Maturation-specific polyadenylation: in vitro activation by p34^{cdc2} and phosphorylation of a 58-kD CPE-binding protein

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During *Xenopus* oocyte maturation, poly(A) elongation controls the translational recruitment of specific mRNAs that possess a CPE (cytoplasmic polyadenylation element). To investigate the activation of polyadenylation, we have employed oocyte extracts that are not normally competent for polyadenylation. Addition of cell lysates containing baculovirus-expressed cyclin to these extracts induces the polyadenylation of exogenous B4 RNA. The involvement of p34^{cdc2} kinase in cyclin-mediated polyadenylation was demonstrated by p13-Sepharose depletion; removal of the kinase from oocyte extracts with this affinity matrix abolishes polyadenylation activation. Reintroduction of cell lysates containing baculovirus-expressed p34^{cdc2}, however, completely restores this activity. To identify factors of the polyadenylation apparatus that might be responsible for the activation, we employed UV cross-linking and identified a 58-kD protein that binds the B4 CPE in oocyte extracts. In polyadenylation-proficient egg extracts, this protein has a slower electrophoretic mobility, which suggests a post-translational modification. A similar size shift of the protein is evident in oocyte extracts supplemented with lysates containing baculovirus-expressed cyclin and p34^{cdc2}. This size shift, which is reversed by treatment with acid phosphatase, coincides temporally with cyclin-induced polyadenylation activation. We propose that p34^{cdc2} kinase activity leads to the phosphorylation of the 58-kD CPE-binding protein and that this event is crucial for the cytoplasmic polyadenylation that occurs during oocyte maturation.

[Key Words: Polyadenylation activation; p34^{cdc2} kinase activity; CPE-binding protein; oocyte maturation]

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Growing evidence suggests that cytoplasmic polyadenylation is a major mechanism that regulates maternal mRNA expression. In several developing systems, mRNA polyadenylation and deadenylation are closely correlated with polysomal recruitment and polysomal release, respectively (Rosenthal et al. 1983; Dworkin et al. 1985; Rosenthal and Wilt 1986; Huarte et al. 1987; Hyman and Wormington 1988; Paynton et al. 1988; Paris and Philippe 1990; Varnum and Wormington 1990). The cause-and-effect relationship between polyadenylation and translation recently has been established for some mRNAs in mouse and *Xenopus*. One example is tissue plasminogen activator (tPA) mRNA, which undergoes cytoplasmic poly(A) elongation and translation during mouse oocyte maturation (Huarte et al. 1987). In this case, the important parameter governing polysomal recruitment is poly(A) tail length; specifically, the number

of adenylate residues normally found on the message in the mature oocyte (Vassalli et al. 1989).

The situation in *Xenopus* may be somewhat more complex. A maternal mRNA called G10 is also polyadenylated and recruited onto polysomes during oocyte maturation. However, translation of this message requires the dynamic process of poly(A) elongation that occurs during maturation and not any particular length of poly(A) tail (McGrew et al. 1989; Richter et al. 1991). Conversely, the translation of another *Xenopus* maternal mRNA during oocyte maturation, designated B4 RNA, is stimulated by a poly(A) tail of the "mature" size rather than by the process of poly(A) elongation (Paris and Richter 1990). These observations indicate that the *Xenopus* oocyte polyadenylation apparatus might be able to discriminate among RNAs, which should be reflected in the *cis* elements that govern polyadenylation. An analysis of all *Xenopus* RNAs studied to date has shown that two sequences are required for maturation-specific polyadenylation: the polyadenylation hexanucleotide AAUAAA

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and a U-rich cytoplasmic polyadenylation element (CPE). The CPEs that have been determined experimentally for various RNAs are UUUUUUAUAAAG (G10 RNA; McGrew and Richter 1990), UUUUUAAU (B4 RNA; Paris and Richter 1990), UUUUAAAU (mouse HPRT RNA, examined in *Xenopus* oocytes; Paris and Richter 1990), UUUUAU (c-mos RNA; Paris and Richter 1990), and UUUUUAAU (D7 RNA; Fox et al. 1989). Although similar in sequence, it is likely that the differences among the CPEs are responsible for the differential regulation of polyadenylation.

In some of our initial studies to examine this regulation, we noted that cyclin mRNA, as well as progesterone, the natural stimulus of maturation, could induce polyadenylation in injected oocytes (McGrew and Richter 1990). Cyclin induces maturation by activating p34^{cdc2}, a serine/threonine kinase that is responsible for regulating the transition from G₂ to M phase of the cell cycle (Draetta et al. 1989; Labbe et al. 1989; Murray and Kirschner 1989; Gautier et al. 1990; Minshull et al. 1990; Solomon et al. 1990). p34^{cdc2} and cyclin form M-phase-promoting factor (MPF), also known as maturation-promoting factor, which is found in mitotic cells from a wide variety of eukaryotic species. Thus, we were curious whether the induction of polyadenylation during oocyte maturation and following cyclin mRNA injection was also mediated by activated p34^{cdc2}. To assess this, we have employed a *Xenopus* oocyte extract to examine the activation of B4 RNA polyadenylation.

