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Further Analysis of Cytoplasmic Polyadenylation in *Xenopus* Embryos and Identification of Embryonic Cytoplasmic Polyadenylation Element-Binding Proteins

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Early development in *Xenopus laevis* is programmed in part by maternally inherited mRNAs that are synthesized and stored in the growing oocyte. During oocyte maturation, several of these messages are translationally activated by poly(A) elongation, which in turn is regulated by two *cis* elements in the 3′ untranslated region, the hexanucleotide AAUAAA and a cytoplasmic polyadenylation element (CPE) consisting of UUUUUAU or similar sequence. In the early embryo, a different set of maternal mRNAs is translationally activated. We have shown previously that one of these, Cl2, requires a CPE consisting of at least 12 uridine residues, in addition to the hexanucleotide, for its cytoplasmic polyadenylation and subsequent translation (R. Simon, J.-P. Tassan, and J. D. Richter, Genes Dev. 6:2580–2591, 1992). To assess whether this embryonic CPE functions in other maternal mRNAs, we have chosen Cl1 RNA, which is known to be polyadenylated during early embryogenesis (J. Paris, B. Osborne, A. Couturier, R. LeGuellec, and M. Philippe, Gene 72:169–176, 1988). Wild-type as well as mutated versions of Cl1 RNA were injected into fertilized eggs and were analyzed for cytoplasmic polyadenylation at times up to the gastrula stage. This RNA also required a poly(U) CPE for cytoplasmic polyadenylation in embryos, but in this case the CPE consisted of 18 uridine residues. In addition, the timing and extent of cytoplasmic poly(A) elongation during early embryogenesis were dependent upon the distance between the CPE and the hexanucleotide. Further, as was the case with Cl2 RNA, Cl1 RNA contains a large masking element that prevents premature cytoplasmic polyadenylation during oocyte maturation. To examine the factors that may be involved in the cytoplasmic polyadenylation of both Cl2 and Cl1 RNAs, we performed UV cross-linking experiments in egg extracts. Two proteins with sizes of ~36 and ~45 kDa interacted specifically with the CPEs of both RNAs, although they bound preferentially to the Cl2 CPE. The role that these proteins might play in cytoplasmic polyadenylation is discussed.

Maternally inherited mRNAs are in large part responsible for the pattern of early development in many organisms. These transcripts are synthesized and stored in a translationally dormant form in the growing oocyte and are activated during oocyte maturation, at fertilization, or in early embryogenesis. Although no single mechanism regulates the translation of maternal mRNAs (29), it is clear that *cis* elements in the 3′ untranslated region (3′UTR) play an important role in this process. Such *cis* elements have a variety of functions that effect mRNA translation, such as message localization, masking, and polyadenylation. In *Drosophila melanogaster*, for example, the correct localization of certain mRNAs is crucial for their translation and consequent pattern formation (reviewed in references 19 and 37). In one case, *nanos* RNA, a maternal transcript that is normally confined to the posterior pole of the embryo, contains an element in its 3′UTR that is necessary for this localization (8). When this element is deleted, the RNA is distributed throughout the embryo. Although this nonlocalized RNA is stable, it is not translated. This could be due to the action of a specific translational repressor, which may be removed by the posterior pole localization apparatus and thus allow *nanos* mRNA translation in that region of the embryo (9).

Translational control also plays an important role in the development of *Caenorhabditis elegans* (1, 2, 6, 11). *tra-2* mRNA, whose product is required for oocyte development in hermaphrodite animals, contains an element in its 3′UTR that can be negatively regulated. Under such regulation, no *tra-2* protein is produced; as a consequence, the germ cell lineage's end product is sperm rather than oocyte production. The sequence within the *tra-2* mRNA 3′UTR that is under negative control is composed of two 28-base direct repeat elements, whose interaction with a specific factor may effect translational repression (11).

