Translational control by poly(A) elongation during *Xenopus* development: differential repression and enhancement by a novel cytoplasmic polyadenylation element

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One characteristic of oocyte maturation and embryogenesis in *Xenopus laevis* is the activation of translationally repressed (masked) maternal mRNAs by cytoplasmic poly(A) elongation. At maturation, poly(A) elongation is controlled by two cis-acting elements in the 3' untranslated regions (UTRs) of responsive mRNAs, the hexanucleotide AAUAAA and the cytoplasmic polyadenylation element (CPE), consisting of UUUUUAU or other similar sequences. To investigate poly(A) elongation and translational activation during embryogenesis, we have focused on C12 RNA, a representative transcript that undergoes these processes. By injecting radiolabeled C12 RNA into fertilized eggs and allowing development to proceed, we found that maximal polyadenylation of this RNA is reached by the 4000-cell blastula stage and that it requires two cis-acting elements, the hexanucleotide AAUAAA and a novel CPE, which is dodecauridine. Interestingly, a shortening of the distance between the two elements changes the timing of maximal polyadenylation to the four-cell stage. The injection of a chloramphenicol acetyl transferase (CAT)–C12 chimeric RNA into fertilized eggs not only results in embryonic polyadenylation of the transcript but also 5- to 15-fold more CAT activity compared with eggs injected with CAT RNA or CAT–C12 chimeric RNA that is prevented from undergoing poly(A) elongation by a mutation in the polyadenylation hexanucleotide. However, eggs injected with a CAT–C12 chimeric RNA that is preadenylated but that cannot undergo further poly(A) elongation contain no more CAT activity than eggs injected with the same RNA without a poly(A) tail, suggesting that the process of poly(A) elongation, and not poly(A) tail length, is important for translation. Finally, we show that precocious poly(A) elongation of C12 RNA during oocyte maturation is prevented by a large "masking" element that includes the dodecauridine CPE. The dual role of the CPE in both repression and activation of poly(A) elongation is discussed.

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nalyzed during maturation and concomitantly exit polysomes (Hyman and Wormington 1988).

Using mRNA-injected oocytes, maturation-specific poly(A) elongation has been shown to involve two cis-acting elements. The first, which is virtually ubiquitous for all eukaryotic mRNAs, is the nuclear pre-mRNA cleavage and polyadenylation hexanucleotide AAUAAA; the second is U-rich and has the general structure of UUUUUUAUU (Fox et al. 1989; McGrew et al. 1989; McGrew and Richter 1990; Paris and Richter 1990, Huarte et al. 1992). The latter sequence, termed the cytoplasmic polyadenylation element (CPE), usually resides <15 bases 5′ of the hexanucleotide. However, neither this sequence, termed the cytoplasmic polyadenylation element (CPE), usually resides <15 bases 5′ of the hexanucleotide. However, neither this spacing (McGrew and Richter 1990; Paris and Richter 1990) nor the 5′-CPE–hexanucleotide–3′ organization (Fox et al. 1989) is necessarily required for polyadenylation. On the other hand, the number of bases downstream of the 3′-most element that do not consist of poly(A) can inhibit this process (McGrew and Richter 1990).

Employing Xenopus oocyte and egg extracts that faithfully polyadenylate RNAs in a manner similar to intact cells, UV cross-linking experiments have revealed some information about the factors that interact with the CPEs of two different mRNAs. The CPE of G10 RNA, which is UUUUUUAUAAG, is bound by an 82-kD protein that is detected in egg, but not oocyte, extracts (McGrew and Richter 1990). This might suggest that the 82-kD protein is synthesized de novo during oocyte maturation and is required for polyadenylation. Indeed, protein synthesis during the first hour of maturation is necessary for G10 polyadenylation (McGrew and Richter 1990). The CPE of B4 RNA [UUUUUUAUU], on the other hand, is bound by a 58-kD protein in both oocyte and egg extracts. In this case, the phosphorylation of the 58-kD protein, as well as the activation of polyadenylation, appears to be regulated by p34cde2 (Paris et al. 1991). Thus, given these observations, cytoplasmic polyadenylation during maturation is likely to be controlled at multiple levels.