Polyadenylation is activated in oocyte extracts when they are supplemented with insect cell lysates containing baculovirus-expressed cyclin. Cyclin-induced polyadenylation requires p34^{cdc2} activity because extracts depleted of this kinase with p13-Sepharose do not polyadenylate exogenous RNA. Furthermore, polyadenylation is restored to these depleted extracts upon addition of lysates containing active baculovirus-expressed p34^{cdc2}. A molecular substrate of p34^{cdc2} kinase appears to be a 58-kD B4 CPE-binding protein, whose phosphorylation may be a key event in the activation of polyadenylation.

Results

Induction of polyadenylation by cell lysates containing baculovirus-expressed cyclin in vivo and in vitro

A mixture of mRNAs encoding clam cyclins A and B induces polyadenylation in injected *Xenopus* oocytes (McGrew and Richter 1990). To determine whether polyadenylation could be activated in vitro by cyclin, we employed Sf9 insect cells infected with recombinant baculovirus vectors expressing high levels of clam cyclin A or B (Parker et al. 1991). Lysates from these cells were tested for their ability to stimulate polyadenylation in injected oocytes and oocyte extracts. The RNAs that we examined for polyadenylation are shown in Figure 1A. sGb/B4 is a 110-base chimeric RNA composed of a part of the *Xenopus* β -globin 5'-untranslated region (UTR) linked to a portion of the B4 RNA 3' UTR. The B4 CPE

and polyadenylation hexanucleotide are boxed, and a cryptic CPE that can stimulate low levels of adenylation in vivo is underlined. A 119-base RNA, sR47, was used in some in vitro experiments as a control; it includes globin sequences identical to sGb/B4, followed by a 3' UTR containing only a polyadenylation hexanucleotide.

By using a retarded electrophoretic migration as an indication of polyadenylation (McGrew et al. 1989; also Fig. 1D), we found that sGb/B4 RNA injected into oocytes was polyadenylated very poorly (compared with noninjected RNA) irrespective of whether oocytes were injected with a lysate from Sf9 cells infected with wild-type baculovirus (Fig. 1B, lanes 2,3). However, this RNA was polyadenylated extensively when oocytes were also injected with a lysate from cells infected with baculovirus-expressing clam cyclin B (lane 4) and cyclin A (data not shown). For comparison, the polyadenylation of sGb/B4 RNA in cyclin-injected oocytes was similar to that which occurred in maturing, progesterone-treated oocytes (Fig. 1B, lane 6).

Next, we determined whether baculovirus-expressed cyclin could induce polyadenylation when added to an oocyte extract. Figure 1C shows that an oocyte extract supplemented with a lysate derived from cells expressing wild-type baculovirus did not polyadenylate added RNA to any appreciable extent (lanes 3 and 4). However, incubation of the extract with a lysate containing cyclin B prior to the addition of the RNA elicited a substantial polyadenylation response (lane 5). Moreover, this cyclin-induced polyadenylation was similar to that observed in egg extracts and in progesterone-matured oocytes because it required an RNA with a CPE (Fig. 1C, cf. lanes 5–8; see also McGrew and Richter 1990; Paris and Richter 1990). Finally, we determined that the cyclin-induced mobility change in sGb/B4 RNA was reversed by treatment with oligo(dT) and RNase H (Fig. 1D), which demonstrates that this RNA was indeed polyadenylated. Thus, the polyadenylation apparatus can be activated in vitro by the addition of a cyclin-containing lysate.

Cyclin-induced polyadenylation requires tyrosine phosphatase activity

Cyclin forms a dimer with and activates the serine/threonine kinase p34^{cdc2} (for review, see Nurse 1990). The activation of p34^{cdc2} occurs in several steps, including phosphorylation/dephosphorylation modifications, and requires the action of a protein-tyrosine phosphatase (Dunphy and Newport 1989; Gautier et al. 1989; Gould and Nurse 1989; Morla et al. 1989; Solomon et al. 1990). To determine whether cyclin-induced polyadenylation also requires protein-tyrosine phosphatase activity, we have supplemented oocyte extracts with sodium orthovanadate, a potent and specific inhibitor of this class of enzyme (Swarup et al. 1982; Tonks et al. 1988; Solomon et al. 1990). When a concentration as low as 10 μ M of this drug was added to oocyte extracts prior to the addition of a cyclin-containing lysate and sGb/B4 RNA, polyadenylation was virtually completely inhibited (Fig. 2A, left). In contrast, the drug had no effect on polyadenylation

Figure 1. Activation of polyadenylation by baculovirus-expressed cyclin in vivo and in vitro. (A) The salient features of two DNAs used for in vitro transcription are shown. psGb/B4 contains a CPE, a polyadenylation hexanucleotide (each boxed), and a cryptic CPE (underlined). psR47 contains only a polyadenylation hexanucleotide (boxed). (B) Oocytes were injected with [³²P]UTP-labeled sGb/B4 RNA and, in some cases, with a lysate from Sf9 cells infected with wild-type baculovirus (Bac-WT) or a recombinant virus expressing clam cyclin B (Bac-cyclin B). (Ni) Noninjected marker RNA; (Control) oocytes injected with radioactive RNA only. Some RNA-injected oocytes were also induced to mature with progesterone (Prog). RNA was extracted and resolved

by denaturing PAGE and autoradiography. (C) Oocyte extracts were supplemented with lysates containing either baculovirus wild-type proteins or baculovirus-expressed cyclin B and incubated for 1 hr. They were then mixed with either sGb/B4 or sR47 RNA and incubated for an additional hour before the RNA was extracted and analyzed. For comparison, polyadenylation assays with egg extracts are also shown. (NR) Nonreacted RNA markers. (D) RNA was isolated from oocyte extracts supplemented with lysates containing baculovirus-expressed cyclin and treated with RNase H in the presence or absence of oligo(dT). The resultant RNA was then analyzed as in B.

when added to an egg extract even at a concentration as high as 1 mM (Fig. 2A, right). Thus, this drug inhibited the activation of polyadenylation and not the ongoing process of adenylate polymerization and strongly suggests that polyadenylation activation by cyclin requires tyrosine dephosphorylation.