Although the *cis* elements governing mRNA localization and repression of translation vary widely (reviewed in references 19 and 35), those activating translation by polyadenylation appear to be more conserved. For example, a number of mRNAs that are translationally dormant in oocytes contain poly(A) tails that are relatively short (20, 39). Following the induction of oocyte maturation, the poly(A) tails are elongated to 150 nucleotides or greater, and the RNAs then assemble into polysomes (20, 26, 36). Generally, the sequences controlling maturation-specific polyadenylation, at least in *Xenopus laevis* and mice, are the hexanucleotide AAUAAA and UUUUUAU or a similar cytoplasmic polyadenylation element (CPE) (7, 20, 26, 31). Other maternal mRNAs in *X. laevis* are translated in the early embryo (18, 24, 25), and in at least one case, it also is controlled by cytoplasmic poly(A) elongation. Cl2 RNA begins to undergo poly(A) addition about 1.5 h after fertilization and continues up to the blastula stage 6 h later (33). Polyadenylation of this RNA is regulated by both a CPE consisting of a poly(U) sequence and a hexanucleotide sequence in the 3′UTR. Two additional features of Cl2 RNA determine when this RNA is polyadenylated and translated during development. First, the number of nucleotides, but not

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a specific sequence, between the CPE and the hexanucleotide determines the time during embryogenesis when the RNA is polyadenylated; the shorter the distance is, the earlier polyadenylation occurs after fertilization. Second, a masking element spanning up to 468 nucleotides prevents precocious polyadenylation during oocyte maturation. When this element is deleted, the RNA is prematurely polyadenylated. Thus, the factors that promote poly(A) elongation of Cl2 RNA are present and active during oocyte maturation but are inhibited by the masking element (33).

In this study, we have sought to determine whether the embryonic poly(U)-type CPE and masking element present in Cl2 RNA also control polyadenylation in other RNAs. Here we show that Cl1 RNA (38) has a poly(U)-type CPE that is necessary for cytoplasmic polyadenylation in *Xenopus* embryos. As is the case with Cl2 RNA, the distance between the CPE and the hexanucleotide sequence has an important function in determining the timing of Cl1 polyadenylation. Finally, Cl1 RNA also contains a large masking element in its 3′UTR that prevents polyadenylation during maturation. Furthermore, we have identified two proteins with sizes of 36 and 45 kDa that photo-cross-link specifically to the CPEs of both Cl1 and Cl2 RNAs. How these proteins might be involved in the regulation of cytoplasmic polyadenylation is discussed.

MATERIALS AND METHODS

Plasmid constructs. Plasmids Cl2Δ1-74 (containing all regulatory *cis* elements), Cl2Δ1-454 (containing the wild-type CPE and the hexanucleotide), Cl2Δ1-470,488-504 (12 uridine residues in the CPE), Cl2Δ1-470,486-504 (10 U's), Cl2Δ1-470,485-504 (9 U's), Cl2Δ1-504 (no CPE), and Cl2Δ1-454,515-559AAUAAA (closer proximity of CPE and hexanucleotide) are described in reference 33. Plasmid Cl2Δ1-454,515-559 was constructed by digesting plasmid Cl2Δ1-454, substitution 515-535AAUAAA(a), a construct with nonspecific sequences between the CPE and the hexanucleotide (35), with *Nco*I (located between the CPE and the hexanucleotide) and *Eco*RI (located downstream in the vector); the larger fragment containing the CPE sequence was filled in with the Klenow enzyme and religated. Plasmid Cl2Δ1-454,480-559 was made by digesting plasmid Cl2Δ1-454 with *Nhe*I (just upstream of the CPE) and *Eco*RI (located downstream in the vector); the larger fragment, which contained only a few Cl2 sequences upstream of the CPE, was filled in with Klenow enzyme and religated. Plasmids Cl1-3′UTR, Cl1Δ1-179, Cl1Δ1-219, and Cl1Δ235-505AUUAAA were constructed by PCR using the following oligonucleotides and a subclone of the Cl1 cDNA (38), encoding the 1,071 3′ nucleotides, as a template: Cl1-3′UTR, 5′ oligonucleotide CCAAGCTTCATAGTGGAAG CC (oligonucleotide 1) and 3′ oligonucleotide CGGATCCG AGGATTACACTTTAATGTCTG (oligonucleotide 2); Cl 1Δ1-179, 5′ oligonucleotide CCAAGCTTGGTTCCTGTAT TTTAAG (oligonucleotide 3) and oligonucleotide 2; and Cl1Δ235-505AUUAAA, 5′ oligonucleotide 1 and 3′ oligonucleotide CGGATCCTTTAATCCATAGTATAAAGTT (oligonucleotide 5). All Cl1 constructs were cloned into the *Hin*dIII-*Bam*HI sites of the Bluescript KS vector. Plasmid Cl1Δ287-468 was constructed by digesting plasmid Cl1-3′UTR with *Eco*RV and *Pst*I (both restriction sites located between the CPE and the hexanucleotide), and the gel-purified large fragment was treated with T4 DNA polymerase to create blunt ends and religated.