In addition to oocyte maturation, coincident poly(A) elongation and polysomal recruitment of mRNAs also have been observed during embryogenesis in Xenopus (Paris et al. 1988; Paris and Philippe 1990). What, then, determines whether an mRNA is polyadenylated and translated during embryogenesis but not during oocyte maturation? To investigate this, we have focused on Cl2 RNA, which is known to be polyadenylated during embryogenesis in Xenopus (Paris et al. 1988; Paris and Philippe 1990). By injecting fertilized eggs with a Cl2 3′ untranslated region (UTR) and examining the structure of the RNA at several subsequent developmental periods, we show that this transcript is maximally polyadenylated by the fine blastula stage. The sequences that are required for this event are the hexanucleotide AAUAAA and a novel CPE, which is dodecauridine. Interestingly, alteration of the distance between the CPE and the hexanucleotide has a profound effect on the timing and extent of polyadenylation. The injection of fertilized eggs with a chloramphenicol acetyl transferase (CAT)–Cl2 fusion RNA results in the embryonic polyadenylation of this transcript, as well as CAT activity levels that exceed those in eggs injected with wild-type CAT RNA by up to 15-fold. However, CAT activity levels are similar in embryos injected with a CAT–Cl2 chimeric RNA which already contains a poly(A) tail but which cannot undergo further elongation, and CAT–Cl2(AAGAAA) RNA, which is refractory to polyadenylation. This suggests that the process of poly(A) elongation, but not the poly(A) tail itself, is necessary for translational activation. Finally, we show that Cl2 RNA polyadenylation is inhibited during oocyte maturation by a large “masking” element that includes the dodecauridine CPE.

Results

Cytoplasmic polyadenylation of a maternal mRNA during early embryogenesis

A clone for Cl2 RNA was isolated by probing an unfertilized Xenopus egg cDNA library with radiolabeled cDNAs derived from poly(A) + RNA from unfertilized eggs and 3-hr [64 cells] embryos (Paris and Philippe 1990). Developmental Northern blots probed with labeled Cl2 DNA revealed that Cl2 RNA was mostly devoid of a poly(A) tail in eggs but acquired a poly(A) tail during embryogenesis. In contrast, other RNAs became deadenylated at this time (Paris et al. 1988). To investigate the specific sequences that are responsible for the cytoplasmic polyadenylation of Cl2 RNA, we subcloned 559 bases [Δ0], 415 bases [Δ1–144], 235 bases [Δ1–324], and 105 bases [Δ1–454] corresponding to the 3′ end of Cl2 RNA behind the T7 promoter (Fig. 1A). The DNAs were linearized, transcribed in vitro in the presence of [32P]UTP, and injected into Xenopus eggs ~1 hr after fertilization. At 0 [1 cell] 1.5 [four-cell stage], 3.0 [large-cell blastula stage, 64 blastomeres], 4.5 hr [medium-cell blastula stage, ~500–1000 cells], and 6.0 hr [fine-cell blastula stage, >4000 cells; stages according to Nieuwkoop and Faber 1956] after injection, five embryos were collected and the RNA was extracted and analyzed on a 5% denaturing polyacrylamide gel. Figure 1A shows that all four RNAs migrated with a slower electrophoretic mobility beginning 1.5 hr after injection. The mobility of each RNA slowed gradually as a function of time after fertilization. The slowest migrating RNAs correspond to an increase in length of ~80 residues. These results suggest that sequences involved in altering the structure of RNA, possibly by polyadenylation, are located within the smallest fragment of the Cl2 3′ UTR. This sequence specificity also is indicated by the observation that injected SR47 RNA [100 bases], which contains the hexanucleotide AAUAAA but no other known regulatory sequence, did not have an altered electrophoretic mobility during embryogenesis (Fig. 1A).

To show that the increase in size of Cl2 RNA was the result of poly(A) elongation, we injected radiolabeled Δ1–454 RNA into fertilized eggs, extracted RNA 6 hr after injection, and treated the RNA with RNase H in the absence or presence of oligo(dT). Figure 1B shows that in
the presence of oligo(dT), the size of the RNA was reduced to the size of the noninjected RNA [M], confirming that the size shift of Cl2 RNA during early embryogenesis was the result of polyadenylation.

Although the Cl2 RNAs were injected into the egg cytoplasm, it was formally possible that the RNA entered embryonic nuclei where it was adenylated through the nuclear polyadenylation apparatus. To assess this, we fixed, embedded, and sectioned radiolabeled RNA-injected embryos and localized the RNA by autoradiography (data not shown). At all stages, the vast majority of silver grains was located above cytoplasmic areas. This result and the fact that SR47 RNA containing only the AAUAAA hexanucleotide is not polyadenylated following cytoplasmic injection strongly suggest that injected Cl2 RNA is polyadenylated in the cytoplasm and not in the nucleus.

The hexanucleotide AAUAAA is involved in cytoplasmic polyadenylation of Cl2 RNA during early embryogenesis

All RNAs of higher eukaryotes investigated so far require the hexanucleotide AAUAAA (or very similar sequence) for polyadenylation, whether it is nuclear or cytoplasmic. Cl2 RNA contains the sequence AAUAAA 18 nucleotides from the 3' end. To examine whether it is involved in the cytoplasmic polyadenylation of Cl2 RNA, we substituted a G for the U in the hexanucleotide of RNA Δ1-454 [RNA Cl2(Δ1-454,536-559AAAGAAA)] and injected the RNA into fertilized eggs. In contrast to Cl2 RNA with the wild-type hexanucleotide (Δ1-454), Cl2(Δ1-454,536-559AAAGAAA) RNA was not polyadenylated during early embryogenesis (Fig. 2), which demonstrates the requirement for this element. We also show that the 18 bases downstream of the hexanucleotide are not important for the polyadenylation process [Cl2(Δ1-454,543-559), Fig. 2].

