

Polyadenylation of maternal mRNA during oocyte maturation: poly(A) addition *in vitro* requires a regulated RNA binding activity and a poly(A) polymerase

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Specific maternal mRNAs receive poly(A) during early development as a means of translational regulation. In this report, we investigated the mechanism and control of poly(A) addition during frog oocyte maturation, in which oocytes advance from first to second meiosis becoming eggs. We analyzed polyadenylation *in vitro* in oocyte and egg extracts. *In vivo*, polyadenylation during maturation requires AAUAAA and a U-rich element. The same sequences are required for polyadenylation in egg extracts *in vitro*. The *in vitro* reaction requires at least two separable components: a poly(A) polymerase and an RNA binding activity with specificity for AAUAAA and the U-rich element. The poly(A) polymerase is similar to nuclear poly(A) polymerases in mammalian cells. Through a 2000-fold partial purification, the frog egg and mammalian enzymes were found to be very similar. More importantly, a purified calf thymus poly(A) polymerase acquired the sequence specificity seen during frog oocyte maturation when mixed with the frog egg RNA binding fraction, demonstrating the interchangeability of the two enzymes. To determine how polyadenylation is activated during maturation, we compared polymerase and RNA binding activities in oocyte and egg extracts. Although oocyte extracts were much less active in maturation-specific polyadenylation, they contained nearly as much poly(A) polymerase activity. In contrast, the RNA binding activity differed dramatically in oocyte and egg extracts: oocyte extracts contained less binding activity and the activity that was present exhibited an altered mobility in gel retardation assays. Finally, we demonstrate that components present in the RNA binding fraction are rate-limiting in the oocyte extract, suggesting that fraction contains the target that is activated by progesterone treatment. This target may be the RNA binding activity itself. We propose that in spite of the many biological differences between them, nuclear polyadenylation and cytoplasmic polyadenylation during early development may be catalyzed by similar, or even identical, components.

Key words: early development/poly(A)/poly(A) polymerase/oocyte maturation/translation

Introduction

Translational control of specific maternal mRNAs is critical during early development (Woodland, 1982; Dworkin and

Dworkin, 1990). Changes in the translational activity of specific mRNAs often can be correlated with changes in the length of their poly(A) tails (reviewed in Wickens, 1990b). In general, mRNAs that receive poly(A) become translationally active, whereas those that lose poly(A) become translationally quiescent. These correlations are widespread, occurring in species as diverse as clam and mouse, and involving many different mRNAs (Dworkin and Dworkin, 1990; Jackson and Standart, 1990; Wickens, 1990b). For at least certain mRNAs, the change in poly(A) length causes the change in translational activity (Hyman and Wormington, 1988; McGrew *et al.*, 1989; Vassalli *et al.*, 1989; Varnum and Wormington, 1990; Sheets, M.D., Fox, C.A., Hunt, T., VandeWoude, G. and Wickens, M., submitted).

Poly(A) addition and removal reactions have been characterized during oocyte maturation. In frogs, maturation is the process in which an oocyte, arrested in meiotic prophase I, advances through meiosis and arrests in second meiotic metaphase, as an 'egg'. During maturation, which can be induced by progesterone *in vitro*, some cytoplasmic mRNAs receive poly(A) while others lose it (reviewed in Wickens, 1990b). At least two sequences are required for poly(A) addition during maturation: the highly conserved AAUAAA sequence, which also is required for polyadenylation in the nucleus, and a second more flexible sequence (e.g. UUUUUAU). This second element, sometimes referred to as a CPE (cytoplasmic polyadenylation element), usually lies several nucleotides upstream of AAUAAA (Fox *et al.*, 1989; McGrew *et al.*, 1989).

Maturation-induced polyadenylation requires only cytoplasmic components since it is unimpaired in oocytes from which the nucleus has been removed (Fox *et al.*, 1989). The activities in the egg cytoplasm that catalyze poly(A) addition have not been identified or characterized. Although specific proteins can be cross-linked by ultraviolet light to 3' untranslated regions (UTRs) that contain a CPE (Paris *et al.*, 1991), their role in polyadenylation has not been demonstrated or has the site to which they bind been identified. Poly(A) removal is a default pathway: it is prevented by addition of poly(A), but requires no specific sequences in the mRNA (Fox and Wickens, 1990; Varnum and Wormington, 1990).

Most mRNAs, including those in the oocyte, initially receive a 250–300 nucleotide poly(A) tail in the nucleus, in a polyadenylation reaction that is coupled to endonucleolytic cleavage of an mRNA precursor. The cleavage reaction acts on a pre-mRNA that extends well past the polyadenylation site of the mature mRNA, and leaves a 3'-OH group to which poly(A) is then added (Moore *et al.*, 1986; Sheets *et al.*, 1987). In metazoans, the cleavage reaction requires AAUAAA and a less conserved U-rich or UG-rich element downstream of the poly(A) addition site (reviewed in Manley, 1988; Wickens, 1990a; Wahle and Keller, 1992; Wahle, 1992).

Although nuclear polyadenylation is tightly coupled to cleavage *in vivo*, it can be uncoupled *in vitro* using RNA substrates that end at their cleavage sites. Uncoupled polyadenylation requires AAUAAA (Zarkower *et al.*, 1986) and is catalyzed by a poly(A) polymerase and a cleavage/polyadenylation specificity factor (CPSF; Christofori and Keller, 1988; Takagaki *et al.*, 1988). The poly(A) polymerase becomes specific for AAUAAA only in the presence of CPSF, a multi-protein complex that binds directly to AAUAAA (Wahle, 1991b; Bienroth *et al.*, 1991; Keller *et al.*, 1991). Together, CPSF, poly(A) polymerase and the RNA form a productive ternary complex. A poly(A) binding protein (PAB II) stimulates elongation of the poly(A) tail (Wahle, 1991a) and participates in the AAUAAA-

independent second phase of the reaction (Sheets and Wickens, 1989).

Nuclear and maturation-induced polyadenylation differ in several important respects. First, maturation-induced polyadenylation occurs in the cytoplasm and requires no nuclear components. Secondly, maturation-induced polyadenylation is not coupled to a cleavage reaction. Rather, the natural mRNA substrates for maturation-induced polyadenylation, which are cytoplasmic, have already been cleaved in the nucleus and possess short poly(A) tails. Thirdly, whereas nuclear polyadenylation appears to require only AAUAAA (Wigley *et al.* 1990; Bardwell *et al.*, 1991), maturation-induced polyadenylation requires additional sequences (Fox *et al.*, 1989; McGrew *et al.*, 1989; Fox and

