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Translational control by cytoplasmic polyadenylation during *Xenopus* oocyte maturation: characterization of *cis* and *trans* elements and regulation by cyclin/MPF

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The expression of certain maternal mRNAs during oocyte maturation is regulated by cytoplasmic polyadenylation. To understand this process, we have focused on a maternal mRNA from *Xenopus* termed G10. This mRNA is stored in the cytoplasm of stage 6 oocytes until maturation when the process of poly(A) elongation stimulates its translation. Deletion analysis of the 3′ untranslated region of G10 RNA has revealed that two sequence elements, UUUUUUAU and AAUAAA were both necessary and sufficient for polyadenylation and polysomal recruitment. In this communication, we have defined the U-rich region that is optimal for polyadenylation as UUUUUUAUAAAG, henceforth referred to as the cytoplasmic polyadenylation element (CPE). We have also identified unique sequence requirements in the 3′ terminus of the RNA that can modulate polyadenylation even in the presence of wild-type *cis* elements. A time course of cytoplasmic polyadenylation *in vivo* shows that it is an early event of maturation and that it requires protein synthesis within the first 15 min of exposure to progesterone. MPF and cyclin can both induce polyadenylation but, at least with respect to MPF, cannot obviate the requirement for protein synthesis. To identify factors that may be responsible for maturation-specific polyadenylation, we employed extracts from oocytes and unfertilized eggs, the latter of which correctly polyadenylates exogenously added RNA. UV crosslinking demonstrated that an 82 kd protein binds to the U-rich CPE in egg, but not oocyte, extracts. The data suggest that progesterone, either in addition to or through MPF/cyclin, induces the synthesis of a factor during very early maturation that stimulates polyadenylation. A candidate for such a factor is the 82 kd protein.

Key words: translational control/cytoplasmic polyadenylation/meiotic maturation/cyclin/MPF

Introduction

Messenger RNAs and proteins that are inherited by the egg at the time of fertilization direct early development at least until transcription of the embryonic genome, which in *Xenopus* does not occur until the 4000 cell midblastula stage (Newport and Kirschner, 1982). The components required for this maternal programming accumulate during oogenesis, but for the most part are not expressed until the oocytes are stimulated to complete the first meiotic division by progesterone (Smith and Richter, 1985). During this maturation stage, some 'masked proteins, such as the maturation promoting factor (MPF) constituent $p34^{cdc2}$, are activated (Gautier *et al.*, 1989). Conversely, other developmentally important proteins must be synthesized *de novo* from mRNAs that are newly recruited into polysomes. This may be inferred from experiments in which the injection of antisense sequences for messengers encoding c-mos (Sagata *et al.*, 1988) and D7 (Smith *et al.*, 1988), for example, inhibited or delayed maturation. Although maturation-specific translation is probably controlled at several levels (Richter, 1987), it is clear that the expression of at least some mRNAs is regulated by polyadenylation.

Changes in polyadenylation during the early development of *Xenopus* (Darnbrough and Ford, 1979; Sagata *et al.*, 1980; Colot and Rosbash, 1982) and sea urchins (Wilt, 1973), have been known for some time, but it was the studies of Rosenthal *et al.* (1983) using *Spisula* material that made the important connection between mRNA polyadenylation and translational recruitment. They noted that mRNAs that were adenylated following fertilization entered polysomes whereas those that were deadenylated were no longer translated. Similar observations were subsequently made in a variety of organisms (Dworkin and Dworkin-Rastl, 1985; Dworkin *et al.*, 1985; Rosenthal and Wilt, 1986; Rosenthal and Ruderman, 1987; Huarte *et al.*, 1987; Hyman and Wormington, 1988; Paynton *et al.*, 1988; Strickland *et al.*, 1988). Whether this relationship was one of coincidence or cause and effect, however, was unclear.

Recent experiments in mouse and *Xenopus* have demonstrated that polyadenylation is required for the translation of specific mRNAs. Tissue plasminogen activator (tPA) mRNA is polyadenylated and translated during mouse oocyte maturation (Huarte *et al.*, 1987). Injection experiments with this message have shown that poly(A) tail length controls polysome recruitment (Vassalli *et al.*, 1989). Similarly, G10, a polyadenylated maternal message stored in the cytoplasm of *Xenopus* oocytes, undergoes poly(A) elongation and translational recruitment during maturation. In this case, however, the process of polyadenylation, rather than the length of the poly(A) tail, appears to stimulate message recruitment (McGrew *et al.*, 1989).

Analysis of G10 mRNAs lacking various portions of the 3′ untranslated region has revealed that two sequence elements, UUUUUUAU and AAUAAA, are required for poly(A) elongation and polysomal recruitment (McGrew *et al.*, 1989). Moreover, the polyadenylation of D7 mRNA during maturation requires nearly identical sequences (Fox *et al.*, 1989). To understand the regulation of polyadenylation and translation, we have examined several G10 RNAs harboring mutations in the U-rich region described above and have defined the optimal cytoplasmic polyadenylation element as UUUUUUAUAAAG. In addition, we show that other sequences residing at the 3′ terminus of the RNA can have a profound effect on polyadenylation. To identify factors that might regulate polyadenylation, we have employed extracts from oocytes and eggs that correctly

adenylate exogenously added RNA. A protein with a molecular mass of 82 kd crosslinks to RNA in egg, but not oocyte, extracts and only when presented with an intact UUUUUUAUAAAG sequence. Finally, we demonstrate that polyadenylation is stimulated by cyclin and MPF as well as by progesterone, and that at least with the latter two agents, protein synthesis is required.

Results

Mutational analysis of sequences responsible for maturation-specific polyadenylation

Deletion mutation analysis of the 3′ untranslated region of G10 mRNA revealed that two sequence elements were required for maturation-specific polyadenylation and subsequent translation (McGrew *et al.*, 1989). The first of these was the highly conserved sequence AAUAAA, which is also known to be important for nuclear pre-mRNA polyadenylation. The second sequence was UUUUUUAU, which resides at the 5′ side of the polyadenylation hexanucleotide (Figure 1). To define more precisely these elements, we have generated a series of mRNAs that contain nucleotide substitutions within these regions and have assessed their effects on polyadenylation during oocyte maturation. Double-stranded oligodeoxynucleotides containing the desired mutations were fused to the coding region of *Xenopus* β-globin, which serves as a convenient reporter sequence. The DNAs were linearized at a *Sal*I site downstream of the polyadenylation hexanucleotide. Radiolabeled run-off transcripts were then synthesized using SP6 polymerase and were injected into the cytoplasms of stage 6 oocytes, some of which were induced to mature with progesterone. Following maturation, as assessed by the appearance of a white spot at the animal pole, RNA was extracted and analyzed by gel electrophoresis and autoradiography.

