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Substrate-specific regulation of RNA deadenylation in *Xenopus* embryo and activated egg extracts

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ABSTRACT

The poly(A) tail of mRNAs plays an important role in translational control. In *Xenopus laevis* matured oocytes, maternal mRNAs that contain a cytoplasmic polyadenylation element (CPE) are polyadenylated, whereas CPE deficient mRNAs are deadenylated by a default process. Eg mRNAs are maternal transcripts that are poly(A)$^+$ in matured oocytes and rapidly deadenylated after fertilization. This post-fertilization deadenylation of Eg mRNAs requires specific sequence information. Such a deadenylation element has been identified previously in the 3′UTR of Eg2 mRNA. In this study, we show that cell-free extracts made from embryos or activated eggs contain two kinetically distinct deadenylation activities, with different substrate specificities. One, responsible for the slow deadenylation of RNAs that are devoid of a functional CPE, has the characteristics of a default PAN activity. The other effectuates the rapid deadenylation of RNAs containing a deadenylation element. The in vitro system described here will allow the characterization of factors controlling the deadenylation of Eg mRNAs in embryos.

Keywords: cell-free extracts; maternal mRNA; poly(A); poly(A) nuclease

INTRODUCTION

In *Xenopus* as well as in mice, the cytoplasmic polyadenylation of several maternal mRNAs during oocyte maturation has been shown to require both the nuclear polyadenylation signal (AAUAAA) and an additional *cis*-element located in the 3′ untranslated region (3′UTR) (Fox et al., 1989; McGrew et al., 1989; Bachvarova, 1992; Huarte et al., 1992). This element, named a cytoplasmic polyadenylation element (CPE), was shown to bind specific factors (CPE-binding proteins) which, at least in one case, are phosphorylated during oocyte maturation (Paris et al., 1991). Concomitantly, maternal mRNAs that do not contain a CPE are deadenylated by a process known as a default deadenylation (Fox & Wickens, 1990; Varnum & Wormington, 1990).

In fertilized eggs, similar changes in the polyadenylation status of the inherited maternal mRNAs are observed (Paris et al., 1988; Paris & Philippe, 1990). However, the substrate specificities of poly(A) nuclease (PAN) and poly(A) polymerase (PAP) activities must change after fertilization. Maternal mRNAs that are polyadenylated after fertilization have been identified (Paris & Philippe, 1990). In these mRNAs, the CPE is only functionally active after fertilization (Simon et al., 1992; Simon & Richter, 1994).

In comparison, maternal Eg mRNAs are maintained polyadenylated in mature oocytes and rapidly deadenylated after fertilization (Paris & Philippe, 1990). This implies that during oocyte maturation, Eg mRNAs were protected by a CPE against the default deadenylation. This idea is substantiated by previous experiments performed to characterize the *cis*-acting elements responsible for the deadenylation of Eg2 mRNA. Such an element, of ca. 20 nt, was found in the 3′UTR of the Eg2 mRNA. Chimeric transcripts containing the wild-type 3′UTR of Eg2 mRNA are rapidly deadenylated when injected into two-cell embryos (Legagneux et al., 1992). Deleting this element not only abolishes the deadenylation of Eg2 chimeric transcripts in embryos, but promotes or allows the polyadenylation of these transcripts (Bouvet et al., 1994). Two different mechanisms could account for this observation as well as those made by Stebbins-Boaz and Richter (1994) for the Eg1-cdk2 mRNA. One is the silencing of the CPE by the de-

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adenylation element, leading to default deadenylation. Alternatively, the deadenylation element could participate in the activation of the PAN and/or target this enzyme onto Eg mRNAs. In this latter case, the PAN would presumably interact with specificity factors. These mechanisms are not mutually exclusive and they could even have additive effects if one considers that the PAN and the PAP can compete for the same RNA substrate as proposed by Wickens (1990).

In the present study, the substrate specificity of deadenylation was analyzed in extracts made from embryos or activated eggs, as a first step in the biochemical characterization of this process. This has allowed us to address the question of whether the deadenylation of Eg-mRNAs in embryos is due to CPE silencing or by PAN activation and/or targeting.

RESULTS

A prerequisite for the biochemical characterization of the nuclease activities that deadenylate maternal mRNAs is the development of an in vitro system that faithfully reproduces the in vivo behavior. The in vitro system used here was based on extracts made from eggs or embryos according to Murray (Murray & Kirschner, 1989; Murray et al., 1989). The validity of these extracts as a convenient model to study the activity that deadenylates Eg mRNAs was tested by comparing the behaviors of chimeric RNAs incubated in extracts with the already described in vivo behaviors. The different chimeric RNAs used in this study are described in Figure 1.