Dodecauridine functions as a CPE for Cl2 RNA

Studies on several Xenopus RNAs have shown that a sequence resembling UUUUUAU, termed the CPE, in addition to the hexanucleotide, is important for cytoplasmic polyadenylation during oocyte maturation (for review, see Richter et al. 1990; Wickens 1990; Richter 1991; Bachvarova 1992). Cl2 RNA does not contain an exact copy of this sequence, but 31 nucleotides upstream
Translational control during *Xenopus* development

**Figure 2.** The hexanucleotide AAUAAA is required for cytoplasmic polyadenylation of C12 RNA. Δ1-454, Δ1-454,543-559, and Δ1-454,536-559AAGAAA RNAs were synthesized in vitro in the presence of [32P]UTP and injected into fertilized eggs. Five embryos were collected at 0, 1.5, 3.0, 4.5, and 6.0 hr after injection, and the RNA was extracted and analyzed on a 5% denaturing gel, followed by autoradiography. The poly(A) tail sizes were estimated by comparison to an RNA ladder of sizes ranging from 0.6 to 1.77 kb. (Open box) Sequence present; (solid box) sequence deleted. (M) Marker RNA.

of the hexanucleotide is a poly(U) region composed of 27 uridine residues interrupted by only 2 guanosine residues (Fig. 3). Because we suspected that this region could contain the C12 RNA equivalent of a CPE, we deleted portions of the poly(U) region, injected in vitro-synthesized RNAs into fertilized eggs, and examined the extent of polyadenylation. RNAs that contained 18 (Δ1-470,495-504; Fig. 3B) or 12 (Δ1-470,486-504; Fig. 3C)
uridine residues were polyadenylated similar to wild-type RNA (Fig. 3A). However, C12 RNAs with only 11 (Δ1-470,487-504; Fig. 3D) or 10 (Δ1-470,486-504; Fig. 3E) uridine residues showed a drastic decrease in the extent of their polyadenylation. Further deletions of uridine residues to 9 (Δ1-470,485-504; Fig. 3F) or no uridine residues (Δ1-504; Fig. 3G) abolished polyadenylation of C12 RNA. These data demonstrate that at least 12 uridine residues, combined with the hexanucleotide AAUAAA, are necessary for effective polyadenylation of C12 RNA. Therefore, we define dodecauridine as comprising the CPE for the C12 RNA.

Element spacing controls the timing and extent of polyadenylation

To determine whether spacing between the two elements of C12 RNA affects polyadenylation, we shortened the distance by deleting 21 [nucleotide 515-559] of the 31 nucleotides between the CPE and the hexanucleotide (RNA B, Δ1-454,515-559AAUAAA, Fig. 4a). Following injection into fertilized eggs, this RNA showed a dramatic change in the timing and extent of polyadenylation. Not only was nearly all of this RNA polyadenylated 1.5 hr after injection [compared with only one-half of the wild-type RNA (Δ1-454,536-559AAUAAA) 3 hr after injection], it also showed an increase in the number of added adenosine residues. Wild-type C12 RNA was adenylated with 40-80 residues, whereas RNA (Δ1-454,515-559AAUAAA) was adenylated with 60-130 residues. To determine whether the distance, the sequence, or a possible secondary structure was involved in this regulation of polyadenylation, we constructed additional mutations [Fig. 4a, RNAs C–F]. Even by deleting 11 (Δ1-454,525-559AAUAAA; RNA C) or 9 (Δ1-454,528-559AAUAAA; RNA D) nucleotides of the original sequence, the RNAs were still polyadenylated earlier and to a greater extent than wild-type RNA (RNA A). Deletion of only 5 (Δ1-454,531-559AAUAAA; RNA E) or 2 (Δ1-454,534-559AAUAAA; RNA F) of the 31 nucleotides restored the normal timing of polyadenylation. However, these RNAs were still polyadenylated to a greater extent compared with wild-type RNA (RNA A). Because the sequence between the two elements could form a hairpin structure, we substituted the original sequence with a random sequence derived from a poly linker that also could form a hairpin structure (RNA G) and a sequence that could not form a hairpin (RNA H).