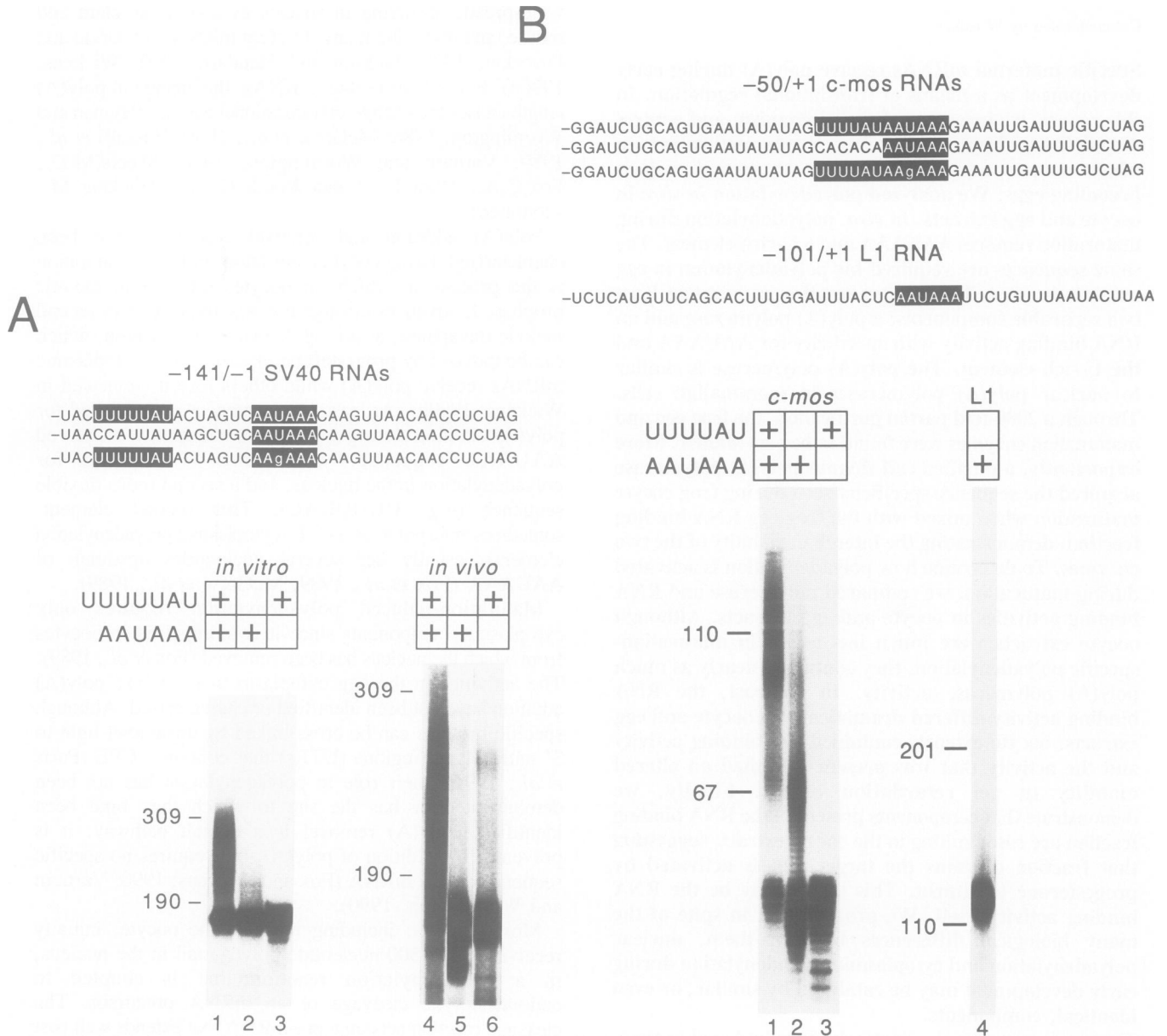


Fig. 1. An ammonium sulfate fractionated egg extract contains maturation-specific polyadenylation activity. **A.** Each substrate is based on -141/-1 SV40 RNA. The UUUUUUAU and AAUAAA sequences are highlighted with black. Each RNA was either incubated 30 min at 25°C in 20 µg of 0-30% ammonium sulfate egg extract (lanes 1-3) or injected into the cytoplasm of oocytes that were subsequently incubated in the presence of progesterone until maturation was complete (lanes 4-6). Lanes 1 and 4, -141/-1 SV40 RNA containing both AAUAAA and UUUUUUAU; lanes 2 and 5, -141/-1 SV40 RNA containing AAUAAA, but not UUUUUUAU; lanes 3 and 6, -141/-1 SV40 RNA containing UUUUUUAU and a point mutation in AAUAAA. **B.** The substrates are -50/+1 *c-mos* RNA and -101/+1 L1 RNA. Each RNA was incubated with 30 µg of 0-40% ammonium sulfate egg extract for 25 min at 25°C.

Wickens, 1990; McGrew and Richter, 1990). Fourthly, whereas nuclear polyadenylation appears to be constitutive, with each mRNA that has been tested behaving identically, maturation-induced polyadenylation is highly regulated. Not only does it appear upon induction of maturation only, but different mRNAs receive different lengths of poly(A), and at different times during maturation (Sheets *et al.*, submitted).

In this report, we use a fractionated egg extract to demonstrate that maturation-specific polyadenylation requires a separable poly(A) polymerase and an RNA binding activity that recognizes AAUAAA and UUUUUAU. We propose that in spite of their many differences, nuclear and maturation-induced polyadenylation are catalyzed by common components. By analyzing oocyte extracts, which are inactive, and egg extracts, which are active, we demonstrate that the RNA binding activity is probably the target that is activated by progesterone addition.

Results

Polyadenylation of maternal mRNAs, which occurs during oocyte maturation, can be reconstituted in frog egg extracts prepared by the method of Murray and Kirschner (1989) and Paris and Richter (1990). In this report we assay polyadenylation in this extract using short substrates (50–100 nts) that contain the terminal region of the 3' UTR of a maternal mRNA. The poly(A) addition site is designated as +1. Thus $-50/+1$ *c-mos* RNA contains the last 50 nucleotides of *c-mos* RNA's 3' UTR, ending at its normal poly(A) addition site.

An ammonium sulfate-fractionated egg extract displays maturation-specific polyadenylation activity

To begin fractionating the polyadenylation activity of the egg extract, we analyzed polyadenylation of SV40 RNA substrates in ammonium sulfate fractions. The activity was recovered in high yield in a 0–30% ammonium sulfate

fraction. To determine whether the recovered activity faithfully reproduced polyadenylation *in vivo*, we compared its sequence specificity with that observed *in vivo* after injecting the same RNAs into the oocyte cytoplasm and inducing maturation with progesterone (Figure 1A).

In vitro, $-141/-1$ SV40 RNA into which UUUUUAU has been inserted receives poly(A) efficiently, whereas SV40 RNAs that lack either UUUUUAU or AAUAAA do not (Figure 1A, lanes 1–3). This closely parallels the behavior of these RNAs *in vivo*, after injection (Figure 1A, lanes 4–6; Fox *et al.*, 1989).

Analysis of several other RNA substrates confirmed that the ammonium sulfate fraction accurately reconstituted *in vivo* polyadenylation. For example, *c-mos* mRNA receives poly(A) during maturation (Sheets *et al.*, submitted) and also receives poly(A) in the *in vitro* system (Figure 1B, lane 1). Its polyadenylation again requires a U-rich element (UUUUUAU) and AAUAAA (Figure 1B, lanes 1–3). In the absence of the U-rich element, polyadenylation still occurs, but less of the substrate reacts and the poly(A) tails formed are 2–3 times shorter than with wild type RNA. Ribosomal protein L1 mRNA, which does not receive poly(A) during maturation because it lacks a U-rich element (Varnum and Wormington, 1990), also fails to receive poly(A) *in vitro* (Figure 1B, lane 4). We conclude that polyadenylation during oocyte maturation is faithfully reconstituted in the 0–30% ammonium sulfate fraction.

Polyadenylation requires at least two separable factors

DEAE-Sepharose chromatography was used to fractionate the polyadenylation activity of the 0–30% ammonium sulfate material. A fraction was obtained that flowed through the column in 0.1 M KCl. Three additional fractions were obtained by elution with 0.2, 0.3 and 0.5 M KCl. A mixture of the 0.1 and 0.3 M fractions polyadenylated $-82/+2$ *c-mos* RNA much more efficiently than did either fraction on its own (Figure 2A, lanes 1–3). The reconstituted

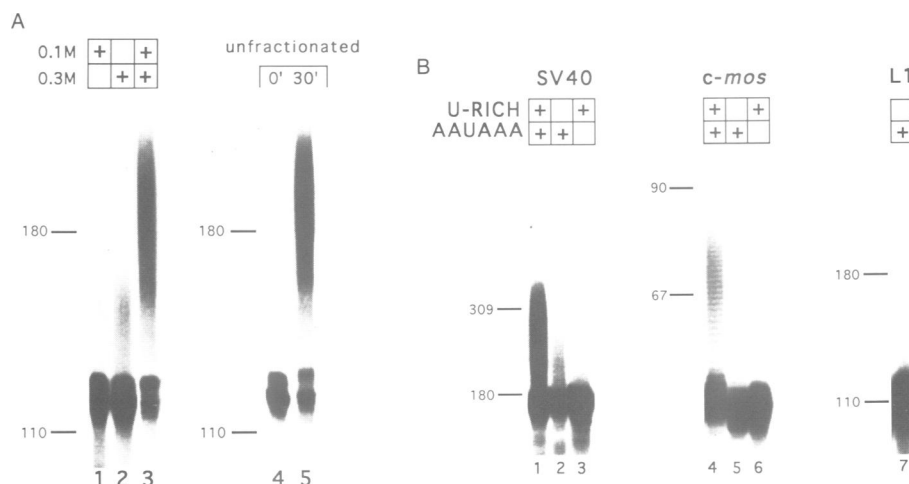


Fig. 2. Polyadenylation requires two separable factors. **A.** $-82/+2$ *c-mos* RNA was incubated with: lane 1, 2.5 μg of 0.1 M DEAE egg fraction; lane 2, 1.8 μg of 0.3 M DEAE egg fraction; lane 3, a mixture of 2.5 μg of 0.1 M DEAE and 1.8 μg of 0.3 M DEAE egg fractions; lane 4, no protein; lane 5, 25 μg of unfractionated 0–30% ammonium sulfate egg extract. RNA was incubated for 30 min at 25°C. The DEAE fractions in this experiment were generated from the same 8 mg of 0–30% ammonium sulfate egg extract. **B.** The reconstituted reaction has the same specificity as the unfractionated extract. Lanes 1–3, $-141/-1$ SV40 RNAs incubated with the same mixture of 0.3 and 0.1 M DEAE fractions in (A); lanes 4–7, $-50/+1$ *c-mos* RNAs and $-104/+1$ L1 RNA were incubated with mixtures of 0.1 M DEAE and 0.3 M DEAE fractions for 25 min at 25°C. A different preparation of 0.3 and 0.1 M DEAE fractions were used for these experiments. Between 150–250 units (see Materials and methods) of poly(A) polymerase activity in a 0.1 M DEAE fraction and 4 μg (0.8 units) of RNA specificity activity in a 0.3 M DEAE fraction were used in lanes 4–6. In lane 7, ~2 units of RNA specificity activity was used. Units of activity are defined in Materials and methods.