In vitro transcription. All Cl1 plasmids were linearized with *Bam*HI, and Cl2 plasmids used to synthesize sense RNAs were linearized with *Eco*RI. Cl2 plasmids used for antisense RNAs were linearized as follows: Cl2Δ1-454 with *Nhe*I; Cl2Δ1-504 with *Bam*HI; and Cl2Δ1-454,515-559 and Cl2Δ1-454,480-559 with *Sac*I. ^{32}P-labeled RNA was synthesized in vitro as described by Krieg and Melton (16), with the following changes: 500 μM CTP, 500 μM ATP, 100 μM UTP, 50 μM GTP, 500 μM GpppG, and 30 μCi of [^{32}P]UTP (800 μCi/mmol) per 10-μl assay mixture. Unlabeled RNAs used in the competition experiments were synthesized with an Ambion Megascript kit. Cl1 sense RNAs and Cl2 antisense RNAs were generated by T3 RNA polymerase, and Cl2 sense RNAs were generated by T7 RNA polymerase.

Microinjection of eggs and oocytes. Manually defolliculated stage VI oocytes were injected with ~10 to 20 nl of RNA solution (50 to 100 ng/μl) and incubated in the presence or absence of 1 μg of progesterone per ml in Barth's medium. Oocytes were collected as soon as a white spot was visible at the animal pole, which is indicative of meiotic maturation, or at most 6 h after the addition of progesterone. Ovulated eggs were fertilized and dejellied in 2% cysteine (pH 7.5), transferred to 5% Ficoll in 0.1× Barth's medium, and microinjected with ~10 to 20 nl of RNA solution. Five eggs or embryos were collected at 0 (one cell), 1.5 (four-cell stage), 3 (large-cell blastula, 64 blastomeres), 4.5 (medium-cell blastula), 6 (fine-cell blastula, >4,000 cells), 9 (initial gastrula stage), or 12 (blastopore stage; stages defined by Nieuwkoop and Faber [23]) h after injection.

RNA from oocytes, eggs, and embryos was extracted as described by McGrew et al. (20) and analyzed on a 5% urea–polyacrylamide gel and then by autoradiography. The extent of polyadenylation was determined by scanning densitometry of autoradiographs.

UV cross-linking. Egg extracts were prepared as described by Murray and Kirschner (22). ^{32}P-labeled RNA was added to *Xenopus* egg extracts in the presence of heparin (final concentration, 5 μg/μl) and incubated for 30 min at room temperature. The samples were transferred to a microtiter plate, placed ~4 cm from a UV light source (Black-Ray XX-15 lamp), and irradiated for 10 min at 4°C with 254-nm light. RNase A (final concentration, 2 mg/ml) was added, and the mixture was incubated for 20 min at 37°C. An equal volume of sodium dodecyl sulfate (SDS) sample buffer was added, and the samples were boiled for 3 min and analyzed on an SDS–10% polyacrylamide gel and then by autoradiography. For the competition experiments, 10 ng of ^{32}P-labeled RNA was mixed with a 10-, 20-, or 50-fold excess of unlabeled RNA and then added to the egg extract. The samples were then exposed to UV light and processed as described above. For the antisense competition experiments, RNAs were incubated at 80°C for 5 min and cooled to room temperature before being added to the egg extract.

RESULTS

A poly(U) CPE is required for cytoplasmic polyadenylation of Cl1 mRNA during early *Xenopus* embryogenesis. We have shown previously that a dodecauridine CPE is required for cytoplasmic polyadenylation and subsequent translational activation of the maternal Cl2 mRNA during early *Xenopus* embryogenesis (33). To investigate whether additional maternal mRNAs require a poly(U) CPE for embryonic polyadenylation, we focused on Cl1 mRNA. This RNA, like Cl2 RNA, contains a large poly(U) region in its 3′UTR that consists of 18 U residues. To determine whether the 18 U residues function as a CPE, we injected fertilized eggs with in vitro-synthesized RNA encoding 505 nucleotides of the most distal region of the 3′UTR (Cl1-3′UTR; Fig. 1), as well as RNAs lacking specific