Figure 4. Element spacing controls the timing and extent of cytoplasmic polyadenylation of C12 RNA during early embryogenesis. (a) Radiolabeled RNA derived from deletion mutants Δ1-454,536-559AAUAAA (A), Δ1-454,515-559AAUAAA (B), Δ1-454,525-559AAUAAA (C), Δ1-454,528-559AAUAAA (D), Δ1-454,531-559 AAUAAA (E), Δ1-454,534-559AAUAAA (F), and substitution mutants Δ1-454, substitution 515-559(a) (G), and Δ1-454 substitution 515-559(b) (H), was injected into fertilized eggs and analyzed for embryonic polyadenylation as described in Fig. 1A. (b) The denoted RNAs were analyzed for embryonic polyadenylation as above except that they were resolved on the same denaturing polyacrylamide gel. (Open box) Sequence present; (solid box) sequence deleted. [M] Marker RNA.
but, in both cases, kept the correct distance of 31 nucleotides. Both RNAs injected into fertilized eggs were polyadenylated to the same extent as wild-type C12 RNA (RNA A), suggesting that distance, but not the actual sequence or a possible secondary structure, influences the timing and extent of polyadenylation.

To further demonstrate that poly(A) tail length is a function of the distance between the CPE and the hexanucleotide, we have performed an additional experiment (Fig. 4b). Two RNAs with the normal 31 nucleotides between the CPE and the hexanucleotide (RNAs a and b), an RNA with only 10 nucleotides in this space (RNA c), and an RNA without a CPE (RNA d) were injected into fertilized eggs, extracted after 6 hr of development, and analyzed on the same denaturing polyacrylamide gel. It is clearly evident that RNA c acquired a much longer poly(A) tail than either RNA a or b. As expected, RNA d was not polyadenylated. Thus, Figure 4 shows that element spacing controls the timing and extent of polyadenylation during embryogenesis.

Polyadenylation-dependent translation of C12 RNA

Previous studies have shown that translational recruitment of certain mRNAs requires poly(A) elongation during oocyte maturation (Huarte et al. 1987; McGrew et al. 1989; Paris and Richter 1990). To determine whether recruitment of C12 RNA also requires polyadenylation, we cloned nucleotides 454–559 of the C12 3’ UTR (CAT–Cl2), containing the CPE and the wild-type hexanucleotide, and the AAGAAA mutant [CAT–Cl2|AAGAAA] behind CAT-coding sequences. In vitro-synthesized RNAs were injected into fertilized eggs, and RNA was prepared from embryos collected at different times during early embryogenesis. RNA transcribed from the CAT–Cl2 construct was polyadenylated in a manner similar to that observed with C12 RNA where ~80 adenosine residues were added (Fig. 5A). RNAs derived from CAT or CAT–Cl2|AAGAAA DNA did not gain a poly(A) tail during early embryogenesis (Fig. 5A). To determine whether the addition of a poly(A) tail had an effect on the translation of CAT–Cl2 RNA, we performed CAT assays with cell extracts from embryos of different stages. A quantitative analysis of these data shows that CAT–Cl2 RNA-injected embryos had 5-fold (3- to 15-fold in other experiments) more CAT activity than either CAT or CAT–Cl2|AAGAAA RNA-injected embryos (Fig. 5B). We infer from these data that polyadenylation of C12 RNA is important for its efficient translation during embryogenesis.

We then determined whether the polyadenylation process itself or the length of the poly(A) tail was important for the translation of CAT–Cl2 chimeric RNA. To do this, the CAT–Cl2|AAGAAA mutant was cloned into a...
Bluescript SK vector that contained 73 adenosine residues in the polylinker sequence. RNA derived from this clone contains 73 adenosine residues at the 3' end but, because it has an AAGAAA hexanucleotide, cannot be further polyadenylated in embryos. This RNA [CAT-C12(AAGAAA)p473] and CAT-C12(AAGAAA) RNA were injected into fertilized eggs, and RNA and protein were prepared at different times during embryogenesis. As expected, neither of the two RNAs showed any change in poly(A) tail size during 6 hr of incubation (Fig. 6A). In addition, the CAT activity from embryos injected with these two RNAs was virtually indistinguishable (Fig. 6B). This result suggests that ongoing polyadenylation, but not the poly(A) tail itself, is necessary for efficient translation of C12 RNA.

Inhibition of C12 RNA polyadenylation during oocyte maturation

There are a number of possibilities to explain why endogenous C12 RNA is polyadenylated after fertilization but not during oocyte maturation. One of the first is that a C12 CPE-specific polyadenylation factor is synthesized or activated only after fertilization. Alternatively, perhaps a C12 CPE-specific polyadenylation factor is also present and active during oocyte maturation but is prevented from interacting with the RNA in the appropriate manner. To address these possibilities, we injected C12(Δ1–454) RNA into stage 6 oocytes, some of which were induced to mature with progesterone. Surprisingly, this RNA was polyadenylated with ~80 residues (Fig. 7A). Moreover, this RNA required the dodecauridine CPE to be polyadenylated during maturation (Δ1–504, Fig. 7A) and was adenylated with more residues [up to 140] when the CPE and the hexanucleotide were in close proximity (Δ1–454, 515–559AAUAAA). Thus, these results with C12 RNA polyadenylation during oocyte maturation are similar to those observed during early embryogenesis.