The behavior of Gb-chimeric transcripts containing the 3′UTR of Eg2 mRNA plus a poly(A) tail of 65 residues (Gb-Eg2-800-A65) was analyzed in extracts made from unfertilized eggs (UFE) or from embryos taken 4 h after fertilization. Data presented in Figure 2 (lanes 1–3) show that this transcript does not markedly change in size when incubated in UFE extracts. In contrast, the same transcript is rapidly shortened when incubated in embryo extracts, giving a discrete band that comigrates with the control poly(A)$^-$ transcript (Fig. 2, lanes 4–6).

The initial differential screening of an egg cDNA library identified nine maternal Eg mRNAs that are deadenylated rapidly after fertilization (Paris & Philippe, 1990). To confirm that the activity responsible for the rapid deadenylation of Eg2 chimeric RNAs was not restricted to this mRNA, a chimeric transcript containing the 3′UTR of Eg5 mRNA was also tested. This RNA (3′Eg5-A65) was also observed to be rapidly shortened in embryo extracts (Fig. 3A). As for the chimeric Eg2 RNAs, after only 2 h of incubation, almost all of the Eg5 derived RNAs were shortened to a discrete size that migrated at a position corresponding to the poly(A)$^-$ RNA. As a further control of specificity, a Gb-chimeric

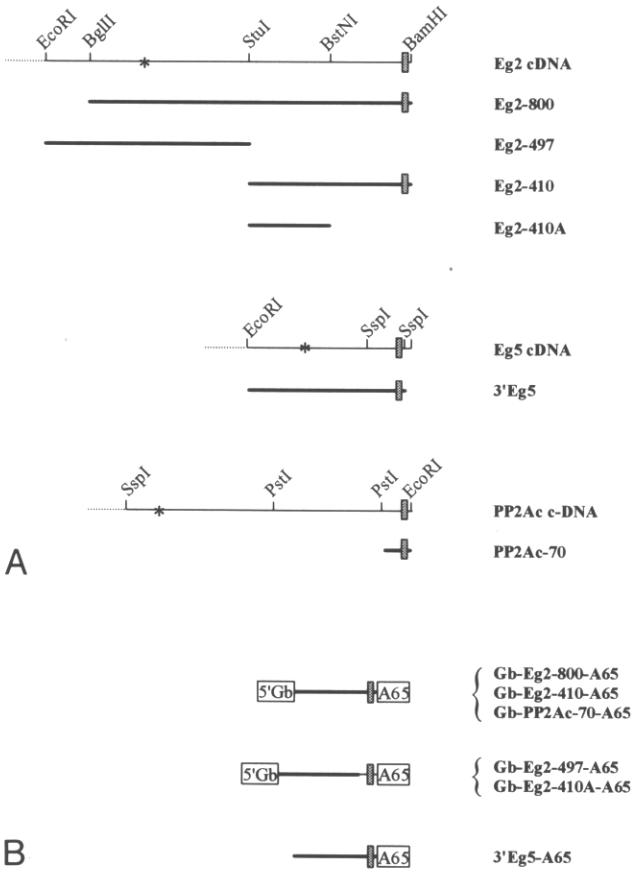

FIGURE 1. Schematic diagram of the different chimeric transcripts used. **A:** Restriction maps of the cDNA 3′ ends are represented by thin lines. Stop codons are represented by stars and polyadenylation signals (AAUAAA) by stippled boxes. The cDNA portions inserted in chimeric plasmids are represented by thick lines under each restriction map. **B:** General structures of the chimeric transcripts. The 5′UTR of the globin cDNA and the 65-residue poly(A) track are represented as open boxes. The cDNA specific portions that are inserted are represented by thick lines. Note that for the Gb-Eg2-497-A65 and Gb-Eg2-410A-A65 transcripts, an exogenous polyadenylation signal has been inserted in the plasmids.

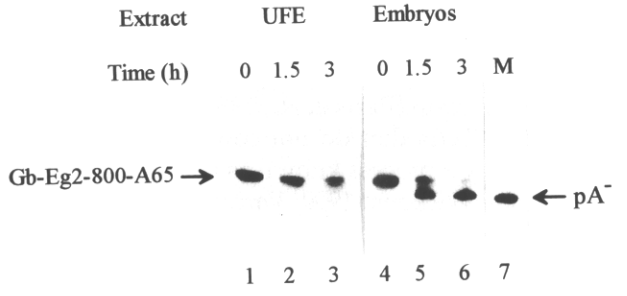

FIGURE 2. Incubation of Gb-chimeric transcripts containing the 3′UTR of Eg2 mRNA in extracts made from unfertilized eggs (UFE) or embryos. The capped, ^{32}P-labeled Gb-Eg2-800-A65 transcripts were incubated at 22 °C in embryo or unfertilized egg extract for the times (in hours) indicated above each lane. At the end of the incubation period, RNA was extracted and analyzed on a denaturing 4% polyacrylamide/urea gel. The gel was dried and autoradiographed. M corresponds to in vitro synthesized poly(A)$^-$ Gb-Eg2-800 RNA.