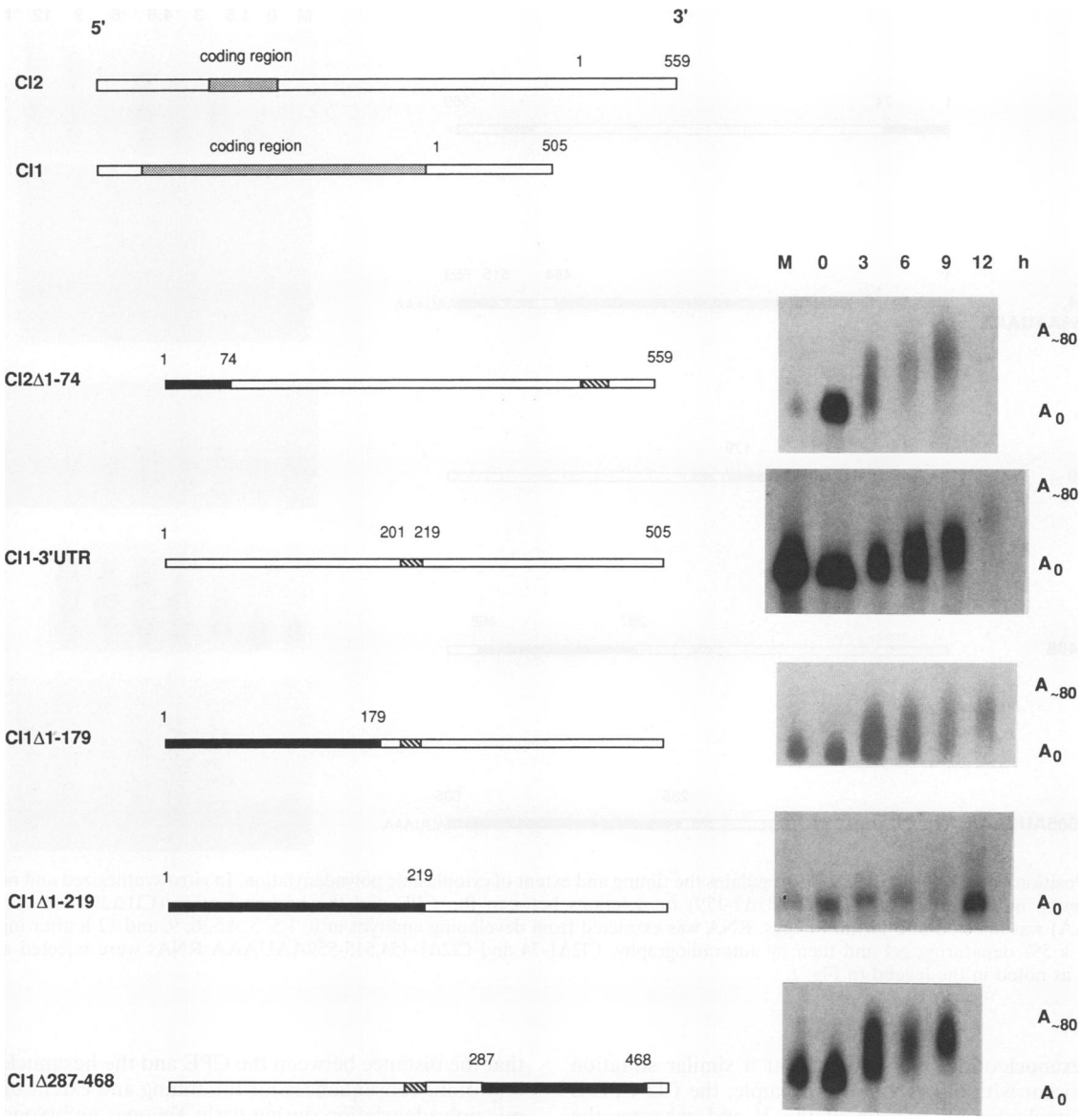

FIG. 1. Analysis of cytoplasmic poly(A) elongation of Cl1 RNA during early *Xenopus* embryogenesis. In vitro-synthesized and radiolabeled RNA containing 505 nucleotides (Cl1-3'UTR), 326 nucleotides (Cl1Δ1-179), or 286 nucleotides (Cl1Δ1-219) of the most distal region of the 3'UTR of Cl1 RNA and an RNA with a deletion in the most distal portion of the 3'UTR (Cl1Δ287-468) were injected into fertilized eggs. RNA was extracted from five eggs or embryos at 0, 3, 6, 9, and 12 h after injection and analyzed on a 5% denaturing gel and then by autoradiography. Cl2Δ1-74 was injected as a control RNA. M, marker RNA; open box, sequence present; solid box, sequence deleted; striped box, poly(U) sequence.