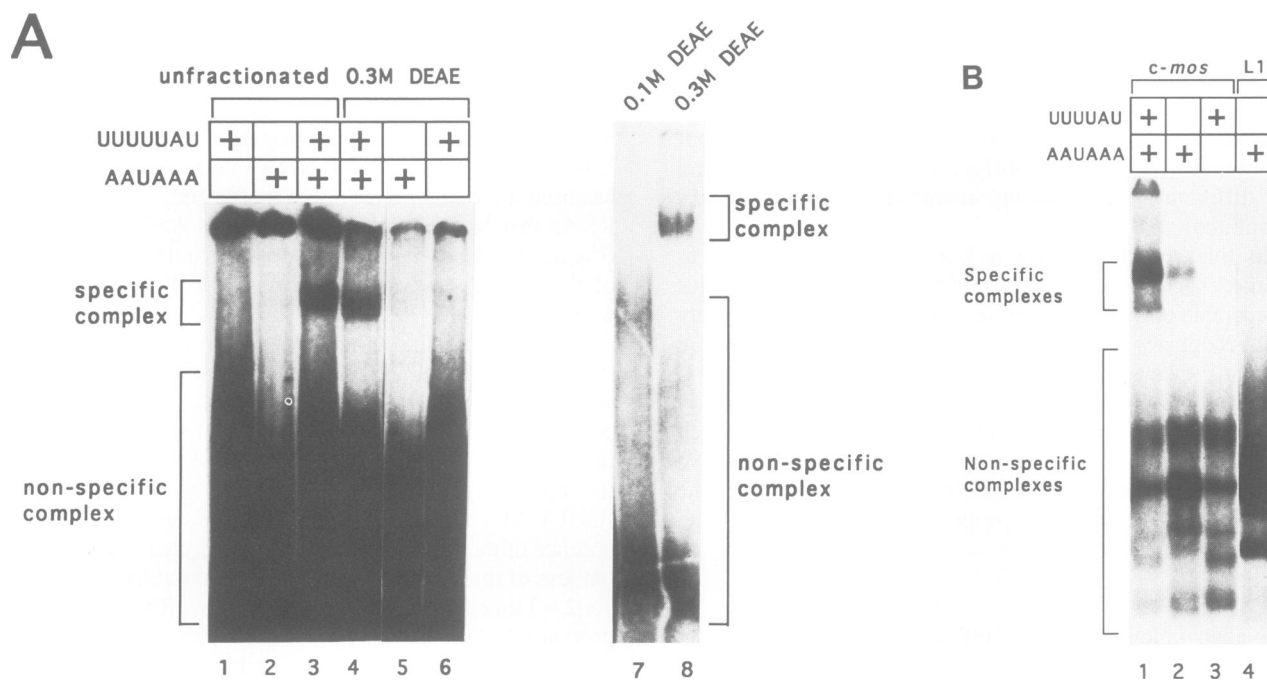


Fig. 3. The 0.3 M DEAE egg fraction contains a specific RNA binding activity. **A.** -141/-1 SV40 RNAs were incubated in the 0.3 M fraction (4 mg) or 0-30% ammonium sulfate fraction (19 mg) for 10 min at 25°C and analyzed by native PAGE. The substrate used is indicated above each lane. Sequences are presented in Figure 1. Lanes 1-3, ammonium sulfate fraction; lanes 4-6, 0.3 M DEAE egg fraction; lane 7, -141/-1 SV40 RNA containing both UUUUUUAU and AAUAAA incubated with 5 µg of the 0.1 M DEAE fraction; lane 8, -141/-1 SV40 RNA containing both UUUUUUAU and AAUAAA incubated with 4 µg of the 0.3 M DEAE fraction. **B.** The 0.3 M DEAE egg fraction (10 mg, 2.5 units) was incubated with various substrates and complexes were analyzed by native gel electrophoresis. Lane 1, -50/+1 *c-mos* RNA containing both UUUUUUAU and AAUAAA; lane 2, -50/+1 *c-mos* RNA containing AAUAAA; lane 3, -50/+1 *c-mos* RNA containing UUUUUUAU; lane 4, -101/+1 L1 RNA.

reaction was very similar in efficiency to that seen before chromatography (Figure 2A, lanes 4 and 5).

To test whether the mixture of 0.1 and 0.3 M fractions displayed the proper sequence specificity, we analyzed the same RNAs as in Figure 1. As in unfractionated material, a U-rich element and AAUAAA were required for efficient polyadenylation (Figure 2B).

From these results, we conclude that the 0.1 and 0.3 M DEAE fractions contain the activities necessary to reconstitute maturation-specific polyadenylation.

The 0.3 M KCl fraction contains a specific RNA binding activity

To determine whether a sequence-specific RNA binding activity was present in either DEAE fraction, we employed a gel retardation assay. Labeled -141/-1 SV40 RNA was added to the 0.3 M DEAE fraction. After incubation, this material was analyzed by gel electrophoresis under non-denaturing conditions. A complex with highly retarded mobility was formed both in the 0.3 M fraction and in unfractionated extract (Figure 3, lanes 3 and 4). The formation of this complex is sequence-specific, since it was detected only on substrates that contained both UUUUUUAU and AAUAAA (Figure 3). Note that we did not detect an activity analogous to mammalian CPSF, a nuclear polyadenylation factor that requires only the AAUAAA sequence for binding (Wigley *et al.*, 1990; Bienroth *et al.*, 1991; see Discussion).

The electrophoretic mobility of the specific complex was slightly faster in the 0.3 M fraction than in unfractionated extract (Figure 3A, lanes 3 and 4). No other DEAE fraction, including the 0.1 M fraction, formed specific complexes

(Figure 3A, lanes 7 and 8; data not shown). The faster migrating complexes that were formed in the 0.1 M fraction did not require AAUAAA or UUUUUUAU and so are termed non-specific (not shown).

The amount of complex formed on various wild type and mutant RNAs incubated with the 0.3 M KCl fraction mirrors the efficiencies with which they receive poly(A) (Figure 3B). Wild type *c-mos* RNA was polyadenylated efficiently in the mixture of 0.1 and 0.3 M fractions and formed the highest level of specific complexes. The *c-mos* mutant in which UUUUUUAU had been changed to CACACA decreased, but did not abolish both complex formation (Figure 3B, lanes 1 and 2) and polyadenylation activity (Figures 1B and 2B). Similarly, a *c-mos* RNA carrying a mutation in AAUAAA, which has no detectable polyadenylation activity, forms no complex (Figure 3B, lane 3). Ribosomal protein L1 RNA, which is inactive in polyadenylation (Figure 1), forms no complex (Figure 3B, lane 4). Taken together, these data strongly suggest that the RNA binding activity present in the 0.3 M DEAE fraction participates in maturation-specific polyadenylation.

The 0.1 M KCl fraction contains a poly(A) polymerase

We assayed the fractions obtained by DEAE-Sepharose chromatography for poly(A) polymerase activity (Edmonds, 1989). For this, we included Mn^{2+} rather than Mg^{2+} in the polyadenylation assay. Under these conditions, mammalian poly(A) polymerases add poly(A) to any RNA, without sequence specificity (Edmonds, 1989). Using this 'non-specific' assay, we tried to detect poly(A) polymerase activity in the egg extract and fractions thereof.

The crude egg extract contains significant polymerase

Table I. Partial purification of the poly(A) polymerase from egg extracts

Fraction	Activity (units $\times 10^{-7}$)	Protein (mg)	Purification (-fold)	Specific activity (units/mg $\times 10^{-3}$)
Extract	2.00	5400	—	4
AmSO ₄	1.80	660	6.6	27
DEAE-Sepharose	2.20	110	50	200
CM-Sepharose	0.45	4.60	240	980
Sepharose-200	0.17	1.20	340	1400
Blue Sepharose	0.08	0.10	2000	7800

Units are defined as pmol ATP incorporated into poly(A) in 10 min, determined as described in Materials and methods. The specific activity and units for crude extract were calculated by assuming that the extract contains 1 mM ATP (Wickens and Gurdon, 1983). Protein concentration was measured by Bradford (1976).

activity; 40–100% of the activity that is recovered in the 0.1 M DEAE-Sepharose fraction (Table I; data not shown). No other DEAE fraction reproducibly had significant activity (not shown). To characterize further the poly(A) polymerase activity, we purified it ~2000-fold, as indicated in Table I. The chromatographic behavior of the egg poly(A) polymerase was very similar to that of the mammalian enzyme (Wahle, 1991b) on DEAE-Sepharose, CM-Sepharose, Sepharose S-200 and Blue Sepharose. The poly(A) polymerase activity eluted as a single major peak from each column in Table I.