The data shown in Figure 7A demonstrate that the C12 CPE-specific polyadenylation apparatus is present and active during oocyte maturation and suggest that injected C12 3' UTR RNA has escaped a negative control of polyadenylation that acts on endogenous C12 RNA. Thinking that additional C12 RNA sequences might be necessary for this negative control, we restored these nucleotides progressively in the 5' direction from the CPE (Fig. 7B). For example, RNA Δ1–144 contains 415 nucleotides but was still polyadenylated during oocyte maturation. Similarly, RNA Δ1–109 (450 nucleotides) was adenylated at this time. However, RNAs Δ1–74 (485 nucleotides), Δ1–39 (520 nucleotides), and Δ0 (559 nucleotides) were not adenylated to any appreciable extent (i.e., <30 bases) during maturation. Therefore, the 5' border of a sequence that inhibits C12 RNA polyadenylation during oocyte maturation, but not embryogenesis, resides within the 35 bases present in Δ1–74 RNA but absent from Δ1–109 RNA.

To determine the 3' border of the inhibitory sequence, we retained the 5' region of the element but deleted the...
Figure 7. Polyadenylation analysis of mutant C2 RNAs during oocyte maturation. (A) Radiolabeled RNAs derived from deletion mutants Δ1–454, Δ1–504, and Δ1–454,515–559, AAUAAA, were injected into stage 6 oocytes, some of which were then incubated in progesterone to induce maturation. Oocytes were collected as soon as a white spot was visible at the animal pole or at least at 6 hr after injection. The RNA was extracted and analyzed for polyadenylation as described previously. (B) RNAs containing the denoted sequences were injected into oocytes, some of which were matured with progesterone. The RNA was then extracted and analyzed for the presence of a poly(A) tail. (C) RNAs containing the denoted sequences were analyzed for polyadenylation in control and mature oocytes and 6-hr embryos. (Open boxes) Sequences present; (solid boxes) sequences that are absent. (M) Marker RNA.

330 (Δ142–470), 210 (Δ265–470), 90 (Δ385–470), and 15 nucleotides (Δ455–470) directly upstream of the CPE. All four RNAs, when injected into stage 6 oocytes, were polyadenylated during oocyte maturation [Fig. 7B], demonstrating that the inhibitory sequence spans at least 405 nucleotides. Moreover, because an RNA with an internal deletion was polyadenylated during oocyte maturation [Δ142–324; Fig. 7B], the inhibitory element could be composed of a continuous sequence. Alternatively, it is possible that two distinct elements are required for repression, one of which is removed in RNA Δ142–324.

The data presented thus far indicate that the 3' bound-
ary of the inhibitory sequence extends at least to a few bases 5' of the CPE. To determine whether sequences 3' of the CPE were also necessary for inhibition, we constructed two additional mutants (Fig. 7B). RNAs Δ515–559AAUA and Δ525–559AAUAAA, which lack 20 and 10 nucleotides between the two elements, respectively, were both polyadenylated during oocyte maturation. Therefore, sequences between the CPE and the hexanucleotide are necessary for the inhibition. However, an RNA lacking 18 bases 3' of the hexanucleotide (Δ543–559; Fig. 7B) was polyadenylated only to a very small extent (<20 bases). Thus, sequences 3' of the hexanucleotide are dispensable for efficient inhibition.

Figure 7C shows clearly the difference in the extent of adenylination during maturation and embryogenesis for four RNAs. It is evident that RNAs Δ1–144 and Δ455–470, which lack a complete inhibitory element (cf. Fig. 7B), are polyadenylated in a nearly identical manner in mature oocytes and in 6-hr embryos. In contrast, RNAs Δ1–74 and Δ0, which contain the full inhibitory element, are adenylated in the normally robust manner in 6-hr embryos but receive very few adenylate residues during maturation.

Finally, we have asked whether the 27 uridine and 2 guanosine residues of the C12 CPE are necessary for the inhibition. When this sequence was replaced by the B4 RNA CPE (UUUUUAUAU) in C12-B4CPE (Fig. 7B), polyadenylation was robust. Therefore, the C12 CPE, which is necessary for polyadenylation during embryogenesis, is also required for the inhibition of polyadenylation during oocyte maturation.