sequences of this region (Cl1Δ1-179, Cl1Δ287-468, and Cl1Δ1-219; Fig. 1). Five embryos were collected at 0 (one-cell stage), 3 (large-cell blastula, 64 blastomeres), 6 (fine-cell blastula, >4,000 cells), 9 (initial gastrula stage), and 12 (blastopore stage; stages defined by Nieuwkoop and Faber [23]) h after injection, and the RNA was extracted and analyzed for polyadenylation on a 5% denaturing gel and then by autoradiography. As a control, we also injected Cl2Δ1-74 RNA, which we have previously shown to be polyadenylated during early *Xenopus* embryogenesis (33). Cl1-3'UTR RNA and Cl1Δ1-179 RNA, which contains 326 3'-terminal nucleotides, both received poly(A) tails of ~80 A residues by 12 h of embryogenesis. This is similar in length to the poly(A) tail received by Cl2 RNA by 6 h of embryogenesis (Fig. 1). However, Cl1Δ1-219 RNA, which lacks 18 U residues but otherwise contains mostly the same sequences as Cl1Δ1-179 RNA, was not polyadenylated (Fig. 1). Deletion of sequences 3' of the poly(U) region (Cl1Δ287-468) did not prevent poly(A) elongation of Cl1 RNA (Fig. 1). Thus, these data show that poly(U)$_{18}$ functions as a CPE for Cl1 RNA during embryogenesis.

The distance between the CPE and the hexanucleotide in the 3'UTR regulates the timing of cytoplasmic polyadenylation during early embryogenesis. Although Cl1 and Cl2 RNAs use poly(U) as a CPE and are polyadenylated to similar extents, there is some difference in the timing of the polyadenylation reaction. Cl1 RNA is polyadenylated only by about 50% at 9 h and finally 100% at 12 h postinjection. Cl2 RNA, on the other hand, is 100% polyadenylated by 6 h (Fig. 1) (33). Because we have shown previously that the timing and extent of polyadenylation are highly sensitive to the distance between the CPE