To delineate first the 3′ boundary of the entire U-rich element a cDNA containing a *Pst*I site four bases 3′ of the UUUUUUAU sequence was constructed and used for RNA synthesis and polyadenylation analysis (XBG10-360, Figure 1). This mRNA was polyadenylated following oocyte maturation and shows that the 3′ boundary of this element extends no more than four bases downstream of the UUUUUUAU. To delineate the 5′ boundary of this same element, a cDNA containing a *Kpn*I site inserted immediately upstream of this sequence (360-45, Figure 1) was used for RNA synthesis and oocyte injection. This mRNA was also polyadenylated following maturation and defines the uridine-rich region as the 5′ boundary of the element. Replacement of the six uridine residues with a corresponding *Kpn*I recognition sequence, however, completely inhibited progesterone induced polyadenylation at maturation (360-47). This observation confirms our earlier report that the uridine-rich region is crucial for polyadenylation (McGrew *et al.*, 1989).

Next we have assessed whether specific uridine residues are essential for polyadenylation by replacing them two at a time with the dinucleotide GG (Figure 1). Substitution of the first four uridine residues significantly reduced polyadenylation, both in terms of the number of molecules that were polyadenylated and the number of adenylate residues added per molecule (360-64 and 360-66). Substitution of the last two uridines, however, completely abolished polyadenylation (360-68). Interestingly, replacement of the following AU dinucleotide, which is completely conserved among several mRNAS that undergo progesterone induced polyadenylation (Fox *et al.*, 1989; McGrew *et al.*, 1989), reduced, but did not eliminate, polyadenylation (360-2).

A final cDNA was synthesized in which the sequences immediately 3′ of the UUUUUUAU were replaced by a

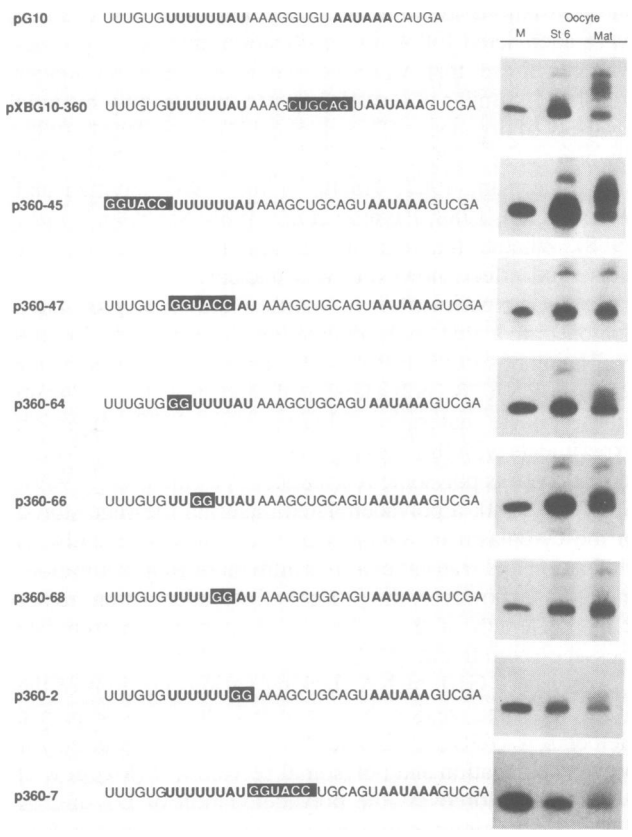

Fig. 1. Polyadenylation analysis of G10 mRNAs harboring nucleotide substitutions. The sequences of the 3′ ends of the injected mRNAs are shown together with the highlighted *cis* acting elements UUUUUUAU and AAUAAA. The black boxes highlight the substituted nucleotides. SP6 derived radiolabeled transcripts were injected into the cytoplasm of stage 6 oocytes that were subsequently incubated in the absence (St 6) or presence (Mat) of progesterone. Following maturation, the RNA was extracted from all oocytes and analyzed by polyacrylamide–urea gel electrophoresis and autoradiography. Uninjected, radiolabeled RNAs were included as markers (M).

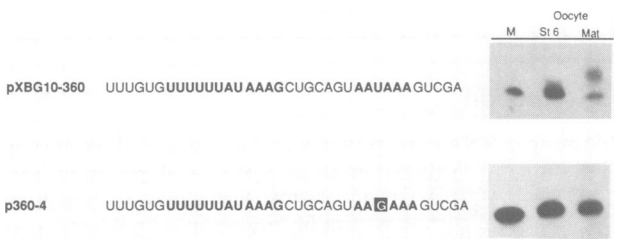

Fig. 2. Polyadenylation analysis of a point mutation in AAUAAA. Sequences of the 3′ termini of the injected RNAs are shown. The positions of the UUUUUUAUAAAG (CPE) and AAUAAA are highlighted (pXBG10-360) as well as the point mutation AAGAAA (p360-4). The polyadenylation of these RNAs before (St 6) and after (Mat) maturation were determined as described in Figure 1.

*Kpn*I site. The message synthesized from this template was not polyadenylated following oocyte maturation (360-7). Therefore, the optimal sequence for this element is the dodecanucleotide UUUUUUAUAAAG, which we henceforth refer to as the cytoplasmic polyadenylation element (CPE).

Several studies have indicated the involvement of the nuclear pre-mRNA cleavage and polyadenylation hexanucleotide AAUAAA in cytoplasmic polyadenylation (Fox *et al.*, 1989; McGrew *et al.*, 1989; Vassalli *et al.*, 1989). We have determined whether a point mutation, AAGAAA, which has been shown to destroy nuclear polyadenylation (Sheets and Wickens, 1989), also disrupts maturation-specific cytoplasmic polyadenylation. An RNA carrying this mutation, 360-4, was not adenylatated during maturation (Figure 2). Therefore, the hexanucleotide AAUAAA plays an integral part in both nuclear and cytoplasmic polyadenylation.

Spacing between the CPE and polyadenylation hexanucleotide does not affect polyadenylation

The spacing between the CPE and polyadenylation hexanucleotide of several mRNAs that undergo polyadenylation during early development varies between four and 13 bases (McGrew *et al.*, 1989). We have determined whether this spacing puts a constraint on cytoplasmic polyadenylation. Wild-type G10 mRNA contains four bases between the CPE and hexanucleotide; XBG10-360 (Figure 3), which contains seven bases between the two elements due to the addition of a *Pst*I site in its corresponding cDNA, is also adenylated at maturation. This same cDNA was digested with *Pst*I and *Sal*I and ligated with oligonucleotides of varying sizes whose sequences were derived from M13. The addition of two to 19 bases, which separated the elements by as much as 26 bases, did not affect polyadenylation. Thus, within these limits, neither the number nor the sequence of nucleotides between the two elements influences maturation-specific polyadenylation.