Discussion

Identification of a novel cis-acting element involved in cytoplasmic polyadenylation during early embryogenesis

In this study we have shown that C12 RNA contains two cis-acting elements in its 3' UTR that are required for cytoplasmic polyadenylation during Xenopus embryogenesis. The first of these is the nuclear pre-mRNA cleavage and polyadenylation hexanucleotide AAUAAA. This same element has been shown previously to also be necessary for cytoplasmic polyadenylation during oocyte maturation in both Xenopus (Fox et al. 1989; McGrew et al. 1989; Paris and Richter 1990) and mouse (Huarte et al. 1987). The second element is composed of 12 uridines and is now designated as the C12 CPE. This CPE differs from those in several mRNAs that are polyadenylated during oocyte maturation by just 1 or 2 nucleotides. The CPE of B4 RNA, for example, is UUUUUAUAU (Paris and Richter 1990). However, the AU dinucleotide for this RNA, as well as G10 RNA, appears to be crucial for efficient polyadenylation during oocyte maturation. A GG substitution for the AU dinucleotide almost completely abrogates the polyadenylation activity of the two mRNAs (McGrew and Richter 1990; Paris et al. 1991) and, at least in the case of B4 RNA, substantially lowers the ability of the 58-kD CPE-binding protein to interact with the RNA (Paris et al. 1991).

Although many RNAs are known to be polyadenylated during oocyte maturation (Fox et al. 1989; McGrew et al. 1989; Paris and Richter 1990; Salles et al. 1992), many fewer have been shown to be polyadenylated specifically during embryogenesis. However, one RNA, for which sequence data are available, C11 RNA (Paris and Philippe 1990; M. Philippe, pers. comm.), contains 18 uridine residues in its 3' UTR. Although it is not known whether this poly(U) sequence constitutes the CPE of this RNA, we speculate that it would.

Developmental control of polyadenylation is determined by element spacing

In C12 RNA, a reduction of the distance between the CPE and AAUAAA hexanucleotide from 31 to 10 or 20 nucleotides results in maximal polyadenylation at the 4-cell stage rather than the 4000-cell fine-blastula stage, as well as an increase in the number of adenosine residues added from 80 to as many as 130. How the spacing between the two elements could control the timing and extent of polyadenylation is unclear. Perhaps the factors that comprise the polyadenylation apparatus are more stable when the two elements are in close proximity, and this results in precocious and robust polyadenylation. Regardless of the mechanism by which this occurs, element spacing could dictate the timing of polyadenylation, and possibly translation, of other RNAs during embryogenesis that have a C12 CPE-like sequence. Thus, regulated developmental expression of mRNAs need not necessarily involve heretofore undescribed elements. In addition, these data also indicate that the factors that control C12 RNA polyadenylation are present and active as early as the four-cell stage.

Translational control by poly(A) elongation

The data in Figure 5 show that the C12 3' UTR can confer polyadenylation and translational stimulation to CAT mRNA. However, there is a pronounced increase in CAT activity only 1.5 hr after CAT–C12 mRNA injection, which is a time when poly(A) elongation is only beginning to occur. Moreover, there is very little accumulation of CAT activity even up to 6 hr after injection when maximal polyadenylation has been reached. This leveling off of CAT activity is not the result of substrate saturation because an assay performed with diluted extract gives nearly the same results as those presented in Figure 5 (data not shown). This might suggest that poly(A) tail length per se is not the determinant for C12 RNA translation, rather, it could be the ongoing process of poly(A) elongation. This phenomenon has been observed with G10 mRNA during oocyte maturation (McGrew et al. 1989). A long poly(A) tail added to this transcript before injection did not stimulate its translation, nor was the RNA translated when it was prevented from undergoing polyadenylation by insertion of a point mutation in the polyadenylation hexanucleotide (Richter et al. 1990) or
by the addition of cordycepin (3'-deoxyadenosine) to the 3' terminus before injection (McGrew et al. 1989). To determine whether CI2 translation also requires ongoing poly(A) elongation, we added a 73-base poly(A) tail to a CAT–CI2 fusion RNA that contained a G for U substitution in the polyadenylation hexanucleotide, which would prevent further polyadenylation following egg injection. The number 73 was chosen because there are ~73 adenosine residues that injected CI2 3' UTR or injected CAT–CI2 fusion RNA acquires in embryos. This RNA was translated to the same extent as CAT RNA only (Fig. 6) and indicates that the process of poly(A) elongation stimulates the translation of CI2 RNA in embryos.

How poly(A) elongation, or even poly(A) tail length, stimulates translation is unclear. Munroe and Jacobson (1990), however, have suggested that the poly(A)-binding protein (PABP) mediates the translational stimulatory effects of the poly(A) tail. Good evidence in yeast suggests that this is the case (Sachs and Davis 1989). However, Xenopus oocytes appear to contain far less PABP than would be predicted based on the amount of poly(A)+ RNA (Zelus et al. 1989). Although this observation does not rule out a role for PABP in translational regulation during early Xenopus development, it might suggest that this factor is not the main mediator of translation.