Vanadate was also added to oocyte extracts either coincident with or following cyclin-containing lysate addition (Fig. 2B). Although the drug was a potent inhibitor of adenylation activation when added with or 20 min after the cyclin-containing lysate, it had no effect when added 1 hr after the cyclin-containing lysate. Therefore, the induction of polyadenylation requires protein-tyrosine phosphatase activity for at least the first 20 min following cyclin-containing lysate addition, which is in good agreement with the time required for cyclin-induced activation of p34^{cdc2} kinase in activated egg extracts (Solomon et al. 1990).

Polyadenylation activation requires p34^{cdc2} kinase

To show direct involvement of p34^{cdc2} in cytoplasmic polyadenylation, we depleted the kinase from oocyte extracts by using the p34^{cdc2} affinity matrix p13-Sephadex (Dunphy et al. 1988) and then tested for cyclin-induced polyadenylation. Figure 3 shows that oocyte extracts

supplemented with lysates containing baculovirus-expressed cyclin alone or cyclin plus p34^{cdc2} adenylated sGb/B4 RNA to the same extent (lanes 2 and 3). When oocyte extracts were mixed with protein A-Sepharose in a mock depletion, the polyadenylation activity induced by cyclin decreased by ~40%, which was probably due to a dilution of the extract. However, depletion of p34^{cdc2} with p13-Sepharose lowered the cyclin-induced polyadenylation by >95% (lane 5). To demonstrate that the loss of cyclin-induced polyadenylation was due specifically to p34^{cdc2} depletion, the depleted extract was supplemented with lysates containing p34^{cdc2} and polyadenylation activation was restored (lane 6). The relative levels of p34^{cdc2} kinase activity in these extracts was monitored by measuring histone H1 phosphorylation, which generally paralleled the polyadenylation activity (Fig. 3, bottom; cf. lanes 1–6). Thus, this experiment establishes that cyclin-induced polyadenylation requires kinase-active p34^{cdc2}.

Identification of a B4 CPE-binding protein

By using gel mobility-shift assays, we have observed similar CPE-dependent polyadenylation complexes on sGb/B4 RNA in oocyte and egg extracts (data not shown; see also Paris and Richter 1990). This suggests that the fac-

Figure 2. Effect of vanadate on polyadenylation. (A) Oocyte and egg extracts were supplemented with various concentrations of sodium orthovanadate and, in some cases, with lysates from cells containing baculovirus wild-type proteins or baculovirus-expressed cyclin. After a 1-hr incubation at 23°C, radiolabeled sGb/B4 RNA was added and polyadenylation assays were performed. (B) Oocyte extracts were supplemented with cyclin at time 0 and 10 μ M vanadate at the denoted times thereafter. sGb/B4 RNA was then added, and polyadenylation assays were performed as described in Fig. 1.

tor(s) involved with maturation-specific polyadenylation are already bound to the CPE in the oocyte but in an inactive form. To identify such CPE-binding factor(s), we have employed UV cross-linking. In vitro-synthesized [32 P]UTP-labeled RNAs containing both the CPE and polyadenylation hexanucleotide (sGb/B4 RNA), the polyadenylation hexanucleotide only (sR47 RNA), or the CPE only (sGb/B4-M4 RNA) were added to oocyte and egg extracts followed by irradiation with 254-nm light. The RNAs were then digested with RNase A, and the proteins that became radioactive by label transfer were examined by SDS-PAGE and autoradiography (Fig. 4A, top). In addition, the RNA from a portion of each sample was extracted before UV irradiation and analyzed for polyadenylation on a denaturing acrylamide gel (Fig. 4A, bottom).

One protein, with an estimated molecular size of 58 kD, cross-linked to RNA in both oocyte and egg extracts but only when the RNA contained an intact CPE (Fig. 4A, top; cf. lane 1 with lane 2 and lane 4 with 5). In addition, small nucleotide replacements within the CPE lowered or destroyed the ability of the 58-kD protein to cross-link to the RNA. In each case, these mutations had exactly parallel effects on polyadenylation activity. For example, substitution of the 3' AU of the CPE with GG diminished the amount of cross-linked 58-kD protein (Fig. 4B, top; cf. lanes 1 and 2) and also lowered polyadenylation (Fig. 4B, bottom). Similarly, replacement of UUA or UUU in the CPE with GGG totally prevented cross-linking of the 58-kD protein and also abolished polyadenylation (lanes 3 and 4). These data strongly suggest that the 58-kD protein binds the B4 CPE and is required for polyadenylation activity.

Because this CPE-binding protein is present regardless of whether polyadenylation is active (Fig. 4A, bottom;

lanes 1,4), one might think that it may not be involved in the regulation of polyadenylation activation. However, we noticed that the CPE-binding protein detected in egg extracts had a slightly slower electrophoretic mobility compared to that from oocyte extracts when compared to molecular weight standards. When cross-linked proteins from these two extracts were compared on the same polyacrylamide gel, it was clear that the egg CPE-binding protein had a different mobility and migrated with an estimated size of 60 kD (Fig. 4C). This suggests that either the 58-kD CPE-binding protein undergoes some post-translational modification during maturation or that it is replaced by an egg-specific 60-kD CPE-binding protein.