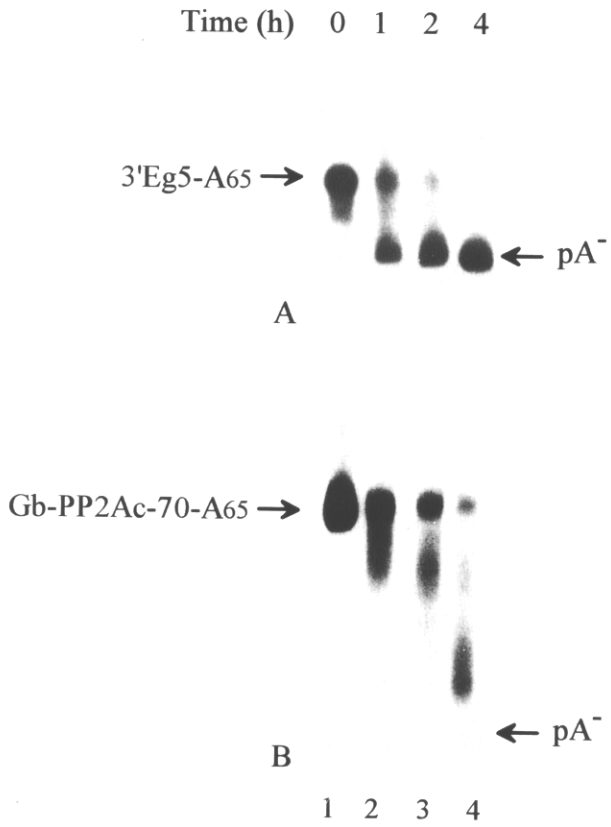

FIGURE 3. Incubation of chimeric transcripts containing a 3′ portion of Eg5 or PP2Ac mRNAs in embryo extracts. Capped, ^{32}P-labeled 3′Eg5-A65 (**A**) or Gb-PP2Ac-70-A65 (**B**) transcripts were incubated at 22 °C in embryo extracts for the times (in hours) indicated above each lane. At the end of the incubation period, RNA was extracted and the samples analyzed on a denaturing 4% polyacrylamide/urea gel as in Figure 2.

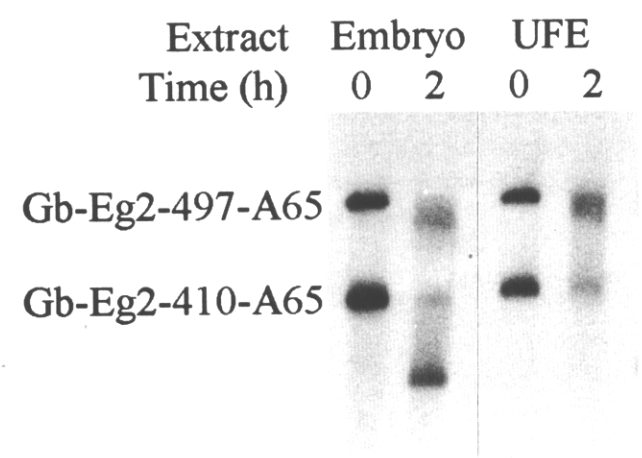

FIGURE 4. Incubation of Gb-chimeric transcripts containing different portions of the Eg2 mRNA 3′UTR in extracts made from embryos or unfertilized eggs (UFE). A mix of the capped, ^{32}P-labeled Gb-Eg2-497-A65 and Gb-Eg2-410-A65 transcripts were incubated at 22 °C in embryo or unfertilized egg extracts for the times (in hours) indicated above each lane. At the end of the incubation period, RNA was extracted and analyzed as in Figure 2.

transcript containing the last 70 nt of the 3′UTR of the maternal mRNA coding for the catalytic subunit of the phosphatase 2A (Gb-PP2Ac-70-A65) was also assayed in the same extract. Chimeric RNAs containing the complete 3′UTR of PP2Ac are adenylated in embryos, whereas this truncated chimeric RNA is not, indicating that the functional CPE has been deleted (unpubl. data). When incubated in embryo extracts, the Gb-PP2Ac-70-A65 RNA was also reduced in size (Fig. 3B). However, the shortening of this RNA was clearly much slower than that of either Gb-Eg2-410-A65 or 3′Eg5-A65. At the 1-h and 2-h time points, a significant amount of partially shortened intermediates of the Gb-PP2Ac-70-A65 RNA were present that were not observed for Eg2 and Eg5 chimeric RNAs.