**Masking prevents CI2 polyadenylation during oocyte maturation**

As stated above, a shortening of the distance between the CPE and the hexanucleotide AAUAAA results in precarious polyadenylation and demonstrates that the factors responsible for this process are present even in two-cell embryos. We were curious as to whether the activity was present even earlier during development, such as oocyte maturation, even though endogenous CI2 is not polyadenylated at this time. By injecting a 105-base CI2 3' UTR into oocytes that were then induced to mature with progesterone, we showed that CI2 polyadenylation activity is present during oocyte maturation and that it is dependent on the CPE. How does injected CI2 3' UTR escape the inhibition that so effectively prevents endogenous CI2 polyadenylation? The answer apparently is that CI2 RNA, in addition to elements that stimulate polyadenylation during maturation and embryogenesis, contains a large masking element that prevents polyadenylation (and probably also translation) during maturation but not during embryogenesis. This masking element spans up to 468 nucleotides. Interestingly, the dodecauridine CPE also is an integral part of the masking element, as its replacement by the B4 RNA CPE relieves the inhibition of polyadenylation at maturation. Thus, the CI2 CPE is required both for repression and stimulation of polyadenylation during Xenopus development.

Standart et al. (1990) have also defined masking boxes that may control translation in Spisula cyclin A and ribonucleotide reductase mRNAs. Although the translation of these RNAs may not be regulated by polyadenylation, the masking boxes reside in the 3' UTR of the messages and span 120–140 nucleotides. Within these large masking elements are two shorter sequences, UUUUA and YAGUG, which are somewhat repetitive, and another sequence, GUGAUAUA, which is found in both elements. Neither these sequences nor UAAUUUAU, which is thought to be a translational inhibitory element in interferon mRNA (Kruys et al. 1989), are present in the CI2 RNA masking element.

Because the CI2 RNA polyadenylation masking element spans >400 contiguous nucleotides, we suspect that some secondary structure is necessary for its function. Secondary structure has also been suggested to be necessary for mRNA localization in Xenopus (Mowry and Melton 1992) and Drosophila (Macdonald and Struhl 1988). In our case, however, we would envision that the putative secondary structure undergoes some change after fertilization so that it no longer inhibits polyadenylation in embryos. Such a change could be mediated by a developmentally regulated RNA helicase. Irrespective of the mechanism of masking and unmasking, a clearer definition will come once the relevant RNA-binding proteins are detected, characterized, and eventually cloned.

**Materials and methods**

**Plasmid constructions**

The 3'-most 559 nucleotides of the CI2 cDNA clone (Paris and Philippe 1990) were amplified by polymerase chain reaction (PCR) using the 5' oligonucleotide GACGGATCCCGTTAT-ATCATATCGG [oligouonucleotide 1] and the 3' oligonucleotide AGCCGAATTCGGAAAGCAATTTCAAAA (2), and cloned into the BamHI–EcoRI sites of Bluescript KS. This clone was used to generate additional constructs by PCR, which were inserted into the same sites in the vector for all 5' deletions of the CI2 DNA, the same 3' oligonucleotide (2) as that described above was used. The 5' oligonucleotides for the 5' deletions were 1–39, GACGGATCCGGAAATGTTCTTATGCTCTCG (3), A1–74, GACGGATCCGTGTTATCTGCTACCT (4), A1–109, GACGGATCCGGAATGTTCTTATGCTACCT (5), A1–144, GACGGATCCGGAAATGTTCTTATGCTACCT (6), A1–324, GACGGATCCGGAAATGTTCTTATGCTACCT (7), A1–454, GACGGATCCGGAAATGTTCTTATGCTACCT (8) and A1–504 (ΔCPE), GACGGATCCGGAAATGTTCTTATGCTACCT (9). Deletion 1–470 was created by cutting construct Δ1–454 with NheI (in the CI2 sequence) and XbaI (in the upstream polylinker), followed by a Klenow fill-in reaction and religation of the DNA. The deletion of nucleotides 543–559 (Δ1–454,543,559), also called Δ1–454,536–559AATAAA in Figure 4A, was generated by using the DNA transcribed from mutant Δ1–454, annealing it with the 3' oligonucleotide described above (2), and treating the hybrid with RNase H. For the mutation of the AAUAAA → AGAAA, we used the 5' oligonucleotide of Δ1–454 (8) and the 3' oligonucleotide AGCCGAATTCGGAAATGTTCTTATGCTACCT (10).