Correlation of polyadenylation activity with the presence of a slow-migrating CPE-binding protein

We have mixed oocyte and egg extracts to determine whether polyadenylation activity would be present and, if so, whether this correlates with the presence of the slow-migrating (60-kD) form of the CPE-binding protein at the expense of the fast-migrating (58-kD) form. Figure 5A (top) shows that only the slower form of the CPE-binding protein was observed in the mixed extract (lane 3). In addition, the mixed extract polyadenylated RNA with an efficiency similar to that of the egg extract alone (Fig. 5A, bottom). This result strengthens the correlation between the slower-migrating form of the CPE-binding protein and active polyadenylation.

Next, we determined whether the slow-migrating CPE-binding protein can be detected by UV cross-linking by using oocyte extracts supplemented with cyclin-containing insect cell lysates. A comparison of lanes 1 and 2 in Figure 5B shows that the slow-migrating CPE-binding

Figure 3. Role of p34^{cdc2} in polyadenylation activation. Some oocyte extracts were primed with lysates from cells containing baculovirus wild-type proteins (lane 1) or baculovirus-expressed cyclin only (lane 2) or lysates containing cyclin plus baculovirus-expressed p34^{cdc2} (lane 3). Other oocyte extracts were depleted of p34^{cdc2} with the affinity matrix p13 (lanes 5,6) or, in a mock depletion, protein A–Sepharose (lane 4). Following depletion, lysates from cells containing baculovirus-expressed cyclin (lanes 4,5) or baculovirus-expressed cyclin and p34^{cdc2} (lane 6) were added. After a 1-hr incubation at 23°C, radiolabeled sGb/B4 RNA was added and polyadenylation assays were performed (*top*). For most of these extracts, their ability to phosphorylate the p34^{cdc2} model substrate histone H1 was also determined (*bottom*). The extracts were diluted and supplemented with total histones plus [³²P]ATP, and phosphorylated histone H1 was resolved by SDS-PAGE and autoradiography.

protein is readily observed in such extracts. Because we had shown that vanadate inhibits the activation of polyadenylation by cyclin-containing lysates in oocyte extracts (Fig. 2), we also determined whether this drug could prevent the appearance of the slow-migrating CPE-binding protein. Figure 5B (lane 3) demonstrates that, in the presence of vanadate, only the fast-migrating 58-kD CPE-binding protein was detected in oocyte extracts supplemented with a lysate-containing cyclin. For comparison, we also show that vanadate treatment of egg extracts, which had no effect on polyadenylation (Fig. 2), had no effect on the ability of the slow-migrating CPE-binding protein to photo-cross-link to RNA (Fig. 5B, cf. lanes 4 and 5).

We have also correlated the timing of the appearance of the slow-migrating form of the CPE-binding protein and the activation of polyadenylation by cyclin in oocyte extracts (Fig. 5C). At 45 min of incubation, the 58-kD CPE-binding protein began to increase in apparent mo-

lecular mass (top, lane 3). At this time, polyadenylation was undetectable (bottom, lane 3). By 60–90 min, when only the 60-kD form of the CPE-binding protein was evident, polyadenylation activity was clearly observed. Thus, there is a strong correlation between the appearance of the slow-migrating CPE-binding protein and the activation of polyadenylation, which supports the notion that this protein is centrally important for this process. In addition, the gradual change in apparent molecular mass of the fast- to the slow-migrating form of the CPE-binding protein argues that this is a single protein species that undergoes a cyclin-induced post-translational modification.

Up to this point, we have employed total soluble insect cell lysates containing cyclin and/or p34^{cdc2} for our polyadenylation assays (for electrophoretic profiles, see Fig. 6A). Although the insect cell lysates containing wild-type baculovirus proteins do not lead to polyadenylation or modification of the CPE-binding protein, we could not rule out the possibility that an insect protein present (or active) only in lysates containing overexpressed cyclin and/or p34^{cdc2} was also required for these events. To assess this, we primed oocyte extracts with SP6-derived mRNAs encoding clam cyclins A and B. These extracts, when mixed with radiolabeled sGb/B4 RNA, exhibited both the slow-migrating form of the CPE-binding protein and active polyadenylation (Fig. 6B). In addition, partially purified *Xenopus* MPF (the cyclin/p34^{cdc2} heterodimer) added to oocyte extracts also promoted the appearance of the slow-migrating CPE-binding protein as well as polyadenylation (Fig. 6C). Therefore, we conclude that overexpressed cyclin and/or p34^{cdc2} are the sole agents in the insect cell lysates that are responsible for the induction of the slow-migrating CPE-binding protein and the activation of polyadenylation.

A post-translational modification of the B4 CPE-binding protein is mediated by p34^{cdc2}

The data presented above do not rule out the possibility that there are actually two B4 CPE-binding proteins: an oocyte-specific protein that is replaced by an egg-specific protein during maturation. To examine this possibility, we have analyzed the size of the B4 CPE-binding protein in extracts that were supplemented with cyclin and p34^{cdc2} after UV irradiation (Fig. 7A). The detection of the slower-migrating form of the CPE-binding protein (lane 3) indicates that it is a modified form of the fast-migrating 58-kD oocyte CPE-binding protein because no new RNA-binding protein would be radiolabeled after UV irradiation.