3′ end sequences can modulate polyadenylation

In our initial injection experiments, we noted that G10 mRNA synthesized from a plasmid that was linearized ~50 bases downstream of the polyadenylation hexanucleotide did not come under endogenous polyadenylation control (McGrew *et al.*, 1989). This suggested that in addition to the CPE and polyadenylation hexanucleotide, other sequences could modulate the polyadenylation reaction. Accordingly, we have linearized a plasmid, pXBG10Δ763-797, which contains both *cis* elements, at different sites in the polylinker as shown in Figure 4. Radiolabeled run-off transcripts were then synthesized from these templates and used for oocyte injection and polyadenylation assays (Figure 4). For example, RNAs synthesized from DNAs linearized with *Sal*I, *Xba*I, *Sma*I and *Eco*RI (pXBG10Δ763-797Δ *Sal*I−*Sma*I), which have from five to 24 bases 3′ of the polyadenylation hexanucleotide, were all polyadenylated at maturation. It should be noted that these different RNAs end in either C, G, A or U, thereby demonstrating that the poly(A) tail may be added to any nucleotide. Surprisingly, an RNA containing 25 bases downstream of the polyadenylation hexanucleotide (*Sac*I, 24 bases plus the final base, a G) was not adenylated at maturation. Similarly, an RNA with 35 bases downstream (*Eco*RI, 34 bases plus the final base, a U) was also not adenylated at maturation. It is important to note that RNAs with >25 bases downstream of the polyadenylation hexanucleotide are adenylated if those 3′-terminal sequences contain poly(A) [e.g. the 3′ end of the G10 mRNA contains 14 bases plus a poly(A) tail of 100 bases, cf. also Figure 5]. Thus, the number and sequence of nucleotides distal to the polyadenylation hexanucleotide can determine whether an RNA is adenylated at maturation.

We have next addressed whether a poly(A) tail beginning 25 bases beyond the polyadenylation hexanucleotide can

Fig. 3. The effect of spacing between the CPE and AAUAAA on maturation-specific polyadenylation. The sequence of the 3′ end of the starting RNA is shown (pXBG10-360). The distance between the CPE and polyadenylation hexanucleotide was increased by insertion of oligonucleotides containing two additional bases (p360-53), nine additional bases (p360-49) or 19 additional bases (p360-51) of M13 derived sequence. The polyadenylation of the injected RNAs was determined before (St 6) and after (Mat) maturation as described in Figure 1.

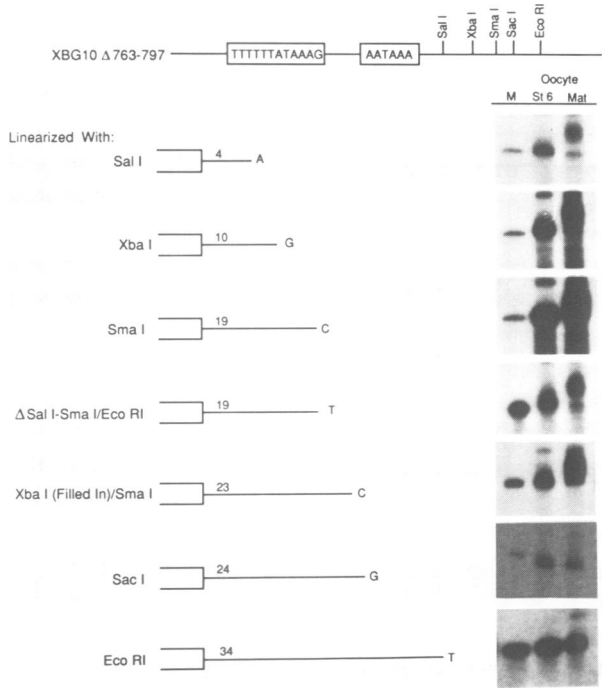

Fig. 4. The effect of the 3′ terminus on maturation-specific polyadenylation. A cDNA containing the *cis* sequences shown at the top was linearized at various regions in the polylinker and used for the synthesis of radiolabeled run-off transcripts. The number of nucleotides 3′ of the polyadenylation hexanucleotide (open box), plus the identity of the ultimate nucleotide, are shown. Polyadenylation analysis was performed as described in Figure 1.

restore polyadenylation during maturation. Figure 5A shows again that an RNA containing 25 bases 3′ of the hexanucleotide (24 bases plus a final G) was not adenylated at maturation. A poly(A) tail of ~100 residues added *in vitro* to that same RNA failed to restore its ability to be polyadenylated. Therefore, the distance of the poly(A) tail from the hexanucleotide is a determining factor for progesterone stimulated polyadenylation.

In a final experiment concerning 3′ end modulation of polyadenylation, we have examined an RNA that contains 20 guanylate residues 3′ to the poly(A) tail to determine if sequences distal to the poly(A) tail can influence this reaction (Figure 5B). Although the control RNA (top) was efficiently adenylated at maturation, the RNA carrying the additional poly(G) tract failed to be polyadenylated. These results therefore suggest that the activity of some factor, possibly a poly(A) polymerase, is disrupted by sequences downstream of the poly(A) tail and indicates that the enzyme must 'read' only adenylate residues for passage to the 3′ end.

Polyadenylation requires protein synthesis

The completion of maturation is usually scored by the presence of a white spot at the animal pole that immediately precedes dissolution of the nuclear envelope (germinal vesicle breakdown). In our experiments described thus far, we have analyzed RNA only after observation of the white spot and, presumably, after germinal vesicle breakdown. To determine when polyadenylation occurs relative to white spot appearance, we have analyzed injected RNA at several time periods after addition of progesterone to the oocytes. Figure 6A shows that >50% of the injected RNA was polyadenylated only 2 h post progesterone, well before the appearance of a white spot (and germinal vesicle breakdown) at 4.5 h. Polyadenylation appeared to be progressive both in terms of number of RNAs adenylated and number of adenylate residues per RNA. These data strongly suggest that adenylation is an early response to progesterone stimulation and that it is unlikely that nuclear contents are required for this process.

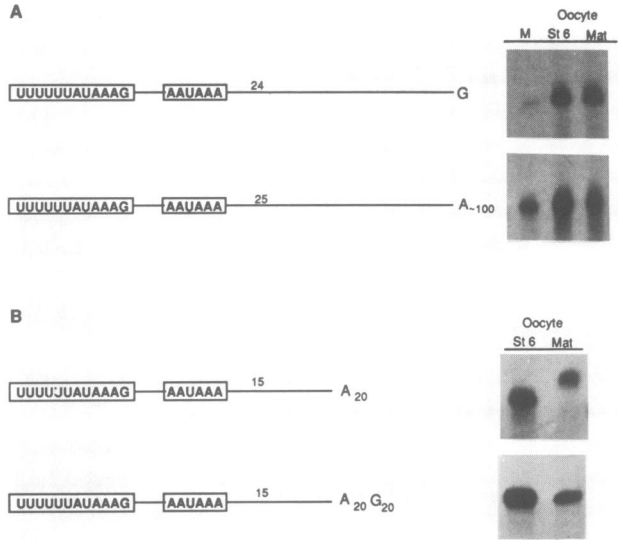

Fig. 5. Analysis of maturation-specific polyadenylation for RNAs with modified 3′ termini. (**A**) pXBG10Δ763-797 linearized at *Sac*I served as a template for the synthesis of a radiolabeled, run-off transcript. A portion of the RNA was incubated with poly(A) polymerase and ATP to add a poly(A) tail of ~100 residues to the 3′ end. These RNAs were analyzed for poly(A) elongation as described in Figure 1. (**B**) The 3′ end of RNA synthesized from pG10 (McGrew *et al.*, 1989) linearized at *Hin*dIII before (bottom) and after (top) treatment with oligo(dC) and RNase H is shown. Polyadenylation analysis following oocyte injecton was as described in Figure 1.