To define the CPE, annealed oligonucleotides were inserted into the XbaI and BamHI sites of mutant Δ1–504. The following oligonucleotides were used: Δ1–470,485–504, 5’-CTAGTCTAAGGCTAGCTTTTTTTTTTTGGATCCAG (11) and 3’-CTGGA-TGATCCAAAAAAGACCTAGCTCTAGACTAG (12), A1–470,486–504, 5’-CTAGTCTAAGGCTAGCTTTTTTTTTTTTCCAG (13) and 3’-GATCCAAAAAAGACCTAGCTCTAGACTAG (14), A1–470,487–504, 5’-CTAGTCTAAGGCTAGCTTTTTTTTTTTG (15) and 3’-GATC-
AGAGCTAGCT[16], Δ1–470,488–504, 5′-CTA-GAGCTAGCTTTTTTITTTTGGGATTCCCAG (191
AAAAAAAAGCTAGCT [18]; A1-470,495–504, 5′-CTAGTCT-
GGACAAAAG (22); Δ1–454,528–559[AAATAA], AGCCGAATTCCTTTATTGCACAGTG (23); Δ1–454,528–559[AAATAA], AGCCGAAT-
TCTTTATTTTCACTGAC (23); Δ1–454,531–559[AAATAA], AGCCGAATTCTTTATTACATTCGTCGACCGTCTGAC (24); Δ1–454,534–559[AAATAA], AGCCGAATTTCTTTATTTCACTGAC (25). For the substitution mutants we used the same 5′ oligonucleotide (8) and the following oligonucleotides for the 3′ end: Δ1–454,515–559 [21 nucleotides inserted and AATAAA; the inserted sequence has no ability to form a hairpin structure], AGCCGAATTTCTTTATTTACTGCAAGCTAGC (26); Δ1–454,515–559 [2 bases inserted for a PstI site and AAATAA, the inserted sequence has the ability to form a hairpin structure], 5′ oligonucleotide GCCATGGCTGCA (29); and 3′ oligonucleotide AGTAACAACTGG (31); and A454-470, CTAGCTAGCAAAA-
GCA (28) and 3′ oligonucleotide GCCCATGGCTGCA (29); and

To examine the influence of sequence between the CPE and the hexanucleotide on polyadenylation, we constructed several mutants by PCR using oligonucleotide (8) for the 5′ primer and 559(AATAAA), AGCCGAATTCTTTATTAGTAGTTGG (21); and 3′-GATCCAAAA-

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In vitro transcription
All DNA templates were linearized with EcoRI with the exception of CAT, which was linearized with KpnI; CAT-C12, and CAT-C12[AAGAAA], which were linearized with EcoRV; and CAT-C12[AAGAAA]pA73, which was linearized with HindIII. RNA was synthesized in vitro as described by Krieg and Melton (1984) with the following changes: 500 μM of CTP, 500 μM of ATP, 100 μM of UTP, 50 μM of GTP, 500 μM of GpppG, and 30 μCi [3H]UTP [800 Ci/mmol]/10μl assay. All RNAs were generated by T7 RNA polymerase, except for mutant CATC12[AAGAAA]pA73, which was generated by T3 RNA polymerase. The RNAs were extracted with phenol-chloroform and were ethanol precipitated and resuspended in water.

Preparation of eggs and oocytes for microinjection
Manually defolliculated stage 6 oocytes were injected with ∼40 nl of RNA solution and incubated in the absence or presence of 1 μg/ml of progesterone in Barth’s medium. Oocytes were collected as soon as the white spot was visible or at least after 6 hr of the addition of progesterone. Ovulated eggs were fertilized, dejellied in 2% cysteine (pH 7.5), transferred to 5% Ficol in 0.1× Barth’s medium, and microinjected with 20–40 nl of solution containing RNA. Five eggs or developing embryos were collected at time 0 (one cell), 1.5 [four-cell stage], 3.0 [large-cell blastula, 64 blastomeres], 4.5 [medium-cell blastula, ∼500–1000 cells], and 6.0 [fine-cell blastula, >4000 cells] hr after injection.

RNA from oocytes, eggs, and embryos was extracted as described in McGrew et al. (1989) and resolved on a 5% urea-polyacrylamide gel followed by autoradiography. The size of the RNAs was determined by an RNA ladder of sizes from 0.6 to 1.77 kb (GIBCO BRL). RNase H/oligo(DT) treatment of RNA was performed as described (McGrew et al. 1989).

CAT assay
Fertilized eggs were injected with radioactive (for polyadenylation assay) or radioinert (for CAT assay) RNA, incubated for 0, 1.5, 3.0, 4.5, or 6.0 hr, and lysed for RNA extraction or preparation of cell extract. RNA from embryos derived from radioactive RNA-injected eggs was analyzed on a denaturing polyacrylamide gel as described previously. The other embryos were prepared for CAT assays by homogenization in 100 μl of 0.2 M Tris-HCl (pH 8.0) and centrifugation to remove the yolk. Five microliters of the supernatant was then used for CAT assays, as described by Jones et al. (1983).

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