A second experiment to demonstrate the relatedness of the oocyte and egg CPE-binding proteins was a comparison of the sizes of fragments generated by partial proteolysis. Extracts from oocytes, cyclin-containing lysate-supplemented oocytes, and eggs were used for UV cross-linking of proteins to Gb/B4 RNA (Fig. 7B, top). The portions of the gel to which the CPE-binding proteins had migrated (denoted as protein A from oocyte

Figure 4. Analysis of a B4 CPE-binding protein. (A) Oocyte and egg extracts were primed with [32 P]-labeled sGb/B4 (contains both *cis* elements), sR47 (contains only the polyadenylation hexanucleotide), and sGb/B4-M4 (contains only the CPE) RNAs, and then irradiated with UV light. The extracts were then incubated with RNase A and the proteins that had become radioactive by label transfer were resolved by SDS-PAGE and autoradiography (top). A portion of these samples was removed just prior to cross-linking and analyzed for polyadenylation activity (bottom). (B) RNAs containing the denoted nucleotide substitutions in the B4 CPE were used for photo-cross-linking as described in A (top) and for polyadenylation analysis (bottom). The arrow denotes the B4 CPE-binding protein. (C) Oocyte and egg extracts primed with sGb/B4 RNA were irradiated with UV light and the cross-linked proteins analyzed as described in A. The arrows denote the two B4 CPE-binding proteins.

extracts, protein B from cyclin-containing lysate-supplemented oocyte extracts, and protein D from egg extracts), as well as nonspecific RNA-binding proteins (denoted as protein C from cyclin-containing lysate-supplemented oocyte extracts), were excised and inserted into the wells of a second SDS-gel together with *Staphylococcus aureus* V8 protease as described by Cleveland et al. (1977). The CPE-binding proteins and the nonspecific RNA-binding proteins were then subjected to partial proteolysis and analyzed by electrophoresis and autoradiography (Fig. 7B, bottom). The proteolytic fragments of the CPE-binding proteins from oocyte, cyclin-containing lysate-supplemented oocyte, and egg extracts were of identical size with two major fragments migrating with apparent sizes of 14 and 25 kD (Fig. 7B, cf. lanes 1,2,7). This proteolytic pattern, as expected, was different from that of the nonspecific RNA-binding proteins (lane 7). On the basis of the data in Figure 7, we therefore conclude that the CPE-binding protein from oocyte, cyclin-containing lysate-supplemented oocyte, and egg extracts is a single protein species.

Phosphorylation of the B4 CPE-binding protein

If p34^{cdc2} kinase interacts directly with the polyadenylation complex, we would expect that the post-transla-

tional modification of the B4 CPE-binding protein is phosphorylation. To examine this, sGb/B4 RNA-primed oocyte extracts supplemented with a lysate containing cyclin were used in a photo-cross-linking assay, a portion of which was subsequently treated with potato acid phosphatase. An inspection of Figure 8 shows that the size shift in the CPE-binding protein induced by cyclin-containing lysates (cf. lanes 1 and 4) was reversed by treatment with the phosphatase (lane 5). The phosphatase buffer had no effect on the cyclin-containing lysate-induced size shift of the CPE-binding protein (lanes 1–6 contained phosphatase buffer). Moreover, the phosphatase was ineffective in reversing the cyclin-containing lysate-induced size shift if the reaction was carried out in the presence of molybdate, which is a specific inhibitor of this enzyme (Jackson et al. 1990) (lane 6). Therefore, the size shift of the B4 CPE-binding protein is due to phosphorylation.

Discussion

Two events required for the activation of p34^{cdc2} are heterodimer formation with cyclin and transient tyrosine phosphorylation, during which time the kinase is nonfunctional. Vanadate, a specific inhibitor of protein-

Figure 5. Correlation of a slow-migrating CPE-binding protein and polyadenylation activity. (A) Separate oocyte and egg extracts or a mixture of the two extracts (equal volume) were incubated at 23°C for 1 hr and primed with radiolabeled Gb/B4 RNA. After a 20-min incubation, a portion of the samples was irradiated with UV light and the cross-linked proteins were analyzed as described in Fig. 4 (top). The remainder of the samples were assayed for polyadenylation activity as described previously (bottom). (B) Oocyte extracts, supplemented with wild-type lysates (no recombinant proteins, lane 1) or cyclin-containing lysates (lanes 2,3) and, in one case with vanadate (50 μ M, lane 3), were primed with sGb/B4 RNA and irradiated with UV light. Egg extracts were also primed with sGb/B4 RNA and, in one case, with vanadate (50 μ M, lanes 4 and 5, respectively). Following UV irradiation, the cross-linked proteins were resolved as described in Fig. 4. (C) Aliquots of oocyte extracts supplemented simultaneously (time zero) with sGb/B4 RNA and baculovirus-expressed cyclin were taken at the denoted time points and analyzed for photo-cross-linked CPE-binding protein (arrows, top) and polyadenylation (bottom).

tyrosine phosphatases (Swarup et al. 1982; Tonks et al. 1988; Solomon et al. 1990), prevents the tyrosine dephosphorylation of p34^{cdc2} and maintains it in an inactive state. We show that vanadate also blocks cyclin-induced polyadenylation in oocytes. This block occurs at the activation stage of polyadenylation because the drug has had no effect on poly(A) elongation 20–60 min after cyclin addition. We infer this to mean that once a component of the polyadenylation complex is phosphorylated by p34^{cdc2}, continued presence of active kinase is no longer required for polyadenylation.