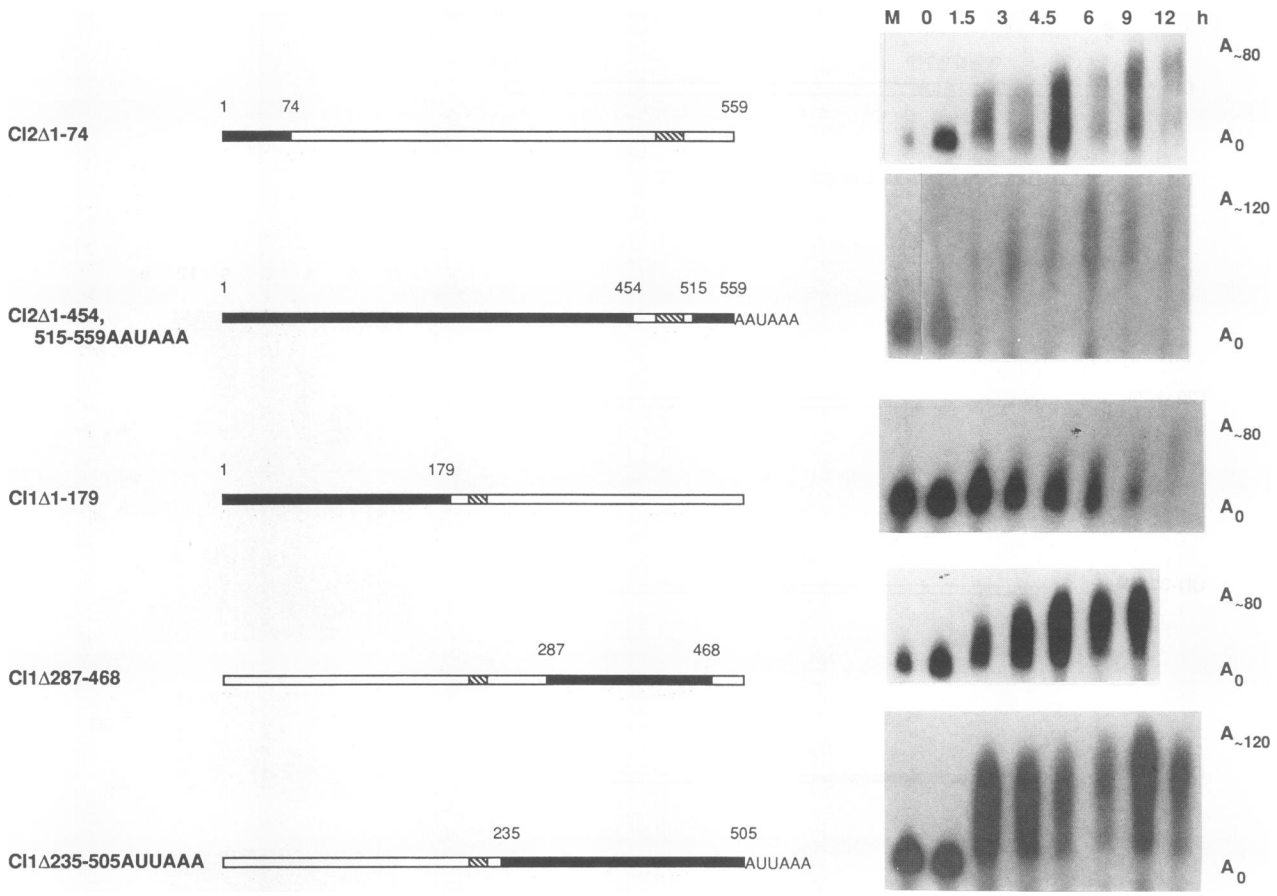

FIG. 2. Position of the CPE in the 3′UTR regulates the timing and extent of cytoplasmic polyadenylation. In vitro-synthesized and radiolabeled RNA containing most of the Cl1 3′UTR (Cl1Δ1-179) or deletions between the CPE and the hexanucleotide (Cl1Δ287-468 and Cl1Δ235-505AUUAAA) was injected into fertilized eggs. RNA was extracted from developing embryos at 0, 1.5, 3, 4.5, 6, 9, and 12 h after injection and analyzed on a 5% denaturing gel and then by autoradiography. Cl2Δ1-74 and Cl2Δ1-454,515-559AAUAAA RNAs were injected as controls. Symbols are as noted in the legend to Fig. 1.

and the hexanucleotide, we thought that a similar situation could also occur with Cl1 RNA. For example, the Cl2 CPE is located 54 nucleotides upstream of the 3′ end, whereas the CPE of Cl1 RNA resides 286 nucleotides upstream of the 3′ end. To assess whether the differences in the timing of polyadenylation between Cl1 and Cl2 RNAs could be due to the distance between the CPE and the hexanucleotide in the 3′ UTR, we deleted 181 (Cl1Δ287-468) and 270 (Cl1Δ235-505AUUAAA) nucleotides between the CPE and the hexanucleotide of the Cl1-3′UTR and injected the in vitro-synthesized RNAs into fertilized eggs. The RNAs were then analyzed at 0, 1.5, 3, 4.5, 6, 9, and 12 h after injection. Cl1Δ287-468 RNA was 50% polyadenylated by 3 h after injection, compared with about 50% at 9 h for the wild-type Cl1 3′UTR (Cl1Δ1-179; Fig. 2). This timing of polyadenylation was similar to the one observed for the wild-type Cl2 3′UTR (Cl2Δ1-74; Fig. 2). Cl1Δ235-505AUUAAA RNA, which contains only 14 nucleotides between the CPE and the hexanucleotide, was 100% polyadenylated by 1.5 h after injection. In addition, this RNA received a poly(A) tail of up to approximately 120 A residues, compared with a maximum poly(A) tail of 80 residues for the wild-type 3′UTR (Fig. 2). Moreover, this extensive polyadenylation was similar to that for Cl2Δ1-454,515-559AAUAAA RNA (Fig. 2), which contains only 10 nucleotides between the CPE and the hexanucleotide. Thus, these results demonstrate that the distance between the CPE and the hexanucleotide has important consequences for the timing and extent of cytoplasmic polyadenylation during early *Xenopus* embryogenesis and is at least partly responsible for the differences in the timing of polyadenylation between Cl1 and Cl2 RNAs.