To examine the poly(A) polymerase activity in more detail, we performed assays of both 'non-specific' and sequence-specific polyadenylation using radiolabeled RNA substrates (Figure 4). Labeled RNAs were incubated with a poly(A) polymerase fraction [CM eluate (Table I)] either in 0.4 mM MnCl₂ (the non-specific assay conditions) or in 1 mM MgCl₂ (the sequence-specific conditions). The results confirm that the more purified material contains a poly(A) polymerase, as when Mn²⁺ is added it adds poly(A) efficiently without sequence specificity: RNAs with or without AAUAAA and UUUUUAU receive ~50 As under the assay conditions used (Figure 4, lanes 1–3). The homogeneous distribution of products formed in Mn²⁺ is a reflection of the lack of processivity of poly(A) polymerase under these conditions (Wahle, 1991b). In contrast, when Mg²⁺ is added, this same partially purified polymerase is inactive on its own (Figure 4, lane 4). However, it becomes active in Mg²⁺ and acquires specificity for AAUAAA and UUUUUAU, when mixed with the 0.3 M DEAE fraction, which contains the specific RNA binding activity (Figure 4, lanes 5–7).

Purified calf thymus poly(A) polymerase substitutes for the egg poly(A) polymerase

A poly(A) polymerase has recently been purified to homogeneity from calf thymus (Wahle, 1991b). This enzyme is required to reconstitute cleavage and polyadenylation of pre-mRNAs *in vitro* and therefore is presumed to be nuclear. Nonetheless, we tested whether pure calf poly(A) polymerase could replace the egg poly(A) polymerase fraction in assays of 'cytoplasmic' maturation-induced polyadenylation. For this, the pure calf thymus poly(A) polymerase was mixed with the 0.3 M DEAE fraction from frog eggs.

The purified calf enzyme substitutes for the egg poly(A) polymerase fraction and reconstitutes maturation-specific polyadenylation (Figure 5; lanes 4–6 versus lanes 7–9). Both UUUUUAU and AAUAAA are required for efficient polyadenylation by the pure enzyme in the presence of the

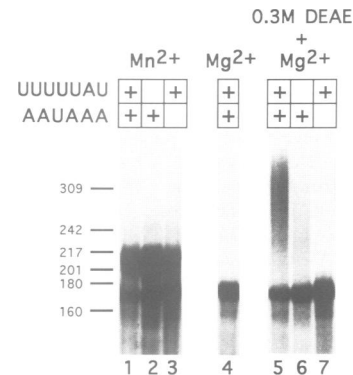


Fig. 4. The 0.1 M KCl fraction contains a poly(A) polymerase. –141/–1 SV40 RNAs were incubated with 1.5 μ g (780 units) of a CM-Sepharose fraction (see Materials and methods). The substrate was 170 nts long. The position of molecular weight markers is given on the left. Lanes 1–3, 0.4 mM MnCl₂; lane 4, 1 mM MgCl₂; lanes 5–7, 1 mM MgCl₂ and 0.3 M DEAE egg fraction. RNA was incubated 25 min at 25°C.

0.3 M DEAE fraction (Figure 5). The same two sequences are necessary for efficient polyadenylation of *c-mos* RNA by the purified polymerase (data not shown). Neither the purified calf polymerase nor the egg extract fractions are active on their own (Figure 5, lanes 1–3).

From these results, we conclude that the calf and frog poly(A) polymerases are functionally interchangeable in supporting maturation-induced polyadenylation. In addition, these results suggest that poly(A) polymerase may be the only activity in the partially purified egg polymerase fraction that is required for maturation-specific polyadenylation.

Oocyte extracts are less active

Before maturation, oocytes contain only a low level of cytoplasmic polyadenylation activity (Fox *et al.*, 1989). After progesterone treatment, polyadenylation activity appears. To determine whether this difference between oocytes before and after progesterone treatment was preserved *in vitro*, we prepared extracts of untreated oocytes using the same protocol we had used with oocytes after maturation was complete. For simplicity, oocytes after maturation are termed eggs and oocytes before maturation are simply called oocytes. Thus, whereas egg extracts are active, oocyte extracts should be inactive.

As expected, *c-mos* is polyadenylated more efficiently in the egg extract than in the oocyte extract (Figure 6, lanes 4–6; see also Figure 9), consistent with previous observations using B4 mRNA (Paris *et al.*, 1991). The *in*

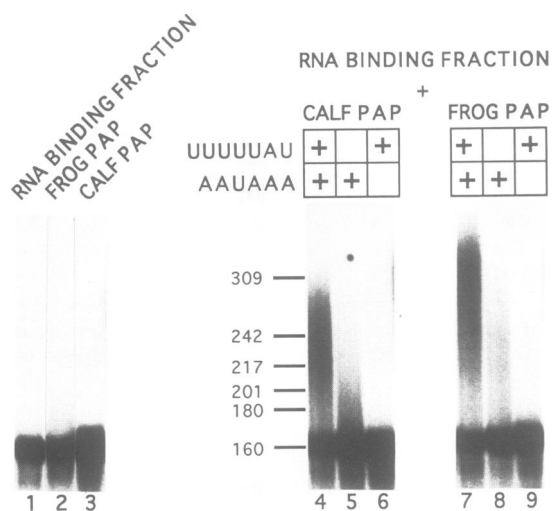


Fig. 5. Purified calf thymus poly(A) polymerase substitutes for the egg poly(A) polymerase. The frog egg RNA binding fraction used in this experiment was further fractionated on a Heparin-agarose column following elution from DEAE-Sepharose. The substrates are -141/-1 SV40 RNA. In lanes 1-3, -141/-1 SV40 RNA containing both UUUUUAU and AAUAAA was used. Lane 1, egg RNA binding fraction; lane 2, 780 units of frog egg poly(A) polymerase in a CM-Sepharose fraction; lane 3, 600 units of purified calf thymus poly(A) polymerase (Wahle, 1991b; fraction 23 from the Mono Q column); lanes 4-6, a mixture of the egg RNA binding fraction and 130 units of purified calf thymus poly(A) polymerase; lanes 7-9, a mixture of the egg RNA binding fraction and 780 units of frog egg poly(A) polymerase in the CM-Sepharose fraction. Units of poly(A) polymerase added were determined using the non-specific (Mn^{2+} containing) assays. In assays containing the same number of units of the calf and egg polymerases, the egg polymerase fraction was slightly less efficient in complementing the egg RNA binding fraction than was the calf enzyme, perhaps due to inhibitors in the impure egg polymerase fraction, or to a difference in the intrinsic specific activities of the frog versus calf enzymes. RNAs were incubated 25 min at 25°C.

in vitro data with *c-mos* are very similar to those obtained *in vivo*, by injecting the same substrate into oocytes (Figure 6, lanes 1-3). Without progesterone addition, the injected RNA receives only short poly(A) tails; after progesterone addition, the RNA receives long poly(A) (Figure 6, lanes 1-3).

The inefficient polyadenylation observed in oocyte extracts exhibits the same sequence specificity as seen in egg extracts (not shown). Thus, although the amount of activity in the oocyte extract is reduced, its specificity is the same as that seen during maturation.

The close resemblance between the *in vitro* and *in vivo* data suggest that proper regulation of polyadenylation activity is preserved in the cell-free systems. In the following experiment we analyze the mechanism by which polyadenylation activity is activated during oocyte maturation by comparing the poly(A) polymerase and RNA binding activities before and after maturation.

Oocyte extracts contain active poly(A) polymerase

Polyadenylation in egg extracts requires both a poly(A) polymerase and a specific RNA binding activity. The inactivity of oocyte extracts could be due to a deficiency in poly(A) polymerase activity, RNA binding activity or both. To determine whether oocytes lacked functional poly(A) polymerase, we performed the experiments described in Table II and Figure 7.

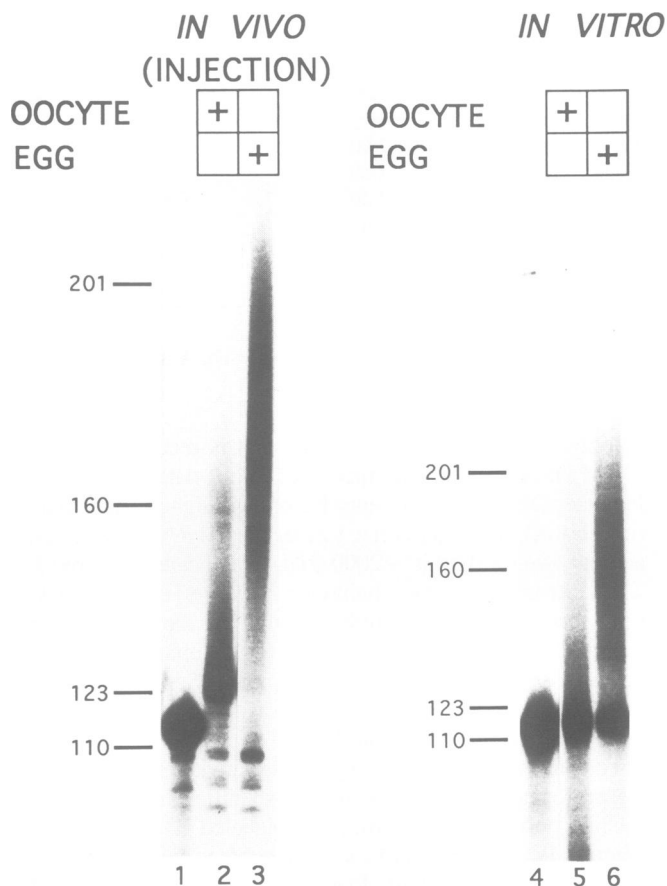


Fig. 6. Oocyte extracts contain less activity than egg extracts. -82/+2 *c-mos* RNA was either injected into oocyte cytoplasm (lanes 1-3) or incubated with a crude extracts (lanes 4-6). Lane 1, RNA that was not injected; lane 2, RNA injected into oocyte cytoplasm and incubated in the absence of progesterone until control oocytes mature; lane 3, RNA injected into oocyte cytoplasm and incubated in the presence of progesterone until maturation is complete; lane 4, RNA incubated without protein; lane 5, RNA incubated with 190 μ g of crude oocyte extract; lane 6, RNA incubated with 190 μ g of crude egg extract. For *in vitro* reactions, RNA was incubated for 25 min at 25°C.