Another line of evidence that strongly implicates p34^{cdc2} in the activation of polyadenylation is that its depletion from oocyte extracts by p13-Sepharose abolished polyadenylation. Importantly, addition of baculovirus-expressed p34^{cdc2} to these depleted extracts completely restored polyadenylation activation. On the basis of these observations, we propose that p34^{cdc2} is centrally important for the activation of polyadenylation.

Although p34^{cdc2} clearly activates polyadenylation *in vitro*, does it do so during the normal course of maturation? The answer to this question is not clear-cut. Polyadenylation is an early event of maturation that is detected 1–2 hr after oocytes are exposed to progesterone, which is the natural inducer of maturation (McGrew and Richter 1990; Paris and Richter 1990). On the other hand, the maturation-specific activation of histone H1 kinase, in which p34^{cdc2} is the catalytic subunit (Arion et al. 1988), occurs at ~4 hr postprogesterone (Shuttleworth et al. 1990). Therefore, these observations alone would suggest that p34^{cdc2} is not the physiological activator of polyadenylation. However, one important consideration should be borne in mind. It is clear that immature oocytes (those that have not been exposed to progesterone) contain a basal level of active p34^{cdc2}, as measured by *in vitro* histone H1 kinase activity (Shuttleworth et al. 1990; J. Paris and J.D. Richter, unpubl.). It is certainly possible that slight changes in p34^{cdc2} kinase

Figure 6. Electrophoretic profiles of baculovirus-expressed cyclin and p34^{cdc2} and the effects of MPF and cyclin mRNA on photo-cross-linking and polyadenylation. (A) Total proteins from Sf9 cells infected for 84 hr with wild-type (WT) or recombinant baculoviruses producing cyclin A, cyclin B, or p34^{cdc2} were analyzed by SDS-PAGE and Coomassie blue staining. The band that represents each of these proteins is denoted by an arrowhead. The migration of molecular mass standards electrophoresed in parallel is indicated at left. (B) Oocyte extracts, in one case primed with an equal mixture of mRNAs encoding clam cyclins A and B (10 ng/ μ l, final concentration), were analyzed for photo-cross-linking of the B4 CPE-binding protein to radiolabeled sGb/B4 RNA (arrows, top) and polyadenylation (bottom). (C) Oocyte extracts, in one case supplemented with partially purified *Xenopus* MPF, were analyzed for photo-cross-linking of the B4 CPE-binding protein (arrows, top) and for polyadenylation (bottom).

activity could lead to the activation of polyadenylation but go undetected when assessed by the histone H1 kinase assay. Moreover, it is possible that changes in the activities of specific phosphatases during the early phases of maturation (Cormier et al. 1990) could lead quickly to the phosphorylation of particular substrates by kinases with low levels of activity, such as the oocyte form of p34^{cdc2}. Thus, the question as to whether p34^{cdc2} is the kinase that activates polyadenylation during oocyte maturation in vivo is, for the moment, unanswered.

If p34^{cdc2} is not the kinase that initiates maturation-specific polyadenylation, what is? Clearly, it would have a substrate specificity that overlaps, at least partially, with p34^{cdc2} and would be activated early during maturation. In this regard, several p34^{cdc2}-like proteins have been described (Simon et al. 1986; Courchesne et al. 1989; Bourouis et al. 1990; Eilon et al. 1990), including two in *Xenopus* (Shuttleworth et al. 1990; Paris et al. 1991). However, the relevance of these or other putative p34^{cdc2}-like kinases (Shuttleworth et al. 1990) to the activation of polyadenylation remains to be demonstrated.

Phosphorylation of a CPE-binding protein

There is a growing list of structural and regulatory proteins that are potential substrates for p34^{cdc2} (for review, see Moreno and Nurse 1990), whose biological functions are likely to be regulated by the activity of this kinase. Our data suggest that the 58-kD B4 CPE-binding protein should be added to this list. However, it does remain a formal possibility that p34^{cdc2} actually phosphorylates

another kinase which, in turn, phosphorylates the CPE-binding protein. For example, phosphorylation of the tyrosine kinase pp60^{c-src} by p34^{cdc2} is thought to have important consequences for its function during mitosis (Morgan et al. 1989; Shenoy et al. 1989). This caveat notwithstanding, it is clear that p34^{cdc2} activates polyadenylation in vitro, probably by phosphorylating the B4 CPE-binding protein.

A family of CPE-binding proteins?