Polyadenylated Gb-chimeric transcripts that contain the 410-nt 3′-end portion of the Eg2 mRNA (Gb-Eg2-410-A65) are specifically and rapidly deadenylated when injected into *Xenopus* embryos (Bouvet et al., 1994). In comparison, transcripts containing the 5′497-nt portion of the Eg2 3′UTR (Gb-Eg2-497-A65), although being partially deadenylated, remain poly(A)$^+$ for up to 3 h after injection. Therefore, the Gb-Eg2-410-A65 and Gb-Eg2-497-A65 RNAs were synthesized in vitro and incubated in extracts made from 4-h post-fertilization embryos. Figure 4 shows that, after 2 h of incubation in embryo extracts, almost all of the Gb-Eg2-410-A65 transcript was shortened to a discrete band corresponding in size to the poly(A)$^-$ transcript. A different behavior was observed for the Gb-Eg2-497-A65 RNA. This transcript was only partially shortened after a 2-h incubation in these extracts, giving a downward shift corresponding to transcripts of heterogeneous sizes.

The data in Figure 4 also show that the activity responsible for the slow deadenylation of Gb-Eg2-497-A65 transcripts was already present in UFE extracts. In contrast, the rapid deadenylation of the Gb-Eg2-410-A65 transcript was not observed in UFE extracts, indicating that this activity is either inactive or silenced in unfertilized egg extracts. That the band corresponding to this transcript is weaker at the end of incubation is due to a slight upward smearing. This was confirmed in other experiments and is probably due to a poly(A) polymerase activity. Polyadenylation of this transcript in UFE extracts would be consistent with the fact that the Eg2 maternal mRNA is poly(A)$^+$ in matured oocytes.

Eggs artificially activated with calcium ionophore reproduce some of the biochemical changes that occur in fertilized eggs (Murray & Kirschner, 1989). Therefore, the behavior of chimeric RNAs was analyzed in extracts made from calcium ionophore activated eggs. Figure 5 (lanes 1–2) shows that the rapid shortening of the Gb-Eg2-410-A65 transcript was as efficient in activated egg extracts as in embryo extracts. Similarly, the

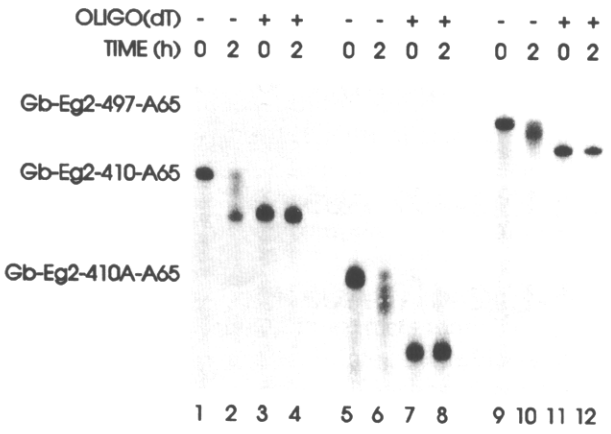

FIGURE 5. Gb-chimeric transcripts are deadenylated in activated egg extracts. The capped, ^{32}P-labeled Gb-Eg2-410-A65 (lanes 1–4), Gb-Eg2-410A-A65 (lanes 5–8) and Gb-Eg2-497-A65 (lanes 9–12) transcripts were incubated at 22 °C for the indicated times in extracts made from activated eggs. RNA was extracted and the samples were divided into two batches. One batch was treated by RNase H in the absence of oligo(dT) (lanes 1–2, 5–6, and 9–10). The other batch was treated by RNase H in the presence of oligo(dT) (lanes 3–4, 7–8, and 11–12). RNA was then reextracted and analyzed as in Figure 2.

slow shortening of Gb-Eg2-497-A65 (lanes 9–10) was clearly observed in activated egg extracts.

That the rapid shortening of the Gb-Eg2-410-A65 transcript as well as the slow shortening of the Gb-Eg2-497-A65 transcript were due to poly(A) nuclease activities, was confirmed by incubating these transcripts in activated egg extracts and subsequently treating the samples with RNase H in the presence or absence of a molar excess of oligo(dT). After 2 h of incubation, a major part of the Gb-Eg2-410-A65 transcript was shortened to a discrete band that comigrated with the transcript deadenylated in vitro by oligo(dT) and RNase H (Fig. 5, compare lanes 2 and 3). A minor part was still polyadenylated as shown by its disappearance when the sample was treated with RNase H in the presence of oligo(dT) (lane 4). Furthermore, the RNase H/oligo(dT) treatment yielded a final product of the same size, irrespective of whether the sample had been preincubated in the extract or not (compare lanes 3 and 4). Therefore, the rapid shortening of Gb-Eg2-410-A65 was due to deadenylation. After 2 h of incubation, the Gb-Eg2-497-A65 transcript was also shortened, giving a number of heterogeneously sized RNAs (lane 10). However, all the transcripts that constitute this smeared signal correspond to RNA molecules that are longer than the transcript that has been fully deadenylated by RNase H plus oligo(dT) treatment (compare lanes 10 and 11). This confirms the partial nature of this shortening activity. Again, the RNaseH/oligo(dT) treatment gave a discrete band of the same size whether the transcripts were preincubated in the extract or not (compare lanes 11 and 12), which shows that the partial shortening of the Gb-Eg2-497-A65 transcript was also due to deadenylation. The same experiment was done with the Gb-Eg2-410A-A65 transcript. The 410A portion of Eg2 3′UTR does not contain the *cis*-acting deadenylation element (Bouvet et al., 1994). As for Gb-Eg2-497-A65, the Gb-Eg2-410A-A65 transcript was deadenylated by a slow process (lanes 5–8).