An identical time course was performed with oocytes incubated in both progesterone and cycloheximide (Figure 6A). This protein synthesis inhibitor completely abolished polyadenylation (and also prevented maturation). This indicates that progesterone stimulation and *de novo* synthesis of a protein(s) are both necessary for subsequent polyadenylation.

To determine whether polyadenylation requires protein synthesis at a specific time during maturation, we have incubated RNA-injected oocytes continuously in medium containing progesterone together with cycloheximide that was added 0−120 min later. All oocytes were then homogenized at 180 min and the RNA was analyzed as described previously (Figure 6B). When cycloheximide was added either at the same time as progesterone or 15 min later, no polyadenylation was detected. Some polyadenylation was observed, however, when cycloheximide was added at 30 or 60 min. Addition of the drug at 90 or 120 min had no effect on polyadenylation. Moreover, cycloheximide treatment between 30 and 180 min post-progesterone also had no effect on polyadenylation (data not shown) indicating that ongoing protein synthesis *per se* is not required for polyadenylation. Thus, there appears to be a critical time during the first 15−30 min of progesterone induced maturation when protein synthesis is required for polyadenylation.

Maturation promoting factor (MPF) is found in the cytosol of eggs and contains the activity necessary to induce maturation when injected into immature (stage 6) oocytes, even if those recipient oocytes are incubated in cycloheximide (Wasserman and Masui, 1975). Thus, MPF obviates the need for protein synthesis at maturation. To assess whether the cycloheximide block to polyadenylation can also be overcome by MPF, we have injected oocytes with G10 RNA and an egg cytosol fraction that has been shown to contain MPF activity, followed by incubation in the absence or presence of cycloheximide. Figure 6C shows that 1.5 h following injection, the MPF/cytosol stimulated polyadenylation, but only when oocytes were incubated in medium free of cycloheximide. Five hours after injection, however, MPF/cytosol-induced polyadenylation was indistinguishable in oocytes incubated in the absence or presence of cycloheximide and probably indicates that the drug did not completely inhibit protein synthesis. Indeed, the concentration of cycloheximide used in this study (10 μg/ml) diminishes protein synthesis by only ~90% (Richter *et al.*, 1987). These surprising results suggest that although MPF can stimulate polyadenylation without progesterone, it cannot 'short-circuit' the requirement for protein synthesis.

Recent studies have shown that active MPF from *Xenopus* eggs is actually a complex of at least two subunits, a 32 kd serine/threonine protein kinase, $p34^{cdc2}$ (Gautier *et al.*, 1990) and a 45 kd subunit that corresponds to a protein found in a wide variety of organisms called cyclin. To assess whether cyclin alone has the capability of inducing polyadenylation, we have injected a mixture of mRNAs encoding clam cyclin A and B, together with G10 mRNA (Figure 6D, Cy). For comparison additional G10 RNA

injected oocytes were cultured in the absence (Figure 6D, C) or presence (Figure 6D, P) of progesterone. Four hours after cyclin mRNA injection or treatment with progesterone, before the appearance of a white spot, polyadenylation was evident in both groups of oocytes. Polyadenylation continued to increase until maturation occurred ~8 h after injection. These data therefore demonstrate that cyclin induces the adenylation of G10 mRNA in a progesterone independent manner. We also note that similar experiments have been performed with baculovirus-expressed clam cyclins A and B (J.D.Richter and K.Swenson). The results show that both of these proteins stimulate adenylation following injection, but neither does so when oocytes are incubated in cycloheximide (data not shown). Thus, these results confirm the data presented in Figure 6 that MPF/cyclin induces adenylation but that it requires protein synthesis.

In vitro characterization of maturation-specific polyadenylation

Ultimately, the mechanisms controlling polyadenylation and translational recruitment at maturation will depend upon the identification and isolation of factors that are responsible for these reactions. Toward this end, we have employed an egg extract similar to that described by Murray and Kirschner (1989) that faithfully polyadenylates exogenously added RNA. Figure 7 shows a time course of polyadenylation in such an extract. Ten minutes after RNA addition, polyadenylation was evident and was complete by ~1 h. To demonstrate that the increased size of the RNA was in fact due to polyadenylation, some RNA was subsequently treated with oligo(dT) and RNase H. This resulted in its enhanced rate of electrophoretic migration and demonstrates that the RNA was indeed polyadenylated.

We tested the fidelity of oocyte and egg extracts with RNAs carrying mutations in the CPE or polyadenylation hexanucleotide sequences. Figure 8 shows that an RNA with the wild-type *cis* elements was polyadenylated in egg, but not oocyte, extracts. RNAs with mutations in either the CPE or polyadenylation hexanucleotide, however, were not adenylated in either extract. Therefore, with respect to polyadenylation, these extracts faithfully represent intact cells.