We first compared the level of poly(A) polymerase activity in oocytes and egg using the non-specific poly(A) polymerase assay (Table II). The specific activities of poly(A) polymerase (pmol of ATP incorporated in 10 min/ μ g protein) were determined from several assays using aliquots of the same oocyte extract used in Figures 6-9. Average values are presented in Table II. The data demonstrate that egg and oocyte extracts contain very similar levels of poly(A) polymerase activity.

In principle, the lack of activity in oocyte extracts may be due to a qualitative rather than quantitative difference in its poly(A) polymerase. In particular, the oocyte poly(A) polymerase may not be able to interact productively with components present in the egg RNA binding fraction. To test this notion, a concentration range of oocyte or egg poly(A) polymerase fractions was added to an excess of the egg RNA binding fraction (Figure 7). Polyadenylation of *c-mos* RNA was then assayed at each concentration. To compare oocyte and egg fractions directly, the same range of polymerase concentrations was added, as determined by units of non-specific poly(A) polymerase activity (assayed in Mn^{2+}). Thus we compared, on a per unit basis, the

Table II. Oocyte extracts contain an active poly(A) polymerase

	Oocyte	Egg
Units/ μ l	74.0 \pm 9.0	93.0 \pm 18.0
Units/ μ g	2.1 \pm 0.2	3.4 \pm 0.7

Non-specific poly(A) polymerase activity measured in either oocyte or egg extracts (see Materials and methods). Protein concentration was measured by Bradford (1976).

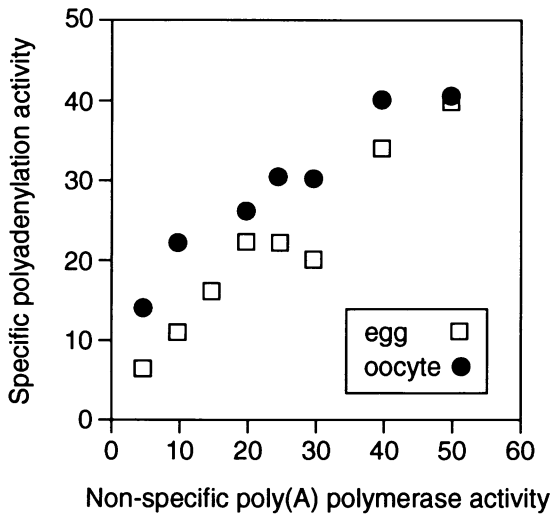


Fig. 7. Oocyte extracts contain an active poly(A) polymerase. Non-specific poly(A) polymerase activity (x axis) as measured in units of ATP incorporated into poly(A) in 10 min versus specific polyadenylation activity (y axis) as measured by the percent of $-50/+1$ *c-mos* RNA converted to polyadenylated product in 15 min at 25°C (see Materials and methods for details). Three units of egg RNA specificity activity in a 0.3 M DEAE fraction was used in this experiment. The specific polyadenylation was calculated using a β emission detector. The egg extract fractions used were the 0.1 and 0.3 M DEAE-Sepharose fractions.

ability of the two polymerases to interact with the RNA binding fraction from eggs. To quantify the level of specific polyadenylation activity, the fraction of the substrate that received poly(A) in the mixture of the polymerase and RNA binding fractions was then determined.

The data demonstrate no significant difference in the two polymerase fractions (Figure 7). The specific polyadenylation activity observed (plotted on the y axis in Figure 7) required both the U-rich element and AAUAAA sequence of the *c-mos* substrate (not shown). We conclude that the oocyte poly(A) polymerase is not defective in its ability to interact with other factors to promote maturation-specific polyadenylation. Since it also is present at levels comparable to those in the egg extract, we infer that it is probably not the key component that differs in oocyte and egg extract.

Differences in the specific RNA binding activities of oocyte and egg extracts

To determine whether the RNA binding activity differed in oocyte and egg extracts, we assayed RNA binding activity present in the ammonium sulfate-precipitated oocyte extracts (Figure 8). We used the same gel retardation assay described in Figure 3, with labeled *c-mos* RNA as a substrate.

The oocyte extract contains a specific RNA binding activity (Figure 8, lanes 1–3). The formation of the specific

complex requires the same sequences, AAUAAA and UUUUAAU, as are required in egg extracts (Figure 8, lanes 1–3 versus lanes 4–6).

Although the RNA binding activities in oocyte and egg extracts are indistinguishable in specificity, the oocyte activity differs significantly in two respects. First, the electrophoretic mobility of complexes formed in oocyte extracts is faster than that of complexes formed in egg extracts. This suggests that either the composition of the complexes differ, or that factors in the complex are differentially modified. Secondly, less complex was detected in oocyte extracts than in egg extracts. The amount of specific complex is difficult to quantify precisely, but appears to be depressed by ~ 10 -fold in oocyte extracts, as determined using a β -array detector. This could reflect a difference in either the amount or affinity of an RNA binding factor. Nevertheless, the data argue that the RNA binding activity differs, qualitatively and quantitatively, between oocyte and egg extracts.

A factor present in the 0.3 M DEAE fraction activates oocyte extracts

The previous results suggest that the lack of polyadenylation activity in oocyte extract is due to a deficiency in the RNA binding activity, not the poly(A) polymerase. This hypothesis predicts that addition of the RNA binding fraction to oocyte extracts should stimulate polyadenylation, while addition of excess poly(A) polymerase should not. To test these predictions, another set of experiments was performed (Figure 9).

Addition of the egg RNA binding fraction (0.3 M DEAE) to unfractionated oocyte extracts stimulates polyadenylation dramatically (Figure 9, lane 4 versus lane 2). The level of activity attained was similar to that seen in egg extract (lane 1) or in a reaction in which the same amount of RNA binding fraction is added to egg poly(A) polymerase (lane 5). The level of non-specific poly(A) polymerase activity present in lane 5 is the same as that present in the oocyte extract (lanes 2 and 4); thus the RNA binding activity is equally capable of stimulating polyadenylation in the oocyte extract and with fractionated polymerase. The amount of the RNA binding fraction that is added determines the level of polyadenylation attained (not shown). These data strongly suggest that the RNA binding fraction is limiting in the oocyte extract.

As predicted, addition of the egg poly(A) polymerase fraction to the oocyte extract had little effect on polyadenylation (Figure 9, lanes 2 and 3). The amount of polymerase added in lane 3 was a substantial (8-fold) excess over that present in the oocyte extract, emphasizing the fact that the polymerase is not rate-limiting. In summary, these data are consistent with those in Figure 7 and strongly suggest that a lack of poly(A) polymerase activity is not responsible for the deficit in specific polyadenylation activity.

In principle, the egg 0.3 M DEAE fraction could stimulate the oocyte extract by supplying an activity that activates previously inert oocyte factors, rather than by supplying an activity that the oocyte lacks. For example, addition of MPF or p34^{cdc2} to oocyte extracts induces maturation and stimulates polyadenylation (Paris *et al.*, 1991). However, whereas activation of oocyte extracts by p34^{cdc2} displays a substantial lag (Paris *et al.*, 1991), activation by the fractionated RNA binding activity does not. Indeed the