Therefore, the kinetics with which RNAs are deadenylated in embryo or activated egg extracts depend on the substrate RNA. Substrates that contain 3′ distal portions of Eg mRNAs are rapidly deadenylated, giving fully deadenylated RNAs as the first major product without an accumulation of partially deadenylated forms. In comparison, other substrates are slowly deadenylated, giving partially deadenylated products well before fully deadenylated RNAs accumulate. It is important to stress that this distinction between slow and rapid deadenylation is an empirical one. If these poly(A) nuclease activities reside within complexes with different regulatory factors, the above distinction may not be correlated with the enzymatic properties of the isolated catalytic subunit. Indeed, at this stage we cannot exclude that both activities share an unique catalytic subunit.

In vivo, the rapid deadenylation activity is dependent on the presence of a deadenylation element (Bouvet et al., 1994). To confirm that this dependence was conserved in the in vitro system, we next compared the deadenylation kinetics for the Gb-Eg2-410-A65 and Gb-Eg2-410Δ2-A65 transcripts in activated egg extracts. The Gb-Eg2-410Δ2-A65 transcript is deleted of a 40-nt portion that contains the deadenylation element. The data in Figure 6 (lanes 6–9) show that this transcript was refractory to rapid deadenylation, illustrating the need for the deadenylation element in vitro as well as in vivo.

These results show that in vitro as well as in vivo, RNAs that do not contain specific *cis*-sequences are deadenylated slowly and that the presence of a *cis*-acting deadenylation element is necessary to promote rapid deadenylation. However, formally, the slow deadenylation observed in these extracts could be due to a compensatory polyadenylation, promoted by cryptic CPEs present in the deadenylation element-deficient transcripts tested in this study. The stimulatory effect of CPEs on polyadenylation requires the presence of a nuclear polyadenylation signal (Fox et al., 1989; McGrew et al., 1989; Simon & Richter, 1994). Eliminating this signal by mutating AAUAAA to AAGAAA leads to silencing of the CPEs. In addition, this mutation renders the poly(A)$^+$ RNA a substrate for default deadenylation (Fox & Wickens, 1990).

Such a mutation was previously shown to have no effect on the rapid deadenylation of Gb-Eg2-410-A65 in vivo (Bouvet et al., 1994). A similar result was obtain in activated egg extracts (Fig. 7).

To determine whether the slow deadenylation of the Gb-Eg2-497-A65 and Gb-Eg2-410A-A65 RNAs was due

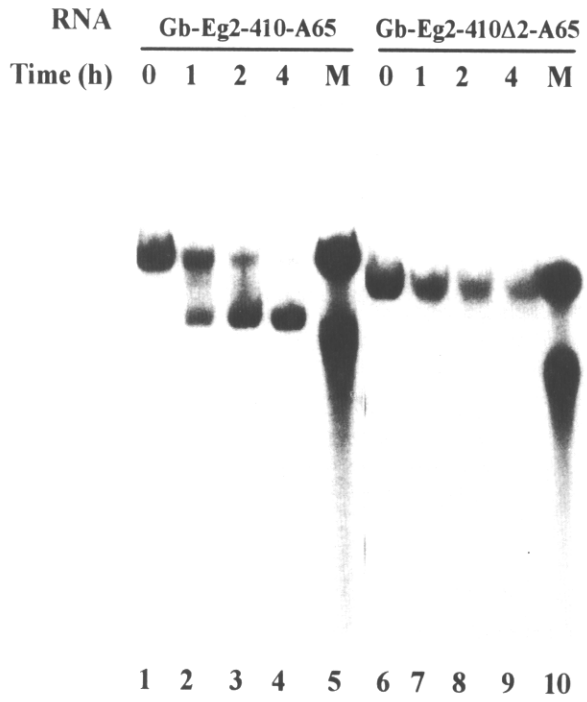

FIGURE 6. Rapid deadenylation of chimeric transcripts requires the presence of a *cis*-acting deadenylation element. The capped, ^{32}P-labeled Gb-Eg2-410-A65 (lanes 1–4) and Gb-Eg2-410Δ2-A65 (lanes 6–9) transcripts were incubated at 22 °C for the indicated times (in hours) in activated egg extracts. RNA was then extracted and the samples were analyzed as in Figure 2. For each series, markers (lanes 5 and 10) contain a mix of the same chimeric transcript synthesized either poly(A)$^+$ and poly(A)$^-$ from plasmids linearized by *Eco*R V or *Bam*H I, respectively.