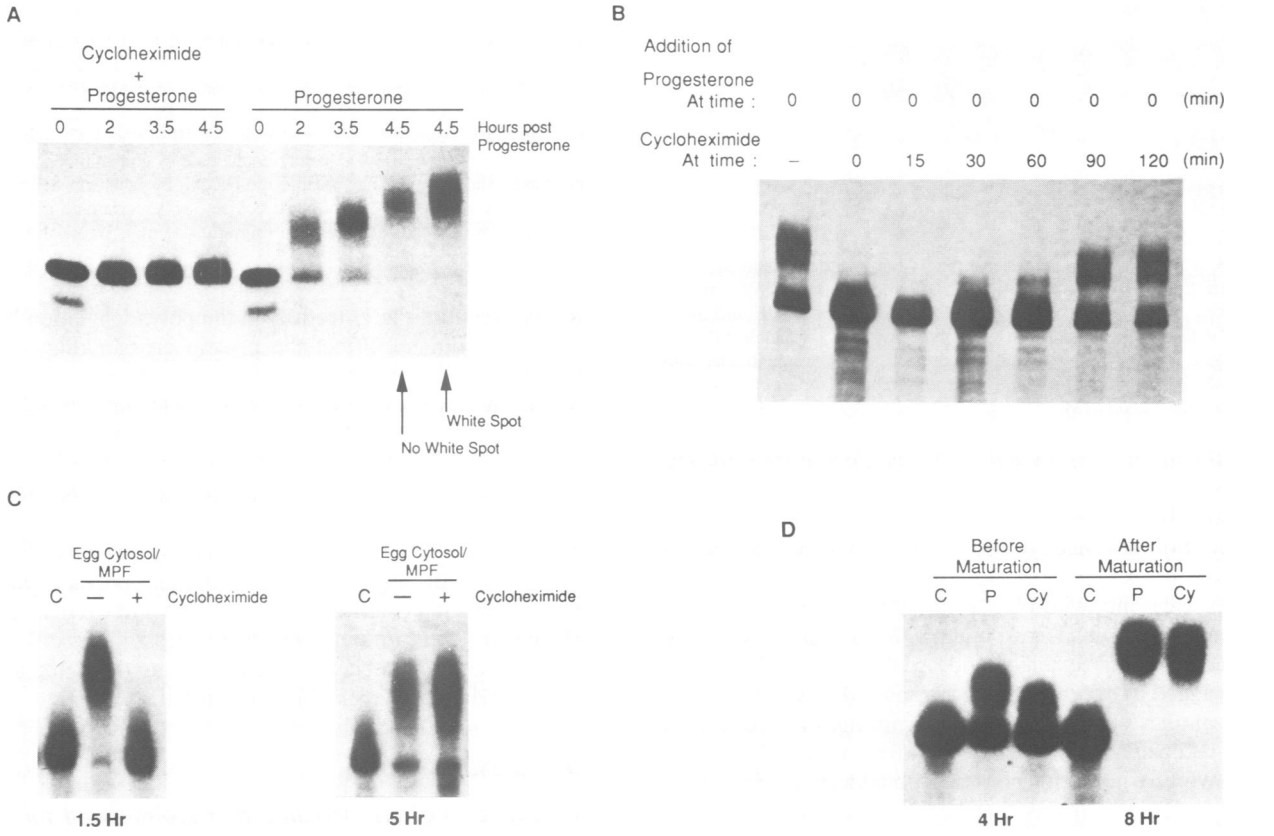

Fig. 6. Time course of adenylation in the presence of cycloheximide, egg cytosol/MPF and clam cyclin mRNA. (**A**) SP6-derived radiolabeled HPG-360, which is a 200 base RNA that contains both wild-type *cis* signals, was injected into stage 6 oocytes, a group of which had been pre-incubated for 2 h in cycloheximide. Both oocyte groups were then incubated in the presence of progesterone, with continued cycloheximide treatment of the first group. Total RNA was isolated from three oocytes at each time point shown after the addition of progesterone. (**B**) Oocytes were injected with HPG-360 RNA and incubated in medium containing progesterone and cycloheximide, the latter of which was added at the denoted times. All oocytes were then processed for RNA analysis at 180 min post-progesterone. (**C**) Injection of egg cytosol/MPF into stage 6 oocytes. SP6-derived radiolabeled HPG-360 RNA was injected into stage 6 oocytes alone (control, C) or with ~40 nl of cytosol taken from unfertilized eggs (egg cytosol/MPF). A portion of the MPF-injected oocytes were pre-incubated for 30 min in cycloheximide (+ cycloheximide) and continued to be incubated in cycloheximide. At 1.5 and 5 h after MPF injection, total RNA was isolated from groups of three oocytes and analyzed as described previously. (**D**) Injection of clam cyclin A and B mRNA into stage 6 oocytes. HPG-360 RNA was injected into oocytes followed by no additional treatment (control, C), treatment with progesterone (P) or injection with ~50 ng of equimolar amounts of clam cyclin A and B mRNAs (Cy). Total RNA from groups of three oocytes was extracted from each sample before white spot formation at 4 h or after white spot formation at 8 h. The RNA was then analyzed by electrophoresis and autoradiography.

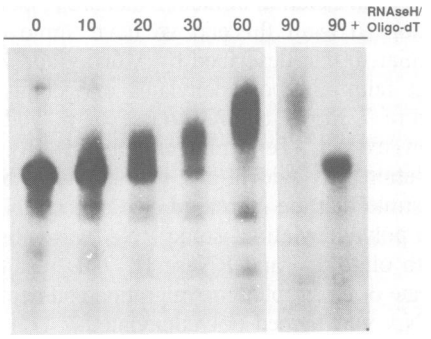

Fig. 7. Time course of adenylation *in vitro*. Radiolabeled GPH RNA containing wild-type CPE and the polyadenylation hexanucleotide was incubated in an egg extract. At several time points (shown in minutes), RNA was extracted and analyzed by electrophoresis and autoradiography. RNA from the 90 min time point was also treated with oligo(dT) and RNase H prior to electrophoresis.

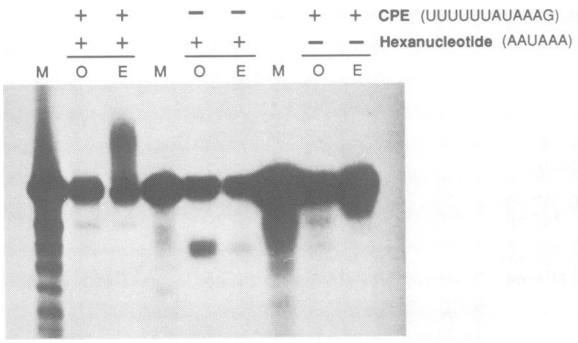

Fig. 8. The effect of mutant *cis* elements on polyadenylation in extracts from oocytes and eggs. SR360 RNA containing wild-type *cis* elements, SR47 RNA containing a replacement of the CPE and SR4 RNA with a point mutation in the polyadenylation hexanucleotide were incubated for 1 h in oocyte and egg extracts. RNA was extracted and analyzed by electrophoresis and autoradiography. Unreacted RNA was included as markers (M).

Identification of a specific CPE binding protein by UV crosslinking

To identify proteins that might bind the CPE or polyadenylation hexanucleotide, we have used oocyte and egg extracts for UV crosslinking experiments. [^{32}P]UTP-labeled RNAs carrying wild-type or mutant *cis* elements (CPE replaced with a corresponding *Kpn*I site, GGUACC; polyadenylation hexanucleotide replaced with AAGAAA) were added to oocyte and egg lysates and incubated for 1 h. The samples were exposed to 254 nm light for 10 min and then incubated with RNase A and micrococcal nuclease. Following stripping of non-specific adducts by formamide treatment, the proteins that became radioactive by label-transfer were resolved by SDS gel electrophoresis and autoradiography. Figure 9 shows that an RNA with both *cis* elements intact crosslinked to several proteins in oocyte extracts, particularly those with relative mobilities of 56 kd (a doublet), 41 kd and 23 kd. In egg extracts, the same proteins were crosslinked, although a new protein with a mobility of 82 kd was evident. Removal of the CPE reduced overall crosslinking in both oocyte and egg extracts, although longer autoradiographic exposure of other gels did reveal the presence of the 56 kd, 41 kd and 23 kd proteins (data not shown). However, the 82 kd protein was not detected.