In contrast to the results presented here, McGrew and Richter (1990) identified an 82-kD protein that photo-cross-links to the G10 CPE in egg, but not oocyte, extracts. We suggest that the 82- and 58-kD CPE-binding proteins could be responsible for the differential regulation of polyadenylation and, consequently, translation, of B4 and G10 RNAs. In the case of B4 RNA, maturation-specific polyadenylation results in a poly(A) tail of ~150 residues during maturation. The tail length of this message is regulated in such a manner that an injected B4 RNA that already contains a poly(A) tail of this mature size will not be adenylated further during maturation (Paris and Richter 1990). In contrast, the poly(A) tail of injected G10 RNA is elongated further during maturation regardless of its initial length (McGrew et al. 1989). Moreover, this differential regulation of polyadenylation has profound consequences for the translation of these mRNAs. Injected B4 RNA is recruited onto polysomes in both immature and mature oocytes only when it has a poly(A) tail of ~150 residues; poly(A) tails either shorter

Figure 7. Post-translational modification of the B4 CPE-binding protein. (A) Oocyte extracts supplemented with lysates from cells containing wild-type baculovirus proteins (lane 1), baculovirus-expressed cyclin and p34^{cdc2} (lane 2), or oocyte extracts alone (lane 3) were primed with radiolabeled sGb/B4 RNA. Following incubation, they were irradiated with UV light and examined for photo-cross-linked proteins as described in Fig. 4. In one case, an oocyte extract primed with radiolabeled RNA was irradiated with UV light before the addition of a lysate containing cyclin and p34^{cdc2} (lane 3). Following a further incubation, the sample was treated with RNase A and the cross-linked proteins were resolved as described above. (B) The proteins that cross-link to radiolabeled Gb/B4 RNA from oocyte, cyclin-containing lysate-supplemented oocyte, and egg extracts were analyzed as described in Fig. 4. The areas of the gel the CPE-binding proteins (A,B,D), as well as nonspecific RNA-binding proteins (C, top), were excised and inserted into wells of a second SDS-polyacrylamide gel together with 0.2 μ g of *S. aureus* V8 protease (bottom). In one case (lane A'), a gel slice containing protein A was loaded into the well without the protease. After electrophoresis, the protein fragments were visualized by autoradiography.

or longer than that number dramatically decrease the translational efficiency of the message (Paris and Richter 1990). Conversely, G10 RNA enters polysomes only when it undergoes the dynamic process of polyadenylation during maturation; the absolute number of adenylate residues does not appear to be of any consequence for the translation of this message (McGrew et al. 1989; Richter et al. 1991). Given these observations, one might ask whether every CPE is recognized by a different protein. Although this cannot be answered at the moment, we do note that the different CPEs in *c-mos* and HPRT RNAs are probably responsible for the unique number of adenylate residues these messages receive during oocyte maturation (Paris and Richter 1990). Thus, it will be of interest to compare the CPE-binding proteins of these RNAs with those of G10 and B4 RNAs.

Activation of polyadenylation by differential protein phosphorylation

We propose that differential protein phosphorylation activates the polyadenylation of some mRNAs during oocyte maturation in *Xenopus*. RNAs that have a B4-like CPE would be bound by the 58-kD protein. Following the induction of maturation by progesterone, p34^{cdc2} kinase or a related kinase phosphorylates the CPE-binding

protein. This phosphorylation, in turn, could induce a conformational change in the protein such that it forms new or different protein-protein interactions that facilitates polyadenylation. What could these other proteins be? Two possible candidates are the polyadenylation hexanucleotide-binding protein, whose existence we infer on the basis of the absolute requirement for an intact AAUAAA for polyadenylation, and the poly(A) polymerase, which surely must be present because its activity can be measured.

One of our next goals is to isolate a cDNA clone for the 58-kD CPE-binding protein. With this in hand, we will be able to determine whether the protein contains a p34^{cdc2} consensus substrate site (Moreno and Nurse 1990) and, if so, whether it is crucial for the activation of polyadenylation. Moreover, the synthesis of large quantities of the CPE-binding protein in a heterologous system and the generation of specific antibody will help us to assess the interactions that it might have with other proteins in the polyadenylation apparatus.

Materials and methods

DNA constructions and RNA synthesis

The construction of plasmids psR47, psGb/B4, and psGb/B4-M4 has been detailed in Paris and Richter (1990) and McGrew and

Figure 8. Analysis of B4 CPE-binding protein phosphorylation. Oocyte extracts supplemented with either lysates containing wild-type baculovirus proteins or baculovirus-expressed cyclin were primed with labeled sGb/B4 RNA and irradiated with UV light. One-third of each sample was then supplemented with phosphatase buffer only (lanes 1,4), buffer plus potato acid phosphatase (lanes 2,5), or buffer plus phosphatase plus molybdate, a phosphatase inhibitor (lanes 3,6). After a 10-min incubation at 23°C, the cross-linked proteins were analyzed as described previously. The arrows refer to the two forms of the CPE-binding protein.

Richter (1990). Plasmids psGb/B4-M5, -M6, and -M7 were constructed with double-stranded oligonucleotides as described for psGb/B4-M4 (Paris and Richter 1990). They all contain a part of the *Xenopus* β -globin 5' UTR fused to different sequences at the 3' end. For psGb/B4, the 3' portion contains the wild-type B4 RNA CPE and a polyadenylation hexanucleotide; sr47 contains a polyadenylation hexanucleotide but no CPE; psGb/B4-M4 contains a B4 CPE but no polyadenylation hexanucleotide; and psGb/B4-M5, -M6, and -M7 contain mutations in the CPE (Fig. 4B). Plasmid DNAs were linearized with *Xba*I and used in SP6 polymerase-directed in vitro transcription reactions in the presence of G(5')ppp(5')G and [³²P]UTP (Krieg and Melton 1984). Clam cyclin-containing cDNA plasmids were a generous gift of Joan Ruderman. Cyclins A and B were generated as described in McGrew and Richter (1990).