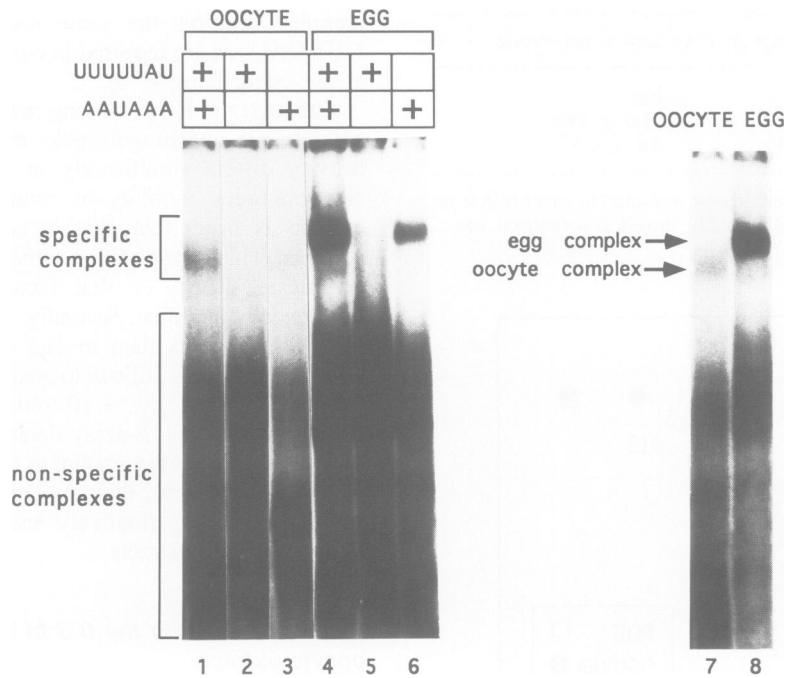


Fig. 8. Oocyte extracts contain an RNA binding activity. $-82/+2$ *c-mos* RNAs were incubated with 50 μ g of 0–40% ammonium sulfate oocyte extract or 30 μ g of 0–40% ammonium sulfate egg extract for 10 min at 25°C and analyzed on a native gel. Lanes 1, 4, 7 and 8, wild type $-82/+1$ *c-mos* RNA with either oocyte extracts (lanes 1 and 7) or egg extracts (lanes 4 and 8); lanes 2 and 5, $-82/+2$ *c-mos* RNA containing a point mutation in AAUAAA with either oocyte extracts (lane 2) or egg extracts (lane 5); lanes 3 and 6, $-82/+1$ *c-mos* RNA containing a deletion of UUUUUAU with either oocyte extracts (lane 3) or egg extracts (lane 6).

appearance kinetics of polyadenylation activity are the same in egg extracts and in supplemented oocyte extracts (not shown). Moreover, a much more highly purified RNA binding activity also activates oocyte extracts effectively (not shown).

These data, as well as those in Figures 7 and 8, strongly suggest that an activity present in the RNA binding fraction and perhaps the RNA binding activity itself, are limiting in oocyte extracts. This activity is probably the target that is activated by progesterone treatment of oocytes, thereby causing polyadenylation activity to appear during maturation.

Discussion

We have used a cell-free system derived from frog eggs to examine the mechanism by which poly(A) is added to specific maternal mRNAs during oocyte maturation. We draw three main conclusions. First, polyadenylation of maternal mRNAs *in vitro* requires at least two separable components: a poly(A) polymerase and an RNA binding activity. The reconstituted *in vitro* reaction requires both AAUAAA and a CPE, and so faithfully recapitulates the polyadenylation reactions observed *in vivo*. Secondly, the egg poly(A) polymerase is closely related to mammalian poly(A) polymerases, which presumably are nuclear. Thirdly, polyadenylation in extracts of oocytes that have not matured is inefficient, probably not because these extracts lack poly(A) polymerase, but because they lack a factor present in the RNA binding fraction of egg extracts. This limiting component may be the RNA binding activity itself.

Separable factors

The poly(A) polymerase(s) in the frog extract is closely related to the poly(A) polymerase previously purified from

egg extract	+				
oocyte extract		+	+	+	
egg 0.1 M DEAE			+		+
egg 0.3 M DEAE				+	+

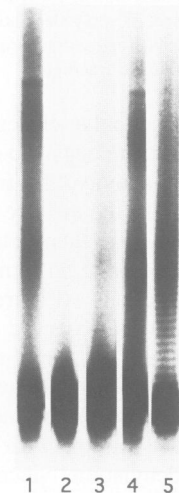


Fig. 9. A factor present in the 0.3 M DEAE fraction activates oocyte extracts. The substrate is $-50/+1$ *c-mos* RNA. Reactions were incubated 20 min at 25°C. Lane 1, 84 μ g of crude egg extract; lane 2, 83 μ g of crude oocyte extract; lane 3, 83 μ g of crude oocyte extract supplemented with 1200 units of egg poly(A) polymerase in the egg 0.1 M DEAE fraction; lane 4, 83 μ g of crude oocyte extract supplemented with 3 units of egg RNA specificity activity in the egg 0.3 M DEAE fraction (1 unit converts 50% of $-50/+1$ *c-mos* RNA to polyadenylated product in the presence of 150 units of poly(A) polymerase in the egg 0.1 M DEAE fraction); lane 5, 150 units of egg poly(A) polymerase in the 0.1 M DEAE fraction with 2 units of egg 0.3 M DEAE fraction.

calf thymus (Wahle, 1991b). Through a 2000-fold purification, involving four different columns, the chromatographic behavior of the frog enzyme is very similar to that of the bovine enzyme. More importantly, a purified calf thymus poly(A) polymerase becomes specific for CPE-containing RNAs when mixed with the RNA binding fraction derived from frog eggs. This strongly suggests that at least that region of the enzyme that interacts with the egg's 'specificity factors' are conserved during evolution.

The ability of the purified mammalian enzyme to catalyze AAUAAA-dependent polyadenylation in the presence of CPSF implies a nuclear location. However, since the same enzyme acquires 'cytoplasmic' specificity when mixed with the egg RNA binding fraction, it may also be cytoplasmic. In somatic cells, similar or identical poly(A) polymerase activities have been reported in cytoplasmic and nuclear fractions (Ryner *et al.*, 1989), consistent with reports of cytoplasmic polyadenylation (reviewed in Edmonds, 1989). The sequence specificity of these cytoplasmic polyadenylation reactions in somatic cells has not been explored. Regardless, our results establish that the same purified enzyme can catalyze polyadenylation with either nuclear or cytoplasmic specificities, depending on the factors with which it interacts. In sea urchin embryos, poly(A) polymerase activity appears to shift from cytoplasmic to nuclear fractions during early embryogenesis (Egrie and Wilt, 1979).

The RNA binding activity present in the 0.3 M fraction is specific for RNAs containing both AAUAAA and a CPE. In nuclear extracts, a multi-protein complex, called CPSF, interacts with AAUAAA (Bienroth *et al.*, 1991; Keller *et al.*, 1991). The role of CPSF in maturation-specific polyadenylation has not been determined, since AAUAAA-binding activity has not yet separated from CPE-binding activity during further purification (C.A.Fox, unpublished data).

It has been proposed that 58 and 82 kDa proteins bind to CPEs and are required for polyadenylation during maturation (McGrew and Richter, 1990; Paris *et al.*, 1991), since these proteins can be cross-linked by UV light to CPE-containing RNAs. However, neither protein has been shown to function in polyadenylation nor to bind to a specific RNA sequence. The observation that a CPE is necessary for cross-linking does not demonstrate binding to that sequence. For example, in nuclear polyadenylation, a 64 kDa protein that is efficiently cross-linked to a variety of AAUAAA-containing substrates does not actually contact that sequence (Keller *et al.*, 1991; Takagaki *et al.*, 1992). However, it is possible that the cross-linked proteins identified previously are present in the RNA binding fraction and that they are critical for the activity of that fraction.

RNAs containing AAUAAA, but lacking a U-rich element, are not polyadenylated efficiently in either oocyte or egg extracts, but are polyadenylated after injection into the oocyte nucleus (Fox *et al.*, 1989). Since the extracts contain both nuclear and cytoplasmic contents, this result implies that nuclear factors are inactive *in vitro*. Nuclear polyadenylation activity (i.e. activity requiring only AAUAAA) also is deficient *in vivo* in eggs injected with RNA, demonstrating that lack of activity *in vitro* is not an artifact (C.A.Fox, unpublished). Rather, the data suggest that 'nuclear' factors may be inactivated during maturation, perhaps at nuclear breakdown due to interactions with

specific cytoplasmic factors or to simple dilution into the large cytoplasmic volume. The inactivation may be mimicked by homogenization of oocytes in preparing extracts.

Cytoplasmic versus nuclear polyadenylation: are different maternal mRNAs polyadenylated by the same factors?

Different RNAs containing CPEs can be cross-linked by UV light to different proteins (Paris *et al.*, 1991). This could mean that different factors are required for polyadenylation of different mRNAs, as suggested by Paris *et al.* (1991). However, failure to detect a protein by UV cross-linking can arise through a variety of circumstances, including not only absence of the protein, but also unfavorable positioning of RNA and protein residues, RNA conformation and the presence of other proteins. These alternative interpretations are particularly pertinent when two different RNA sequences are compared. As a result, the inference that different mRNAs may be polyadenylated by different factors requires further investigation.