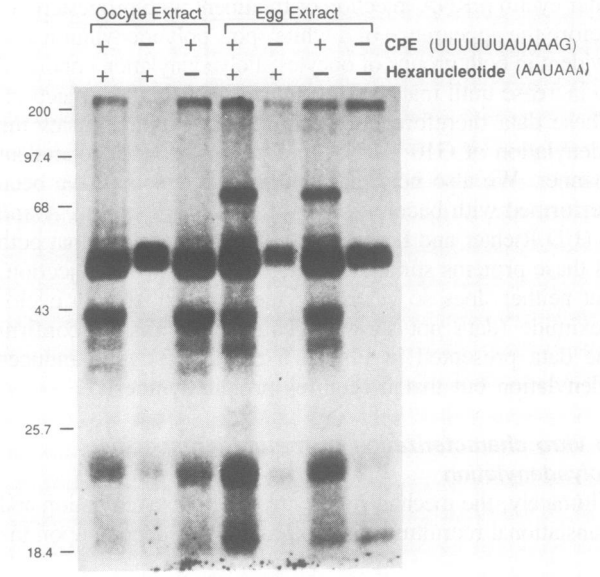

Fig. 9. Protein−RNA photocrosslinking in oocyte and egg extracts. [^{32}P]UTP-labeled HPG-360 RNA carrying wild-type *cis* elements, SR47 RNA carrying a nucleotide substitution in the CPE sequence, HPG-4 RNA carrying a mutant hexanucleotide sequence and HPGΔSH RNA truncated upstream of both *cis* elements were added to oocyte and egg lysates and incubated for 1 h at room temperature. The samples were exposed to UV irradiation for 10 min at 4°C, and digested for 30 min at 37°C with ribonuclease A and micrococcal nuclease. The proteins that became radioactive by label-transfer were analyzed by electrophoresis on a 10% SDS−polyacrylamide gel and visualized by autoradiography. The migration of mol. wt markers is noted.

Replacement of the U for a G in the polyadenylation hexanucleotide did not affect the protein crosslinking pattern relative to wild-type RNA in either oocyte or egg extracts. A truncated RNA that lacked both *cis* elements crosslinked to the 56 kd and 23 kd proteins (albeit poorly), but did not crosslink to the 82 kd protein. These data therefore demonstrate that an 82 kd protein crosslinks to RNA in egg, but not oocyte, extracts and that its crosslinking is abolished when the CPE is removed. We suggest that this protein, whose crosslinking capability parallels polyadenylation activity, is either activated or newly synthesized in eggs. Moreover, this protein appears to interact directly with the CPE and we henceforth refer to it as the cytoplasmic polyadenylation element binding protein (CPEB).

Discussion

Mutagenesis of cis elements that are required for polyadenylation

Although deletion of the UUUUUUAU sequence from G10 RNA abolished polyadenylation (McGrew *et al.*, 1989), it was unclear whether adjacent nucleotides were also important for this reaction. The present study, using RNAs with nucleotide substitutions rather than deletions, reveals that the optimal G10 cytoplasmic polyadenylation element (CPE) is UUUUUUAUAAAG. A comparison of this sequence with those in other RNAs that are thought to undergo polyadenylation (Fox *et al.*, 1989; McGrew *et al.*, 1989) shows that although the UUUUUUAU is highly conserved,

there is no similarity in the AAAG sequence, even though it is essential for G10 adenylation. This diversity in the CPEs of different RNAs might suggest a complex array of interacting factors or differing affinities of the same factor for multiple CPEs. Indirect evidence for this is suggested by the timing of adenylation of different RNAs. For example, the data in Figure 6A show clearly that G10 polyadenylation is an early response to progesterone stimulation and that it is not coordinate with white spot appearance or GVBD. Conversely, a derivative of D7 RNA, whose putative CPE is UUUUUUAU followed by ACUA, undergoes polyadenylation at about the same time as GVBD (Fox *et al.*, 1989), well after G10 adenylation. Moreover, RNAs with different CPEs show wide ranges in their ability to be polyadenylated (J.Paris and J.D.Richter, submitted for publication), which might also point to a diversity of factors and/or affinities. These possibilities are presently under investigation.

Base substitutions within the G10 CPE show that only the UU dinucleotide immediately preceding the AU is absolutely necessary for polyadenylation, although substitution of the other uridines severely affects the reaction. Interestingly, GG substitution for the AU dinucleotide, which is completely conserved among all the CPEs, only reduced polyadenylation. Conversely, a U to G change in the polyadenylation hexanucleotide completely abolished cytoplasmic (Figure 2) as well as nuclear polyadenylation (Fox *et al.*, 1989). Whether the factors that interact with this sequence are the same for both nuclear and cytoplasmic polyadenylations remains to be determined. We note, however, that nuclear contents are not required for maturation-specific polyadenylation (Figure 6; Fox *et al.*, 1989).

3′ end modulation of polyadenylation

The 3′ untranslated regions of several mRNAs are AU-rich and, hence, contain sequences that resemble CPEs. In addition, the observations that the G10 CPE can function in spite of several base changes and that neither spacing nor sequence between the CPE and polyadenylation hexanucleotide are critical for poly(A) elongation might suggest that almost any mRNA would be polyadenylated at maturation. The fact that this does not occur indicates that other constraints are placed upon mRNAs so that they are not polyadenylated (and presumably not translated) during this time of development. One such constraint appears to be the number, but not sequence, of nucleotides between the polyadenylation hexanucleotide and the start of the poly(A) tail. Messages with a random 5−24 bases 3′ of the polyadenylation hexanucleotide are efficiently adenylated at maturation. If the RNA has 25 or more random bases in this region, however, polyadenylation is abolished. How this sharp transition occurs is not clear, although one suspects the existence of a factor that distinguishes poly(A) from a heteropolymer of >25 bases. Of course, the poly(A) binding protein is just such a factor, yet its role in this polyadenylation reaction is unclear since an initial poly(A) tail is not required for poly(A) elongation. In any event, it is important to note that of the several RNAs that are apparently adenylated during maturation whose 3′ sequences have been determined, only a single message has >24 bases between the polyadenylation hexanucleotide and the poly(A) tail (cf. sequences tabulated in Fox *et al.*, 1989 and McGrew *et al.*, 1989). D7 RNA, which has a functional CPE, contains 32 bases in this region (Smith *et al.*, 1988). Whether this RNA is under some unique regulation is unknown, although as mentioned earlier, it is adenylated much later than G10 RNA.

Finally, we have shown that sequences distal to the poly(A) tail have a negative effect on polyadenylation (Figure 5B). We emphasize that this is not due to the number of bases after the polyadenylation hexanucleotide since an RNA with up to 300 adenylate residues at its 3′ end is further polyadenylated at maturation (McGrew *et al.*, 1989). Rather, it is possible that the poly(A) polymerase binds initially to a region near the polyadenylation hexanucleotide and scans down the poly(A) tail until it reaches the end of the molecule where it begins to adenylate. This scanning, however, would be coupled to a function that can only 'read' poly(A) since guanylate residues abort the reaction. Alternatively, the structure of the poly(A) tail could be disrupted by poly(G), which in turn inhibits poly(A) elongation. We presently cannot distinguish between these possibilities.

Polyadenylation is an early event that requires protein synthesis and is induced by MPF/cytosol and cyclin

The observation that ~50% of G10 RNA is polyadenylated by 2 h post-progesterone indicates that it is a relatively early response to steroid stimulation. Interestingly, polyadenylation is sensitive to treatment with cycloheximide which suggests that progesterone induces the synthesis of a new factor that is required for this reaction. One would envisage that such a factor is synthesized during the first 15 min of maturation. This factor could act by binding RNA directly (see below) or by activating other adenylation factors.

