable XFRCPeX was completely derepressed during *cdc2*-induced maturation and was activated by both *mos* and *cdc2* in the absence of the other effector, suggesting that polyadenylation is not required to activate this element by either *mos* or *cdc2* alone. However, in RNAs containing the complete 3'UTR (5XFRUTRx), the nonadenylatable form was not derepressed by *mos* alone. In contrast, these RNAs were completely derepressed during *cdc2*-induced maturation and were activated equally by *cdc2* in the absence of *c-mos*, independent of their ability to be adenylated (Figure 6). From these data, we conclude that *cdc2* does not require polyadenylation to activate the translation of XFGFR through the coding region/CPE, the TIE in the 3'UTR, or the full-length XFGFR RNA.

Our finding that *mos* requires poly(A) elongation to derepress the intact XFGFR RNA was a somewhat unexpected result, because in previous work we had shown that progesterone induces complete adenylation of endogenous XFGFR mRNA in the absence of *c-mos* (Culp and Musci, 1998). Thus, we wondered whether *mos* itself might induce XFGFR polyadenylation. Endogenous XFGFR in immature oocytes contains a discrete poly(A) tail of ~30 adenosine residues (Culp and Musci, 1998). This short poly(A) tail was extended appreciably by either progesterone stimulation or *mos* injection (Figure 7) (Culp and Musci, 1998). Thus, XFGFR polyadenylation may be induced by either progesterone or *mos* activity.

Because *mos* functions through sequences in both the XFGFR coding region and the CPE to activate translation, we wished to determine whether these same sequences are required for *mos* to induce XFGFR polyadenylation. We expressed *mos* in oocytes previously injected with RNA that contained the XFGFR coding region and the CPE and that could be partially activated by *mos* alone (5XFRUTR) or with RNA that contained the XFGFR CPE but no coding region sequences and that were not derepressed at all by *mos* (βgalUTR). Northern analysis demonstrated that both RNAs were adenylated by *mos* (Figure 7). Thus, *mos* induced adenylation through the XFGFR CPE sequences alone and did not require the XFGFR coding region for this process. These experiments suggest that *mos* has distinct targets for translational activation and polyadenylation. Although *mos* activates translation through an inhibitory element

formed by the coding region and the CPE, adenylation by *mos* uses only the CPE.

We conclude from these studies that although *mos* does not require polyadenylation to derepress translation through the coding region/CPE alone, in the presence of the TIE adenylation is required for *mos* to function as a translational activator. These experiments suggest that *mos* has two separate roles in activating XFGFR translation: polyadenylation through the CPE and derepression through the coding region/CPE.

DISCUSSION

Both *c-mos* and *cdc2* Regulate XFGFR Translation

We initiated this study to determine the role of meiotic cell cycle activators in regulating XFGFR translation. We found that both *cdc2* and *c-mos*, key components of the meiotic cell cycle, also serve to regulate XFGFR translation and protein levels during *Xenopus* oocyte maturation. These effectors, although both required for normal levels of XFGFR protein, do not function redundantly, and they cooperate in these processes. Two pieces of evidence indicate that both *c-mos* and *cdc2* increase XFGFR protein levels primarily through translational activation of XFGFR mRNA: *cdc2* derepresses the 3'UTR, therefore functioning at the level of translation (Figure 6); and in *cdc2*-blocked oocytes, polyadenylation of the full-length synthetic XFGFR RNA is required for *mos* to elicit its increase in XFGFR protein levels (Figure 6). In addition, *mos* activity does not greatly affect the steady-state levels of XFGFR protein generated from synthetic XFR RNA (Figure 6), and the stability of XFGFR protein generated from synthetic XFR RNA is quite high in immature oocytes and is only modestly increased by *mos* or *cdc2* (Figure 5C). Although both *mos* and *cdc2* confer stability to XFGFR protein, this activity cannot account for the much larger changes in XFGFR protein levels during oocyte maturation. We had wished to use polysome analysis to assay directly the abilities of *mos* and *cdc2* to activate translation of XFGFR mRNA. However, in both immature and mature oocytes, XFGFR mRNA is associated with a large complex and is not released with reagents that dissociate ribosomes (our unpublished results). We conclude from these studies that both *c-mos* and *cdc2* activate translation of XFGFR mRNA during oocyte maturation.