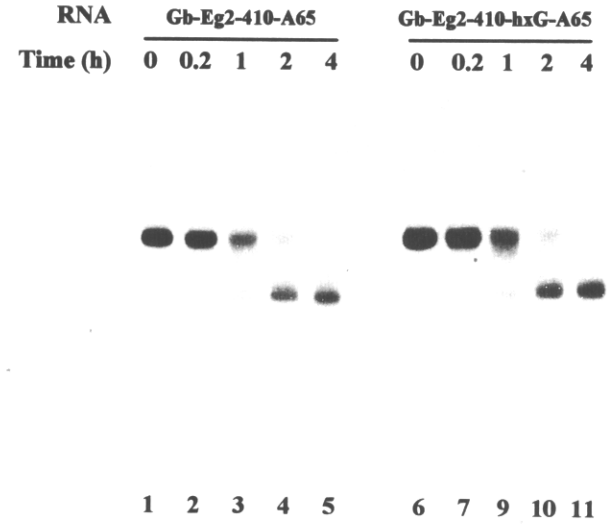

FIGURE 7. Mutating the polyadenylation signal does not affect the rapid deadenylation activity in activated egg extracts. The capped, ^{32}P-labeled Gb-Eg2-410-A65 (lanes 1–5) and Gb-Eg2-410-hxG-A65 (lanes 6–10) transcripts were incubated at 22 °C for the indicated times (in hours) in activated egg extracts. RNA was then extracted and the samples were analyzed as in Figure 2.

to compensatory polyadenylation, the same mutation was also introduced within the polyadenylation signal of these transcripts. The data presented in Figure 8 show that the resulting mutant RNAs (Gb-Eg2-497-hxG-A65 and Gb-Eg2-410A-hxG-A65) are deadenylated at the same rate as the corresponding wild-type RNAs in activated egg extracts. Hence, compensatory polyadenylation, if it exists, does not appear to significantly alter the deadenylation kinetics of the various Eg2 chimeric RNAs studied here.

DISCUSSION

We have used an in vitro system consisting of activated egg or embryo extracts to initiate the characterization of the PAN responsible for the specific deadenylation of Eg mRNAs in embryos. In these extracts, the deadenylation behaviors of chimeric RNAs were very similar to those already described for the maternal mRNAs, as well as for the same chimeric transcripts, in vivo. For instance, transcripts containing a deadenylation element (such as Gb-Eg2-410-A65 and 3′Eg5-A65) were rapidly deadenylated, whereas those devoid of such an element (Gb-Eg2-497-A65, Gb-Eg2-410A-A65, or Gb-PP2Ac-70-A65) were shortened by a slower process. The slow PAN activity is characterized by the appearance of partially deadenylated products before fully deadenylated RNAs accumulate. This activity is present in extracts from both unfertilized and fertilized or activated eggs. The rapid activity, which was absent from UFE extracts, gave fully deadenylated RNAs as the first major products without an accumulation of partially deadenylated RNAs.

The slow PAN activity has a wide substrate specificity, being able to deadenylate all the RNAs tested that are devoid of canonical CPE motifs. These substrates could contain cryptic CPEs able to slow the PAN activity by promoting a compensatory polyadenylation. Therefore, we have mutated the AAUAAA polyadenylation signal to AAGAAA, which renders the RNA a substrate for the default deadenylation activity by preventing CPE-driven polyadenylation. These mutated RNAs were deadenylated with the same kinetics as the corresponding wild-type transcripts in activated egg extracts. Hence, we propose that the slow PAN activity described here, which is present in both UFE and activated egg extracts, corresponds to the already described default PAN (Fox & Wickens, 1990; Varnum & Wormington, 1990).

All Eg maternal mRNAs contain a CPE that is functionally active in matured oocytes because they are poly(A)$^+$ in unfertilized eggs. Concerning the deadenylation of these mRNAs in fertilized eggs, two hypothesis can be proposed. (1) The deadenylation element could act by inactivating the CPE. This would lead to a deadenylation by the default PAN activity. (2) The deadenylation element could act by targeting