An alternative view is that polyadenylation of different mRNAs during maturation is catalyzed by a single set of factors. In one form of this hypothesis, the factors that catalyze polyadenylation during maturation are the same as those that catalyze cleavage and polyadenylation in the nucleus. How could the same factors add poly(A) to all mRNAs in the nucleus, yet act only on CPE-containing RNAs in the cytoplasm? Perhaps a single RNA binding activity interacts with AAUAAA, but does so more effectively in the presence of a CPE. If this activity were present in the nucleus in excess, but not the cytoplasm, then the CPE would be dispensable in the nucleus, but not in the cytoplasm. This same model can explain how different mRNAs receive poly(A) at different times during maturation or receive different numbers of As, as has been observed *in vivo* (Sheets *et al.*, submitted). Ample precedent exists for the critical role of protein concentration gradients and differences in target-site affinities during early development (Driever and Nusslein-Volhard, 1989; Struhl *et al.*, 1989).

Differences in the polyadenylation of different mRNAs could also arise by sequence-specific repressors of a single polyadenylation apparatus. Sequences have been identified in the 3'UTRs of *Caenorhabditis elegans fem-3* and *tra-2* mRNAs (Ahringer and Kimble, 1991; Goodwin, E., Evans, T. and Kimble, J., in preparation) and *Drosophila hunchback* mRNA (Wharton and Struhl, 1991) that are required for repression of these genes (reviewed in Evans *et al.*, 1992; Wharton, 1992). It is possible, though untested, that factors bound to these sequences repress polyadenylation. If so, their selective removal could confer differences between mRNAs. This would make the regulation of polyadenylation during maturation analogous to transcriptional regulation in bacteria, in that a single polymerase would be regulated by sequence-specific repressors whose activity was controlled.

The formation of mRNA 3' termini in the nucleus requires sequences downstream of AAUAAA. These downstream elements are flexible in sequence and in their distance relative to AAUAAA. They are only modestly conserved and are often U-rich (Manley, 1988). In all these respects, they resemble CPEs. It is possible that CPEs and downstream elements have the same function, perhaps enhancing the formation of a complex between CPSF and AAUAAA

(Gilmartin and Nevins, 1989). Similarly, elements upstream of AAUAAA that are required for cleavage and polyadenylation in the nucleus (Valsamakis *et al.*, 1991) may be identical in function to the upstream, U-rich elements required for cytoplasmic polyadenylation during oocyte maturation.

Our working model—that cytoplasmic and nuclear polyadenylation share common factors—is consistent with the observation that a single purified poly(A) polymerase can support both types of reactions, depending on the factors with which it interacts. This model is simple and testable. Conclusive tests will require purification of the RNA binding and poly(A) polymerase activities from egg extracts and a direct comparison to their nuclear counterparts.

Activation of polyadenylation during maturation

In vivo, polyadenylation activity is missing before maturation, but appears after maturation begins. This is preserved in cell-free systems: extracts prepared from oocytes are much less active than extracts prepared from eggs (McGrew and Richter, 1990; Paris and Richter, 1990). Using these two extracts, we have tried to determine whether poly(A) polymerase and RNA binding factor, or both, are responsible for the difference in polyadenylation activity.

The polymerase is not likely to be the primary target through which polyadenylation is activated for three reasons. First, recovery of poly(A) polymerase activity is similar in oocyte and egg extracts. Similarly, in sea urchins, the level of poly(A) polymerase activity does not change significantly during embryogenesis even though the total poly(A) content of the embryo doubles (Egrie and Wilt, 1979). Secondly, the frog oocyte and egg polymerase fractions are equally capable of interacting with the components present in the egg RNA binding fraction. Thirdly, addition of a substantial excess of egg poly(A) polymerase to an oocyte extract does not stimulate its polyadenylation activity significantly, suggesting that polymerase activity is not limiting. In spite of these results, the modest difference in polymerase levels before and after maturation could be important, particularly since cytoplasmic and nuclear enzymes cannot be distinguished in our extracts.

In contrast to the polymerase, the RNA binding fraction—and perhaps the RNA binding activity itself—differs conspicuously in oocyte and egg extracts. In gel retardation assays, oocyte extracts form specific RNA binding complexes less efficiently and form complexes with a different electrophoretic mobility than those formed in egg extracts. The difference in mobility could be due to phosphorylation, consistent with the phosphorylation during maturation of a protein that can be cross-linked to a specific, CPE-containing RNA (Paris *et al.*, 1991). Most importantly, addition of the egg 0.3 M DEAE fraction (containing the RNA binding activity) to oocyte extracts stimulates polyadenylation, demonstrating that factors present in that fraction are very probably responsible for the difference between oocyte and egg extracts.

The increase in the level of RNA binding activity during maturation is probably due to the activation of quiescent protein factors, rather than to their synthesis *de novo*. Addition of p34^{cdc2} kinase to inactive oocyte extracts turns on their polyadenylation activity (Paris *et al.*, 1991). Since translation is quite inefficient in these extracts, this result suggests that the latent activity of pre-existing factors is

stimulated, rather than new factors being synthesized *de novo*. Treatment of oocytes with cycloheximide has been reported to prevent the appearance of polyadenylation activity (McGrew and Richter, 1990), implying that at least one mRNA must be translated after progesterone treatment in order to activate the polyadenylation apparatus. However, this need not be a factor directly involved in catalyzing the reaction.

One attractive interpretation of our results is that eggs contain an RNA binding activity that oocytes lack and that this difference reflects the mechanism by which polyadenylation is activated during maturation. Continued purification of the RNA binding activity and poly(A) polymerase, and of the activity that turns on oocyte extracts, should allow us to test this hypothesis directly and to identify the factors that catalyze polyadenylation during oocyte maturation.

Materials and methods

RNA substrates

The poly(A) site is designated as +1. Thus -141/-1 SV40 RNA contains 141 nts upstream of its poly(A) site and ends one nucleotide before its poly(A) site.

-141/-1 SV40 RNA and -141/-1 SV40 RNAs containing UUUUUUAU insertions or point mutations in AAUAAA. Plasmids containing the sequences for these RNAs have been previously described by Fox *et al.* (1989).

-50/+1 c-mos RNAs and -50/+1 c-mos RNAs containing a UUUUUUAU substitution or a point mutation in AAUAAA. Oligonucleotides encoding the T7 promoter linked to the 3'-most 50 nts of *c-mos* mRNA (Sagata *et al.*, 1988) were annealed to an oligonucleotide complementary to the T7 promoter region. The RNA was prepared by transcription with T7 RNA polymerase. One oligonucleotide generated a -50/+1 *c-mos* RNA with the wild type sequence. A second oligonucleotide generated an RNA with a U to G substitution in AAUAAA. A third oligonucleotide generated an RNA with a UUUUUUAU to CACACA substitution (see Figure 1B for more complete sequence information).

-82/+2 c-mos RNA and -82/+2 c-mos RNAs containing a deletion of UUUUUUAU or a point mutation in AAUAAA. -82/+2 wild type *c-mos* RNA and -82/+2 *c-mos* RNA containing a U to G substitution in AAUAAA were generated as described by Sheets *et al.* (submitted). pSP-82/+2 *c-mos* containing a deletion of UUUUUUAU was generated by site directed mutagenesis (Kunkel, 1985) of *c-mos* cDNA subcloned into M13. The RNA was generated as described for wild type -82/+2 *c-mos* RNA by Sheets *et al.* (submitted).

-101/+1 ribosomal protein L1 RNA. Two complementary oligonucleotides that contained the 3' terminal 101 nucleotides of L1 cDNA and *EcoRI* linkers were annealed, digested with *EcoRI* and cloned into the *EcoRI* site of pGem3z. The plasmid was digested with *AflIII* and transcribed with T7 RNA polymerase to yield -101/+1 L1 RNA.

Transcription in vitro

RNAs were prepared as previously described by Fox *et al.* (1989), using T7 or SP6 RNA polymerases. RNAs possessed specific activities of 4×10^3 – 9×10^3 c.p.m./fmol.

Oocyte microinjections

Injections and incubations of oocytes were performed as described previously (Fox *et al.*, 1989).

Preparation of crude extracts and ammonium sulfate fractions

Oocytes. Ovaries were removed, cut into small chunks and shaken gently overnight at 20°C in a liberal amount of a solution containing 200 mg/l collagenase A (Boehringer Mannheim), 125 mM sodium phosphate pH 7.2 and 0.5× MR buffer (Gerhart *et al.*, 1984).

Eggs. Frogs were injected with 50 units of PMS (CalBiochem) 3–10 days before being injected with 500 units of HCG (Sigma). The frogs laid eggs

in $1 \times$ MR buffer overnight at 20°C. Eggs were dejellied in cysteine as described (Murray and Kirschner, 1989).

Preparation of extract. The extract was prepared from whole cell extracts of oocytes and eggs essentially as described by Murray and Kirschner (1989; see below).

Ammonium sulfate fractionation. The crude extract was clarified by centrifugation at 10 000 *g* for 5–10 min. The clarified extract was diluted with 1/3 vol of a buffer DE-A [50 mM Tris, 15% (v/v) glycerol, 150 μ M EDTA plus 100 mM KCl at pH 8.5 and 5°C]. The diluted extract was brought to 40% saturation with an ice-cold solution of 100% saturated ammonium sulfate. The mixture was incubated on ice for 30 min and then centrifuged at 10 000 *g* for 10–20 min, depending on the consistency of the pellet. The pellet was resuspended in 1–2 vol of the DE-A buffer and then was desalted by gel filtration using Biogel P-6 (Bio-Rad). 1 ml of crude extract at 75 mg/ml generally yielded 0.7 ml of 0–40% ammonium sulfate fraction at 10 mg/ml.