One target of *cdc2* is the 3'UTR inhibitory element, the TIE. Because of the role this element plays in the timing and activation of translation, as elucidated in previous work, when we began this study the TIE seemed a likely target and perhaps the sole target of cellular activation pathways (Robbie *et al.*, 1995; Culp and Musci, 1998). Surprisingly, however, we identified a second inhibitory element that is formed by a combination of sequences in the XFGFR coding region and the CPE. Complete activation of XFGFR translation through the coding region/CPE sequences, or this "second element," requires the activities of both *c-mos* and *cdc2*. The XFGFR regulatory elements we have identified thus far and their known functions are summarized in Table 1. Through these experiments we show that two independent pathways, initiated by *c-mos* and *cdc2*, converge onto a single mRNA, XFGFR, to effect its complete translational activation.

Table 1. RNA regulatory elements

RNA element	Inhibitory in the immature oocyte?	Action during meiosis	Target of which effector(s)
TIE	Yes	Derepression	cdc2
Coding region/CPE	Yes	Derepression, polyadenylation	<i>c-mos</i> /cdc2, <i>c-mos</i>
CPE (in isolation)	No	Polyadenylation	Progesterone/ <i>c-mos</i>

The potential for *mos* and *cdc2* to effect changes in translation has been investigated in several recent studies. Poly(A) polymerase, a protein critical for poly(A) elongation, has been shown to be a direct target of *cdc2* during *Xenopus* meiosis (Colgan *et al.*, 1996). Although phosphorylation by *cdc2* inhibits poly(A) polymerase activity, this inhibition is not achieved until poly(A) polymerase is completely phosphorylated late in oocyte maturation, when the translation of many mRNAs decreases (Colgan *et al.*, 1998). In addition, *cdc2* induces and is required for the phosphorylation of the CPEB, which is critical for the translational activation of a number of RNAs (Paris *et al.*, 1991; Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). Direct phosphorylation of CPEB by *cdc2* has not been shown, although CPEB does contain a *cdc2* consensus phosphorylation site (Hake and Richter, 1994). A dependence on *cdc2* activity for polyadenylation has been shown for some mRNAs, which suggests either an additional link between *cdc2* and translation or the possibility that this dependence may be related to the phosphorylation of CPEB by *cdc2* (Ballantyne *et al.*, 1997; de Moor and Richter, 1997).

In contrast to the demonstrated connections between *cdc2* and translation, the relationship between *mos* and translation is less well defined. *Mos*-dependent polyadenylation has been described for a number of *Xenopus* mRNAs, although *cdc2* may be the more direct effector for at least some of them (Ballantyne *et al.*, 1997; de Moor and Richter, 1997). At present, the only known physiological target of *mos* is *mek*, a member of the MAPK pathway (Nebreda and Hunt, 1993; Posada *et al.*, 1993). However, we show here that the MAPK pathway is not involved in activating XFGFR translation, because inactivation of MAPK had no positive effect on either *mos*- or *cdc2*-activated XFGFR translation. Interestingly, MAPK may negatively regulate XFGFR translation or protein stability, because activation of the MAPK pathway reduced XFGFR protein levels, and depletion of activated MAPK somewhat increased the level of XFGFR protein induced by *cdc2* (Figure 4, B and C). Our finding that the MAPK pathway does not positively regulate XFGFR translation suggests that *mos* functions through an as-yet-unknown pathway to derepress XFGFR mRNA.

Our studies show that neither *mos* nor *cdc2* alone is sufficient to fully derepress the coding region/CPE. Thus, these two effectors may have distinct functions, although whether they act on the same or different RNA target(s) within the coding region/CPE is unclear at this time. Future experiments to define the coding region/CPE inhibitory element and the putative protein(s) that might bind this element will help elucidate whether *c-mos* and *cdc2* function entirely separately to activate XFGFR translation or whether they converge on a common target.