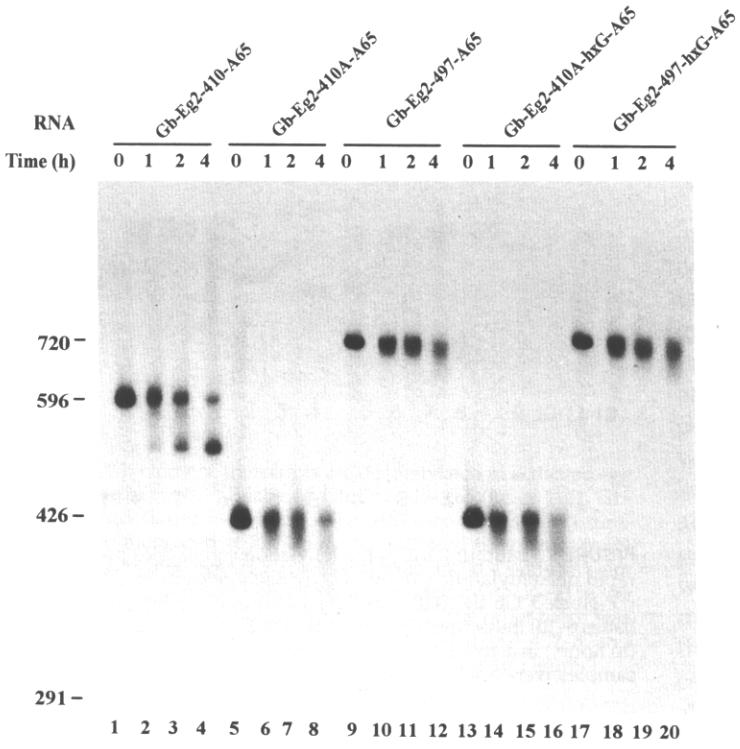

FIGURE 8. Mutating the polyadenylation signal does not affect the slow deadenylation activity in activated egg extracts. The capped, ^{32}P-labeled Gb-Eg2-410-A65 (lanes 1–4), Gb-Eg2-410A-A65 (lanes 5–8), Gb-Eg2-497-A65 (lanes 9–12), Gb-Eg2-410A-hxG-A65 (lanes 13–16), and Gb-Eg2-497-hxG-A65 (lanes 17–20) transcripts were incubated at 22 °C for the indicated times (in hours) in activated egg extracts. RNA was then extracted and the samples were analyzed as in Figure 2.

and/or activating a PAN activity, leading to a rapid deadenylation that overcomes the CPE-dependent polyadenylation.

If the sole function of the deadenylation element was to inactivate the CPE, making the RNA a substrate for the default PAN, there should be no difference in the kinetics with which RNAs that contain a deadenylation element and bona fida CPE-deficient RNAs are deadenylated. However, the time-course experiments described here show that the deadenylation of the deadenylation element-containing RNAs is much more rapid than that of other RNAs, even of those in which the AAUAAA hexanucleotide has been mutated to the nonfunctional AAGAAA sequence. Hence, the function of the deadenylation element is not simply to inactivate the CPE.

We conclude that activated egg extracts constitute an in vitro system that would allow the identification and functional characterization of the factors responsible for embryo-specific deadenylation. Using these extracts, we show here that the deadenylation of Eg maternal mRNAs in embryos is not simply accounted for by a default mechanism switched on by silencing the cytoplasmic polyadenylation elements. Rather, this deadenylation is an active process requiring a *cis*-acting deadenylation element. This active and sequence-specific process could be achieved either by activating the basal activity of the default PAN or by targeting a specific PAN to Eg mRNAs. Both mechanisms would involve a *trans*-acting factor that interacts with the deadenylation element. A candidate for such a factor has been described previously (Legagneux et al., 1992), whose function can now be studied in the in vitro system presented here.

MATERIALS AND METHODS

Plasmid construction and RNA synthesis

The Eg2-410A, Gb-Eg2-497-A65, Gb-Eg2-410-A65, and Gb-Eg2-410Δ2-A65 plasmids have been described (Bouvet et al., 1994).

The Gb-Eg2-410A-A65 plasmid was constructed from the Klenow enzyme-treated *Sma* I–*Hind* III fragment of Eg2-410A, using the procedure described previously for the Gb-Eg2-497-A65 plasmid.

For pGb-PP2Ac-70-A65, the 700-bp *Ssp* I–*Eco*R I 3′ fragment was isolated from the full-length PP2Ac cDNA (Cormier et al., 1991) cloned in the *Eco*R I site of pBluescript KS (Stratagene). After blunt ending with Klenow enzyme, this 700-bp fragment was cloned between the *Hinc* II and *Sma* I sites of pBluescript KS. Plasmids containing this PP2Ac cDNA fragment oriented with the 3′ end toward the *Bam*H I site were selected. These plasmids were cut with *Xho* I, blunt ended with Klenow enzyme, and then digested with *Bam*H I. The resulting fragment (700 bp) was cloned between the blunt-ended *Bgl* II and *Bam*H I sites of the pGb-Eg2-410-A65 plasmid, giving pGb-PP2Ac-700-A65. Note that the Eg2 fragment is removed when the pGb-Eg2-410-A65 plasmid is cut by both *Bgl* II and *Bam*H I enzymes. The pGb-PP2Ac-70-A65 plasmid was made by digesting pGb-PP2Ac-700-A65 with *Xho* I and *Pst* I, blunt ending with Klenow enzyme and T4 polymerase, and religating.