Preparation of baculovirus-infected cell protein

DNA fragments encoding clam cyclins A (Swenson et al. 1986) and B (Westendorf et al. 1989) and the human homolog of p34^{cdc2} were inserted into the vector pVL941 (Luckow and Summers 1988) as described by Parker et al. (1991). *Spodoptera frugiperda* (Sf9) cells, which were cultured essentially as described by Summers and Smith (1987), were mixed with wild-type viral DNA and plasmid DNA and cultured for 5 days. Recombinant viruses were identified and plaque-purified as described (Pawlica-Worms et al. 1990; Roy et al. 1991). Recombinant proteins were identified by immunoblot analysis and quantitated by densitometric scanning of Coomassie blue-stained gels (Roy et al. 1991).

About 3×10^6 infected cells containing 2.5–5 μ g of cyclin or p34^{cdc2} were pelleted by centrifugation and resuspended in 50 μ l of XB buffer [100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 10 mM HEPES (pH 7.7), 50 mM sucrose]. The cells were lysed by brief sonication, and the cellular debris was pelleted by centrifugation. The supernatant from the lysate was injected directly into oocytes or added to oocyte extracts. Although not quantitated for every lysate that was prepared, the approximate concentrations of exogenous cyclin was 30 nM in injected oocytes and 300 nM in extracts. The approximate concentration of exogenous p34^{cdc2} in extracts was 300 nM. The gel profiles shown in Figure 6A represent cell pellets lysed directly in SDS sample buffer and probably represent a greater proportion of recombinant proteins than were injected into oocytes or added to extracts.

Oocyte injection and RNA analysis

Manually defolliculated stage 6 oocytes were injected with ~20 nl of a 0.1-mg/ml solution of radiolabeled RNA and, in some cases, with 20 nl of a solution containing a baculovirus-infected cell lysate. The oocytes were then cultured in the absence or presence of 1 μ g/ml of progesterone for ~3 hr. At that time, the RNA was extracted by phenol and chloroform and analyzed on a 5% polyacrylamide–8 M urea gel (Paris and Richter 1990).

Preparation of oocyte and egg extracts and polyadenylation assays

Xenopus oocyte and egg extracts were prepared as described by McGrew and Richter (1990), following the procedure of Murray and Kirschner (1989).

Polyadenylation reactions, which had a final volume of 9 μ l, contained 5–10 ng of radioactive RNA (10^4 – 10^5 cpm), 3 μ l of extract (total protein concentration of 50–70 mg/ml), and buffer [50 mM sucrose, 100 mM KCl, 0.1 mM CaCl₂, 10 mM HEPES–Na (pH 7.7), 0.4 mM EGTA, 5 mM MgCl₂, 4 mM ATP, and 30 mM creatine phosphate]. In some cases, 2.5 μ l of Sf9 cell lysates (total protein concentration of 2.5–5 mg/ml) containing baculovirus-expressed proteins was added to the extract 1 hr before the test RNA. Incubation was continued for an additional hour at 23°C before the RNA was extracted and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Cleavage of poly(A) tails by oligo(dT) and RNase H was performed according to McGrew et al. (1989).

UV cross-linking and analysis of radiolabeled proteins

Xenopus oocyte or egg extracts, in some cases mixed with lysates containing baculovirus-expressed proteins, cyclin mRNA, or MPF [phenyl-Superose fraction, generously provided by James Maller (Gautier et al. 1990)] were incubated for 1 hr at 23°C, after which time ~5 ng of radiolabeled RNA (sp. act. of 10^4 dpm/ng) was added. After an additional 20 min of incubation at 23°C, an aliquot of this mixture was placed ~4 cm from a UV light source (Black-Ray lamp XX-15) and irradiated for 10 min at 4°C with 254-nm light. Three volumes of 10 mM Tris-HCl (pH 6.8) and RNase A (final concentration of 2 mg/ml) were added, and the mixture was incubated for 30 min at 37°C. An equal volume of SDS sample buffer was then added, the samples were boiled for 5 min, and the proteins were separated on a 8.5% SDS–polyacrylamide gel and autoradiographed.

Phosphatase treatment and partial proteolysis

In some cases, one volume of 2 $\times$ PEM buffer [0.2 M PIPES (pH 6.6), 2 mM EGTA, 2 mM MgSO₄] and potato acid phosphatase (4

mg/ml, 2 U/mg, Boehringer Mannheim grade II) in the presence or absence of 5 mM molybdc acid (Sigma) were added to the polyadenylation mixture (after cross-linking) and incubated for 15 min at 23°C. RNase A was then added, and the cross-linked proteins were analyzed as described above. The analysis of cross-linked proteins by partial proteolysis with *S. aureus* V8 protease was performed exactly as described by Cleveland et al. (1977).

p34^{cdc2} depletion and histone H1 phosphorylation

Recombinant p13^{suc1} was expressed in *Escherichia coli*, partially purified by gel filtration chromatography, and attached to Sepharose CL-4B essentially as described by Brizuela et al. (1987). A 20-μl volume of gravity-packed p13-Sepharose or protein A-Sepharose was washed three times in XB buffer and mixed with one volume of oocyte extract. After intermittent agitation for 15 min at 4°C, the beads were centrifuged briefly and the supernatant was collected carefully. The supernatant was then supplemented with XB buffer and baculovirus-expressed cyclin and, in one case, with a lysate containing p34^{cdc2}, and incubated for an additional hour at 23°C. The extract was then assayed for polyadenylation activity or histone H1 phosphorylation activity. Histone H1 phosphorylation assays were performed exactly as described by Felix et al. (1989).

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