The XFGFR Coding Region and CPE Together Inhibit Translation

Our current work has identified an inhibitory element within the XFGFR mRNA that requires both the XFGFR coding region and CPE to repress translation in the immature oocyte. Because these two regions are at least 1 kb apart on the full-length mRNA, the functioning of this inhibitory element must require the interaction of sequences over a significant distance. However, because deletion of the intervening 1 kb had no effect on the functioning of this element, the absolute distance between these sequences appears not to be critical. Interestingly, polyadenylation of histone B4 is dependent on *c-mos*, and the sequences that confer this *c-mos* dependence are contained in the coding region of the B4 RNA (de Moor and Richter, 1997). Because polyadenylation functions through and requires the CPE in the 3'UTR, the *c-mos* dependence of the B4 RNA must involve an interaction between the coding region and the CPE. Although the ultimate *mos*-dependent effects on XFGFR and histone B4 mRNAs are somewhat different, both involve a necessary interaction between the coding region and the CPE in their respective mRNAs. It will be of interest to determine if the coding region elements contained in these two RNAs exhibit sequence or structural similarities.

We observed that in intact RNA containing the TIE, derepression of the coding region/CPE by *mos* in the absence of *cdc2* requires polyadenylation, a process directed by the CPE. This result suggests that the XFGFR coding region/CPE functions not only to inhibit translation in the immature oocyte but also to activate translation during oocyte maturation. Inhibition and activation within the same element has also been observed in other mRNAs. The clam cyclin A and ribonucleotide reductase mRNAs both contain masking elements within their 3'UTRs and bind a common inhibitory protein, p82 (Standart *et al.*, 1990; Walker *et al.*, 1996). However, it was recently discovered that p82 is a homologue of vertebrate CPEB and likewise is important for cytoplasmic polyadenylation of these clam RNAs during translational recruitment in oocyte maturation (Minshall *et al.*, 1999; Walker *et al.*, 1999). Similarly, the 3' UTR element in *C. elegans tra-2* RNA functions not only to repress translation but also to regulate polyadenylation, an activating function (Goodwin *et al.*, 1993; Jan *et al.*, 1997). The TIE within the XFGFR 3'UTR also has an inhibitory function in the immature oocyte, but once derepressed it appears to enhance translation during oocyte maturation through a mechanism distinct from polyadenylation (Culp and Musci, 1998). Thus, it is becoming clearer that some RNA elements and the proteins with which they interact have components of both inhibitory and activating functions. In contrast, the mouse

prm-1 3'UTR contains at least two repressive elements, both of which are separable from the sequences required for translational activation (Fajardo *et al.*, 1997). The 3'UTR inhibitory element in *oskar* mRNA is also distinct from the element required for derepression, which is located in the 5'UTR (Gunkel *et al.*, 1998). In all of these RNAs, the opposing functions of translational repression and activation can be dissected with the use of translation extracts or chimeric reporter RNAs; we accede, however, that the processes of derepression and activation of masked messages may be mechanistically linked *in vivo*.

What Is the Role of Polyadenylation in Activating XFGFR Translation?

We reported previously that progesterone activates endogenous XFGFR polyadenylation during oocyte maturation, and we have demonstrated here that XFGFR polyadenylation is also activated by *mos*. In contrast, oocytes matured by injecting *cdc2*/cyclin B exhibit little to no XFGFR polyadenylation. However, these oocytes do translate *mos* protein. What does not logically follow then is why XFGFR mRNA is not polyadenylated by the *mos* activity that accumulates in these cells. This apparent paradox potentially provides clues regarding the role of XFGFR polyadenylation by *mos* or progesterone. We hypothesize that in progesterone- or *mos*-induced oocyte maturation, the initial event is elongation of the XFGFR poly(A) tail. Polyadenylation then allows *mos* to partially derepress the coding region/CPE. Upon later *cdc2* activation, complete derepression through both the coding region/CPE and the TIE is achieved, resulting in maximal translation. Conversely, in oocytes matured by *cdc2* injection, because *cdc2* does not require polyadenylation for its activity, derepression of the *cdc2*-dependent elements in the XFGFR mRNA occurs first, followed by translation of *c-mos*. The previous activation of the TIE by *cdc2* allows newly translated *mos* to access and derepress the coding region/CPE, obviating the need for polyadenylation. In this model, either poly(A) elongation or release of the TIE by *cdc2* is the initial event required for *mos* to gain access to the coding region/CPE.

mRNA Recruitment through Modification of RNA-binding Proteins

Through these studies, we hope ultimately to understand how the specificity of translational activation is achieved. Does each repressed mRNA bind a unique inhibitory protein? Do classes of mRNAs defined by their timing of activation bind the same inhibitory protein? If cell cycle effectors regulate translation of individual RNAs by direct modification of RNA binding proteins, these effectors would likely need to interact with diverse targets to effect specific activation. If cell cycle regulators recruit mRNAs indirectly through intermediates, these key effectors would need to interact with only a few general "time-specific" activators, which might then activate specific classes of mRNAs.