Fractionation of ammonium sulfate egg extract

Approximately 1 mg of 0–40% ammonium sulfate extract was applied per ml of DEAE-Sepharose resin, with columns varying from 10–60 ml of resin. Columns were equilibrated in DE-A buffer. Four fractions were collected: a flow-through fraction at 0.1 M KCl and three fractions eluted with DE-A buffer containing a final concentration of 0.2, 0.3 or 0.5 M KCl. After concentration, the 0.1 and 0.3 M fractions contained ~0.7 and 0.3 mg, respectively. When mixed together they reconstituted maturation-specific polyadenylation (see Figure 2A). For certain experiments, fractions were concentrated ~10-fold using Centriprep-30 devices (Amicon), following the manufacturer's instructions.

Polyadenylation assays

To assay sequence-specific polyadenylation, a typical assay contained extract or fractions thereof in a total volume of 6 μ l, combined with 2 units RNasin (Promega), 2.5 mM DTT, 0.6 μ g/ μ l yeast RNA, 1–2 fmol RNA and $1 \times$ energy mix [1 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA (pH 7.7) and 7.5 mM creatine phosphate]. The reactions were carried out in a total volume of 9–10 μ l. Extract or fractions were in DE-A buffer. Assays mixtures were assembled on ice and then transferred to 25°C.

Non-sequence-specific polyadenylation activity, i.e. poly(A) polymerase activity in the absence of any other factors, was assayed in the presence of Mn²⁺, as described by Wahle (1991b).

Analysis of RNA

RNA was extracted and analyzed by electrophoresis through polyacrylamide gels containing 7 M urea, followed by autoradiography of the dried gels. Positions of molecular weight markers (*Msp*I pBR322) were determined either from the same or comparable gels.

Analysis of RNA binding complexes

Reaction mixtures contained the same components as in specific polyadenylation assays, except that no energy mix was added. ATP was not necessary for complex formation. Reactions were incubated at 25°C for 10 min. 1.2 μ g heparin was added and the mixture incubated on ice 5 min. RNA–protein complexes were analyzed by electrophoresis at 180 V for 2 h through 4% native polyacrylamide gels (12 cm \times 15 cm, 4% acrylamide:0.05% bis-acrylamide) at 4°C, followed by autoradiography of the dried gels. Detection of oocyte complexes appeared to be particularly sensitive to the conditions used for electrophoresis.

Purification of the poly(A) polymerase

Extract. The extract was prepared from ~110 frog eggs, which yielded 167 ml of clarified crude extract, as described by Murray and Kirschner (1989), except that protease inhibitors were eliminated from the wash prior to the crushing spin and SW28 tubes (Beckman) were used for the crushing and clarifying spins. 56 ml of DE-A buffer was added to the clarified extract.

Ammonium sulfate precipitation. The diluted crude extract was brought to 40% saturation with ammonium sulfate, incubated on ice 30 min and centrifuged in an SS34 rotor at 10 000 *g* for 30 min. The pellet was resuspended in 60 ml of DE-A buffer and clarified by centrifugation for 10 min at 10 000 *g* at 4°C.

DEAE-Sepharose chromatography. The resuspended ammonium sulfate pellet was desalted using gel filtration (Biogel P-6) into DE-A buffer. 109 ml of desalted ammonium sulfate extract was loaded onto a 500 ml (5 cm \times 25 cm) DEAE-Sepharose column equilibrated in buffer DE-A buffer. The column was run at 1.5 ml/min. The 0.1 M KCl fraction (flow-through),

which contained poly(A) polymerase activity, was reduced to 50 ml using an Ultrafiltration Cell and YM-10 filter (Amicon). The concentration step improved the recovery of poly(A) polymerase activity from 77–100%. This was ~2–2.5 times the typical recovery obtained from smaller columns.

CM-Sepharose chromatography. The 0.1 M DEAE fraction, containing poly(A) polymerase activity, was exchanged into buffer CM-A buffer (50 mM MOPS, 15% v/v glycerol and 150 μ M EDTA) containing 20 mM KCl by gel filtration (Biogel P-6). It was then loaded at 2 ml/min onto an 18 ml CM-Sepharose column (1.6 cm \times 8 cm). The column was developed with a gradient of 20–500 mM KCl in 12 column volumes. Active fractions eluted between 200 and 280 mM KCl and were pooled, concentrated to 6 ml by ultrafiltration and brought to 80% saturation with ammonium sulfate. The slurry was stored at 4°C overnight, centrifuged at 10 000 *g* for 10 min in an SS34 rotor and the pellet resuspended in DE-A buffer.

Sepharose 200 chromatography. The CM-Sepharose fraction in 1.5 ml was loaded onto a Sepharose 200 column (1.6 cm \times 38 cm) equilibrated in DE-A buffer containing 20 mM KCl and run at 0.3 ml/min. The center of the peak of activity eluted corresponding to a molecular weight of ~50 kDa. The active fractions were pooled, concentrated to 4 ml and loaded onto a 4 ml (1 cm \times 5 cm) Blue Sepharose column at a flow rate of 0.13 ml/min. The column was developed with a gradient from 20–500 mM KCl. The active fractions eluted between 290 and 300 mM KCl. These fractions were pooled and concentrated in Centricon-30 devices (Amicon). BSA and NP40 were added to 1 mg/ml and 0.05%, respectively, in fractions that were to be assayed. This material was used to calculate units of activity.

Comparison of oocyte and egg activities

12 ml of egg extract were obtained from the eggs of 10 frogs. 7 ml of oocyte extract were obtained from the oocytes of four frogs. These batches of extracts were used for the experiments in Figures 6–9. To assay non-specific poly(A) polymerase activity in crude extracts, 100 μ l aliquots of crude extracts were exchanged into DE-A buffer using gel filtration with Biogel P-6. This allowed us to assay the extracts in the absence of endogenous ATP. Three types of assays were done for each aliquot, each in duplicate or triplicate. The first assay contained 0.3 μ g/ μ l poly(A) substrate, 0.5 mM ATP and 0.7 mM MnCl₂. This assays the non-specific poly(A) polymerase activity that is stimulated by Mn²⁺. The second assay was the same as the first, but lacked poly(A). The third assay contained 1.2 mM EDTA instead of a divalent cation. Since poly(A) polymerases require a divalent metal, this assay establishes a background level of ATP incorporation due to non-polymerase activities. Assays using the 0.1 M DEAE fractions of oocyte and egg extracts were linear over a 10-fold range of protein concentrations.

To calculate non-specific poly(A) polymerase activity, the pmol of ATP incorporated in the EDTA-containing reactions were subtracted both from the pmol ATP incorporated in the reactions with and without poly(A). The 'non-poly(A)' value was then subtracted from the 'plus poly(A)' value to obtain the level of poly(A)-dependent, Mn²⁺-dependent ATP incorporation, as reported in Table II. Several aliquots were prepared and comparisons were made between aliquots prepared on the same day. Independent preparations of oocyte and egg extracts yielded the same 2-fold difference in oocyte and egg activities reported in Table II, although the absolute values of specific activities (pmol ATP incorporated per mg) varied slightly (~3-fold).

To compare the CPE and AAUAAA dependent activities of the oocyte and egg polymerases (Figure 7), we prepared poly(A) polymerase fractions from both egg and oocyte extracts. 6 ml of each extract were brought to 40% saturation with ammonium sulfate as described above and the pellets were desalted using gel filtration. The desalted ammonium sulfate extracts were in a volume of 4 ml at 10 mg/ml (egg extract) and of 8 ml at 15 mg/ml (oocyte extract). 1 ml of each ammonium sulfate fraction was chromatographed on a 12 ml DEAE-Sepharose column, as described above. The two columns used were poured and equilibrated the same day and run as identically as possible. The non-specific poly(A) polymerase activity was present only in the 0.1 M fraction (flow-through) of both extracts. We recovered ~40% of the total polymerase activity present in the crude egg extract (typical for columns of this size). The egg and oocyte poly(A) polymerases were compared in non-specific assays on several days. The difference between poly(A) polymerase activity recovered per mg of crude egg versus crude oocyte extract was consistently 2-fold (2.1 \pm 0.2), agreeing well with the specific activities measured in crude extracts (Table II).

To quantify the level of AAUAAA- and CPE-specific polyadenylation activity, we added excess RNA binding (0.3 M) fraction derived from the egg extract to varying amounts of the polymerase (0.1 M) fractions from either the egg or oocyte extract. Standard incubation conditions were used. After incubation, RNAs were analyzed by gel electrophoresis. The fraction

of RNA that had received poly(A) was determined using a β detector (Betagen). To determine relative levels of specific polyadenylation, the total amount of RNA present was divided into the amount of RNA that had received three or more adenosines. These values are given in the y axis of Figure 7.

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