MPF, which is activated fairly late in response to progesterone, at ~0.5 relative time to GVBD (Reynhout and Smith, 1974), is known to obviate the requirement for protein synthesis during maturation. Surprisingly, an egg cytosol fraction containing MPF stimulates adenylation even though, as stated above, it is an early event [in other experiments, adenylation is clearly evident by 1 h post-progesterone (0.1 GVBD), J.Paris and J.D.Richter, submitted for publication]. Moreover, this stimulation is also cycloheximide sensitive. This unexpected finding shows that MPF/cytosol cannot short circuit the requirement for protein synthesis. This suggests that MPF/cytosol and progesterone both stimulate the synthesis of a new factor. Moreover, the observation that the late-appearing activity of MPF can act to induce an early event indicates that MPF could have a multitude of activities that are not necessarily coupled to cell cycle progression.

In order to assess whether MPF, rather than another component of the cytosol fraction, was the inducer of polyadenylation, we also injected pure cyclin mRNA, whose encoded protein is one component of MPF. The observation that cyclin mRNA was a potent stimulus of polyadenylation could indicate that cyclin protein was the inducer of polyadenylation in our MPF preparation. Alternatively, cyclin might act via MPF activation, which in turns leads to polyadenylation.

An 82 kd CPE binding protein

To begin to define the factors that are required for polyadenylation, we have used extracts that faithfully adenylate exogenously added RNA. UV crosslinking revealed that several proteins bound RNA in both oocyte

and egg extracts. However, a unique 82 kd protein crosslinked to RNA only in egg extracts and only when RNA contained an intact CPE. It therefore seems reasonable to propose that this protein interacts directly with the CPE. Furthermore, it could be this protein, CPEB, that must be synthesized *de novo* by progesterone or MPF. Alternatively, CPEB might be a masked protein that must be activated during maturation. Experiments are in progress to determine which is the case.

We were unable to detect any protein that crosslinked to the polyadenylation hexanucleotide. In nuclear extracts, a 64 kd protein (Wilusz and Shenk, 1988) crosslinks to this region. It is possible that an egg protein that binds this sequence is not detectable in our crosslinking, or that it binds rather transiently. Other experiments, such as gel shift assays, are required to assess whether a factor does indeed bind the polyadenylation hexanucleotide.

In this communication, we have defined the CPE and have shown that it is bound by a developmentally regulated 82 kd protein. In addition, polyadenylation is stimulated by MPF/cyclin and requires protein synthesis during the first 15−30 min of maturation. Clearly, it is important to purify and clone the 82 kd protein and other factors that are involved in polyadenylation. Furthermore, the possible role played by MPF/cyclin in this reaction requires further definition. We are presently pursuing these goals.

Materials and methods

Plasmid constructions

To construct plasmids containing nucleotide substitutions corresponding to the last 50 nucleotides of G10 mRNA, oligonucleotides specific for the desired sequence were synthesized. Complementary oligonucleotides containing a 5′ *Bst*EII site and a 3′ *Sal*I site, a 5′ *Bst*EII and a 3′ *Pst*I site, or a 5′ *Pst*I site and a 3′ *Sal*I site were annealed at 50°C for 60 min in 0.1 M NaCl and were ligated into the corresponding sites of pSP64XBM (Krieg and Melton, 1984). The plasmid pXBG10-360 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTATTTGTG-TTTTTTATAAAGCTGCAGTAATAAAG annealed to its complement 5′-TCGACTTTATTACTGCAGCTTTATAAAAAACACAAATAA-TATTCTAGTG; p360-45 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTAGGTACCTTTTTTATAAAGCTGCA annealed to its complement 5′-GCTTTATAAAAAAGGTACCTAATA-TTCTAGTG; the plasmid p360-47 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTATTTGTGGGTACCATAAAGCTGCA annealed to its complement 5′-GCTTTATGGTACCCACAAATAATA-TTCTAGTG; the plasmid p360-64 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTATTTGTGGGTTTTATAAAGCTGCA annealed to its complement 5′-GCTTTATAAAACCCACAAATAATA-TTCTAGTG; the plasmid p360-66 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTATTTGTGTTGGTTATAAAGCTGCA annealed to its complement 5′-GCTTTATAACCAACACAAATAATA-TTCTAGTG; the plasmid p360-68 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTATTTGTGTTTTGGATAAAGCTGCA annealed to its complement 5′-GCTTTATCCAAAACACAAATAAT-ATTCTAGTG; the plasmid p360-2 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTATTTGTGTTTTTTGGAAAGCTGCAGT-AATAAAG annealed to its complement 5′-TCGACTTTATTACTGCAG-CTTTCCAAAAAACACAAATAATATTCTAGTG; the plasmid p360-7 contained the oligonucleotide sequence 5′-GTTACCACTAGAATATTA-TTTGTGTTTTTTATGGTACCTGCA annealed to its complement 5′-GGTACCATAAAAAACACAAATAATATTCTAGTG; the plasmid p360-4 contained the oligonucleotide sequence 5′-GTTACCACTAGAA-TATTATTTGTGTTTTTTATAAAGCTGCAGTAAGAAAG annealed to its complement 5′-TCGACTTTCTTACTGCAGCTTTATAAAAAACAC-AAATAATATTCTAGTG; the plasmid p360-53 contained the oligonucleotide sequence 5′-GGCTAATAAAG annealed to its complement 5′-TCGACTTTATTAGCCTGCA; the plasmid p360-49 contained the oligonucleotide 5′-GGCTATCACCTAATAAAG annealed to its complement 5′-TCGACTTTATTAGGTGATAGCCTGC; the plasmid p360-51 contained the oligonucleotide 5′-GGCTATCACCATGATTGA-GGTAATAAAG annealed to its complement 5′-TCGACTTTATTACCT-CAATCATGGTGATAGCCTGCA.

To construct pSR360, the plasmid pXBG10-360 was digested with *Nco*I and *Bst*EII, filled in using DNA polymerase I and religated. This procedure removes all of the globin coding sequences and places the 3′-terminal sequences of G10 mRNA downstream of the SP6 promoter. The plasmids pSR4 and pSR47 were constructed in the same manner using p360-4 and p360-47, respectively.

To construct pXBG10Δ763-797Δ*Sal*I−*Sma*I the plasmid pXBG10Δ763-797 (McGrew *et al.*, 1989) was linearized at the *Sal*I site and filled-in using DNA polymerase I (Klenow fragment), then digested with *Sma*I and religated. pXBG10Δ763-797/*Xba*I (filled-in) was digested with *Xba*I and filled in with DNA polymerase I followed by religation.