In our experiments, we have not addressed the proximity of *cdc2* and *mos* activities to XFGFR translational activation. We previously reported a specific interaction between the TIE and an oocyte cytoplasmic protein, through which we propose that translational repression of this element may be mediated (Robbie *et al.*, 1995). We hypothesize that a similar

RNA-protein interaction may also be responsible for inhibition of the XFGFR coding region/CPE. These RNA-binding proteins are then possible targets for posttranslational modification, which might result in derepression of XFGFR. Isolation and characterization of these proteins will allow us to assess whether these proteins are direct targets of *mos* and *cdc2* and thus how these cell cycle regulators cooperate to activate XFGFR translation.

CPEB is a well-characterized example of an RNA-binding protein important for translational recruitment during meiosis. As might be expected of a protein whose function alters over time, CPEB is phosphorylated during oocyte maturation in response to *cdc2* activity, and this phosphorylation correlates with the timing of polyadenylation of some mRNAs (Paris *et al.*, 1991; Hake and Richter, 1994). Although polyadenylation of some RNAs is dependent on *cdc2*, other RNAs are adenylated independently of *cdc2* activity (Baltantyne *et al.*, 1997; de Moor and Richter, 1997). However, CPEB is thought to be required for cytoplasmic polyadenylation of all mRNAs, irrespective of their *cdc2* dependence (Stebbins-Boaz *et al.*, 1996). Consequently, this single protein is likely not sufficient to provide the specificity for this complex recruitment process. Other factors may associate with CPEB *in vivo* to specify the timing of adenylation and translational activation during oocyte maturation. The possible effect of phosphorylation on CPEB function has recently been suggested. Although in *Xenopus* extracts CPEB interacts with its RNA targets independently of phosphorylation status (Paris *et al.*, 1991), in clam eggs, phosphorylated CPEB is not associated with mRNAs after they are recruited onto polysomes, suggesting an altered function for the phosphorylated form of this protein (Minshall *et al.*, 1999).

Phosphorylation is a common mechanism of translational control in biological systems. Somatic cells that reenter the cell cycle after a period of quiescence imposed by nutritional starvation, serum deprivation, or some other mechanism experience an increase in global translation (Kaufman, 1994; Morris, 1995; Sonenberg, 1996). This is true as well in germ cells, including *Xenopus* oocytes, as they enter meiosis (Wasserman *et al.*, 1982). This general increase in translation is correlated with, and is responsive to, changes in the phosphorylation status of a number of translation initiation factors, including eIF4E, eIF4G, eIF2B, and eIF2 α , the PHAS/4eBP proteins that interact with eIF4E, and the ribosomal protein S6 (Morris, 1995; Sonenberg, 1996; Lawrence and Abraham, 1997). The effect of phosphorylating eIF4E, eIF4G, eIF2B, and S6 and of dephosphorylating eIF2 α is to enhance the rate of initiation, generally thought to be the rate-limiting step in translation. In some cell systems, the mTOR/p70^{s6k} kinase pathway is responsible for effecting these changes in phosphorylation, whereas in other cells, including *Xenopus* oocytes, the effectors responsible for this translational control are not known (Morley and Pain, 1995; Flynn and Proud, 1996; Sonenberg, 1996; Fraser *et al.*, 1999). Intriguingly, phosphorylation enhances the interaction between eIF4E, the cap-binding protein, and eIF4G, which serves as a bridge between eIF4E and poly(A) polymerase (Bu *et al.*, 1993; Morley *et al.*, 1993; Tarun and Sachs, 1996; Fraser *et al.*, 1999). In addition, recent experiments have demonstrated that eIF4G is critical for the polyadenylation-dependent translational recruitment of specific masked mes-

sages (Keiper and Rhoads, 1999). Thus, the translational activation of masked messages may be connected to changes in global translation during *Xenopus* meiosis. How intimately these two activities interlace remains to be discovered.

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