For the 3′Eg5-A65 plasmid, the 3′ fragment between the internal *Eco*R I site and the ultimate *Ssp* I site of the Eg5 cDNA (Le Guellec et al., 1991) was inserted between the *Eco*R I and *Sma* I sites of pBluescript KS, giving pBspt-3′Eg5. The *Kpn* I–*Bam*H I fragment was excised from pBspt-3′Eg5 and inserted into the *Kpn* I and *Bam*H I sites of the M13 mp18 phagemid, giving the mp18-3′Eg5 phagemid. Finally, the *Eco*R V–*Xba* I fragment was excised from mp18-3′Eg5 and cloned between the *Sma* I and *Xba* I sites of the BSDP1400H$^-$-A65 plasmid (Bouvet et al., 1994).

The mutated Gb-Eg2-410A-hxG-A65 and Gb-Eg2-497-hxG-A65 plasmids were obtained by PCR using a sense oligonucleotide corresponding to the 5′ end of the transcribed region and an antisense oligonucleotide overlapping the polyadenylation signal.

Sense oligonucleotide:

5′-GAATTCGAGCTCGGTACCCAG-3′

Antisense oligonucleotide:

5′-GGGGATCCGAGCTTTCTTTTAAAAC-3′.

The point mutation is underlined. The amplified fragment was cut by *Kpn* I and *Bam*H I and inserted between the *Kpn* I and *Bam*H I sites of the BSDP1400-H$^-$-A65 plasmid.

All the constructions were confirmed by sequencing.

To synthesize poly(A)$^+$ transcripts, A65 containing plasmids were linearizing with *Eco*R V. The capped transcripts were synthesized by using the Promega transcription kit, according to the suppliers instructions, in a 20-μL reaction containing 1 μg of the linearized plasmid, 10 nmol of ATP, 10 nmol of CTP, 10 nmol of UTP, 1 nmol of GTP, and 10 nmol of m^{7}G(5′)ppp(5′)G. For radiolabeled transcripts, UTP was reduced to 2 nmol and 20 μCi of α-[^{32}P]UTP were added. Transcripts were purified by ethanol precipitation in the presence of 2.5 M ammonium acetate. Depending on the quality of the transcripts, they were further purified by electrophoresis in a denaturing polyacrylamide/urea gel.

Eggs, embryos, and extracts

All the biological material was maintained or incubated at 22 °C. Unfertilized (cytostatic factor-arrested) eggs were prepared as follows: Female frogs were induced to lay eggs by injection of 800 μ of human chorionic gonadotropin, 18 h before collecting the eggs. Females were placed in boxes containing 1 L of high-salt BARTH buffer (for 1 L: 6.71 g NaCl, 0.075 g KCl, 0.202 g $NaHCO_3$, 0.202 g $MgSO_4$, 0.06 g $CaCl_2$, 0.078 g $CaNO_3$, 1.2 g Tris, pH 7.6, with HCl). Lain eggs were collected and rinsed once in high-salt BARTH and three times in XB-CSF (100 mM KCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM K-EGTA, pH 7.7, with KOH). Eggs were dejellied in XB-CSF containing 2% cysteine, pH 7.8. Eggs were conserved in the XB-CSF buffer until preparation of the extract. For activation, eggs were rinsed in XB (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.7, with KOH) and activated by incubation in calcium ionophore (A 23817 at 0.5 μg/mL in XB). At the appearance of the cortical contraction, eggs were rinsed four times in XB and dejellied in XB containing 2% cysteine, pH 7.8. For fertilized eggs, females were squeezed and eggs were collected in a glass petri dish. Fertilization and dejellying were performed as described previously (Paris et al., 1988). For embryo extracts, dejellied embryos were maintained in F1 medium (10 mM Hepes, 31.25 mM NaCl, 1.75 mM KCl, 1 mM $CaCl_2$, 0.06 mM $MgCl_2$, pH 7.6, with NaOH) and rinsed three times in XB before extraction (4 h of development). Extracts were made according to Murray and Kirschner (1989) and Murray et al. (1989). The buffer was displaced by Versilube oil (General Electric) before the centrifugal rupture of the eggs, except when large quantities of activated egg extracts were made. No differences were found in the behavior of the transcripts incubated in extracts made using either of these two protocols. In all cases, extracts were supplemented by pepstatin, leupeptin, chymostatin at 10 μg/mL, cytochalasin B at 100 μg/mL, and ATP at 1 mM.

Incubation and extraction of chimeric transcripts

The capped transcripts (10–20 fmol) were incubated in extracts at 22 °C. At the end of the incubation, RNA was extracted by 200 μL of extraction buffer (1% SDS, 20 mM Tris, pH 7.5, 100 mM NaCl, 30 mM EDTA) (Harland & Misher, 1988) followed by two extractions with phenol/chloroform. RNA was precipitated with ethanol, solubilized in formamide sample buffer, and denatured 10 min at 100 °C before being loaded onto a 4% polyacrylamide/urea gel. For RNase H/oligo dT treatments, RNA pellets were resuspended in RNase H buffer and processed as described by Osborne et al. (1991).

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