The *Pst*I−*Hin*dIII fragment of pG10 (McGrew *et al.*, 1989) was ligated to pGEM I (Promega), digested with *Pst*I and *Hin*dIII to form pGPH. pSP65pA53 (Slavicek *et al.*, 1988) was digested with *Ssp*I and *Nae*I and then religated to form pSP65ΔSN. This plasmid was digested with *Eco*RI and *Hin*dIII, then ligated to the *Eco*RI−*Hin*dIII fragment from pGPH that contains a portion of G10 coding sequence plus the entire 3′ untranslated region to create pHPG. pHPG was digested with *Sma*I and *Hin*cII followed by religation to form pHPGΔSH. This procedure removes the polylinker region upstream of the G10 coding sequence. The *Ssp*I−*Bgl*I fragment from pXBG10-360 was ligated to the *Ssp*I−*Bgl*I fragment of pHPGΔSH to create pHPG-360. Similarly, the *Ssp*I−*Bgl*I fragment of p360-4 was ligated to the *Ssp*I−*Bgl*I fragment of pHPGΔSH to form pHPG-4.

RNA preparation and injection of oocytes

All of the DNA templates were linearized at a *Sal*I site immediately adjacent to the AAUAAA sequence unless noted otherwise (e.g. see Figure 4). Transcription by SP6 polymerase or T7 polymerase (for pGPH) was carried out essentially as described by Krieg and Melton (1984), except that the reaction mixture contained 100 μM UTP, 50 μM GTP, 500 μM GpppG and 40 μCi of [^{32}P]UTP (800 Ci/mmol, New England Nuclear). Following synthesis, the 3′ termini of GPH and XBG10 (GPH/dT, XBG10/dC) were removed by RNase H digestion in the presence of oligo(dT) or oligo(dC) as described previously (McGrew *et al.*, 1989). Clam cyclin A mRNA was synthesized using the plasmid pAXH$^+$ (Swenson *et al.*, 1986) linearized at the *Hin*dIII site and clam cyclin B mRNA was synthesized using the plasmid pCD102 (Westendorf *et al.*, 1989) linearized at the *Eco*RI site. Cyclin mRNAs were transcribed without the addition of [^{32}P]UTP. All RNAs were extracted with phenol and chloroform and precipitated with ethanol.

The 3′ termini of two RNAs, 360-4 and XBG10Δ763-797, were modified to contain 50−100 adenylate residues by incubation of 5 μg of radiolabeled RNA with 50 μM ATP, 50 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$, 50 μg/ml BSA, 250 mM NaCl, 1 mM $MnCl_2$, 5 mM DTT, 1 U/μl of RNasin for 3 min at 37°C followed by the addition of 0.8 U of poly(A) polymerase (Pharmacia) for 2 min at 37°C. The RNA was immediately extracted with phenol/chloroform and precipitated with ethanol in the presence of 10 μg/ml of tRNA.

Manually defolliculated oocytes were injected with 20−40 ng of radiolabeled RNA (3 × 10^6 c.p.m./μg) and cultured in Barth's medium in the absence or presence of 1 μg/ml progesterone. Typically, within 1 h after the appearance of a white spot at the animal pole, total RNA was extracted from the oocytes and analyzed without further treatment.

Oocytes were also incubated in 10 μg/ml cycloheximide for 2 h prior to the injection of radiolabeled RNA (Figure 6A). After injection, incubation was continued in 10 μg/ml cycloheximide with the addition of 1 μg/ml progesterone. In other experiments, RNA-injected oocytes were incubated in progesterone and cycloheximide, the latter of which was added 0−120 min after progesterone. The oocytes were then collected for RNA analysis 180 min after initial exposure to progesterone (Figure 6B). In a final set of experiments (Figure 6C), defolliculated oocytes were incubated in 10 μg/ml cycloheximide for 30 min prior to injection of radiolabeled RNA. After incubation, cycloheximide treated oocytes and untreated oocytes were injected with ~40 nl of cytosol from unfertilized eggs. For the cycloheximide treated oocytes, incubation was continued in the presence of 10 μg/ml cycloheximide.

Extract preparation and in vitro adenylation

Oocyte and egg extracts were prepared as described by Murray and Kirschner (1989). Eggs were shed into MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM NaEGTA, 5 mM NaHEPES, pH 7.8) and dejellied with 2% cysteine, pH 7.8. The eggs were washed three times with XB buffer (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM KHEPES, pH 7.7, 50 mM sucrose), then held in XB containing 10 μg/ml each of leupeptin, chymostatin and pepstatin and 100 μg/ml cytocholasin

B for 10–15 min. They were then transferred to 0.4 ml polypropylene Eppendorf centrifuge tubes containing 0.1 ml Versilube F-50 oil (General Electric) and 0.3 ml XB buffer plus the protease inhibitors and cytocholasin B. The eggs were centrifuged in an HB-4 rotor for 60 s at 1000 r.p.m. and then 30 s at 2000 r.p.m. at 2°C. All of the buffer and Versilube that was displaced above the eggs was removed and the eggs were then incubated on ice for 10 min. They were crushed by centrifugation at 10 000 r.p.m. in HB-4 rotor for 10 min at 2°C. The cytoplasmic layer was recovered and adjusted to 1 mM ATP, 1 mM $MgCl_2$, 7.5 mM creatine phosphate, 0.1 mM EGTA, pH 7.7, 10 μg/ml protease inhibitors and 100 μg/ml cytocholasin B. The cytoplasmic layer was centrifuged again at 10 000 r.p.m. for 15 min in the HB-4 rotor at 2°C to remove any residual yolk and pigment granules. Aliquots were frozen on dry ice and stored at −80°C prior to use. Stage 5–6 oocytes obtained from a collagenase treated ovary were prepared in a similar manner except that the centrifugations were performed at 16°C. Oocyte and egg cell-free extracts contained ~25–50 mg/ml total protein when assayed by the method of Bradford (1976) using bovine serum albumin as a standard.

In vitro polyadenylation reactions were performed in a total of 10 μl and contained 10–100 ng of radiolabeled substrate mRNA and 90% (v/v) oocyte or egg extract. The mixture was incubated at room temperature (23–26°C) for 1 h and the RNA extracted by phenol/chloroform and separated on 4.5% polyacrylamide/8.3 M urea gels.

UV crosslinking

Oocyte or egg extracts (50% v/v) containing substrate RNA (3×10^6 c.p.m. equivalent to ~12 ng RNA) and 1 mM ATP, 1 mM $MgCl_2$, 7.5 mM creatine phosphate, 0.1 mM EGTA, pH 7.7, were incubated at room temperature for 1 h and then exposed to UV light (Blak-Ray Lamp XX-15) at 254 nm for 10 min at 4°C. The UV light source was supported ~4 cm above the samples and had an intensity of 3.02 mW/cm^2. After crosslinking, an equal volume of 2× ribonuclease buffer (125 mM Tris–HCl, pH 6.8, 1% sodium lauroyl sarcosinate, 20% glycerol, 2% β-mercaptoethanol, 4 mM $CaCl_2$) was added and the RNA was digested with 1 μg/ml ribonuclease A and 0.5 U/μl micrococcal nuclease at 37°C for 30 min. The samples were then adjusted to 0.5% SDS, 0.5 M NaCl and 60% formamide and heated for 3 min at 60°C. The proteins were then precipitated with 2 vol 100% ethanol at −20°C and analyzed by electrophoresis on 10% polyacrylamide–SDS gels and autoradiography.

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