Cl1 RNA contains a masking element to prevent cytoplasmic polyadenylation during oocyte maturation. Although endogenous Cl1 and Cl2 RNAs are polyadenylated during embryogenesis, neither is polyadenylated during oocyte maturation (24, 33). At least for Cl2 RNA, the switch in developmental control of polyadenylation is regulated by a large masking element that precludes precocious polyadenylation and subsequently translation. To determine whether a masking element also prevents the polyadenylation of Cl1 RNA during oocyte maturation, we injected in vitro-synthesized Cl1-3′UTR, Cl1Δ1-179, Cl1Δ1-219, and Cl1Δ287-468 RNAs into stage VI oocytes, which were then incubated in the absence or presence of progesterone, the natural inducer of meiotic maturation. As a control RNA, we injected Cl2Δ1-454 RNA, which lacks the Cl2 masking element and therefore should be polyadenylated during oocyte maturation. The maturing oocytes were collected as soon as a white spot was visible at the animal pole or at most 6 h after injection. RNAs Cl1-3′UTR, Cl1Δ1-179, and Cl1Δ1-219, all of which contained the most distal 3′ region of the 3′UTR, did not gain a poly(A) tail during this time (Fig. 3).

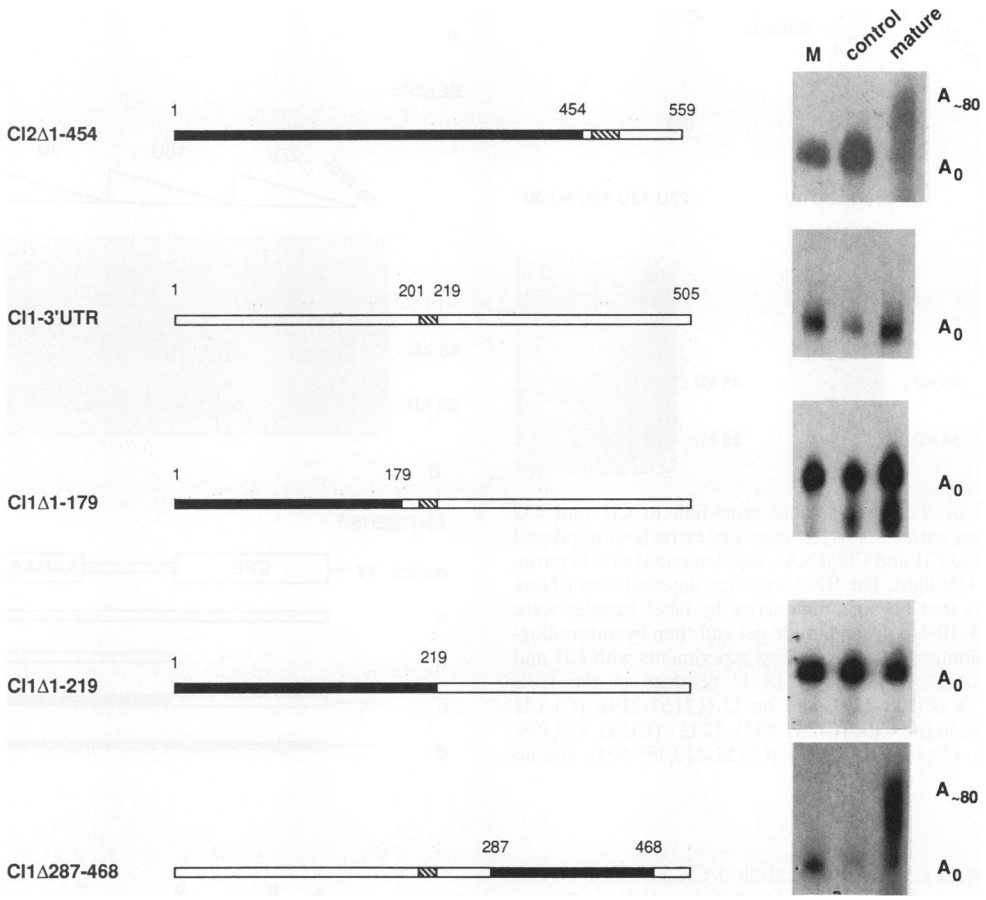

FIG. 3. Analysis of cytoplasmic polyadenylation of Cl1 RNA during oocyte maturation. RNAs encoding Cl1-3'UTR, Cl1Δ1-179, Cl1Δ1-219, and Cl1Δ287-468 were injected into stage VI oocytes, which were subsequently incubated in the absence (control) or presence (mature) of progesterone. Five oocytes were collected as soon as a white spot was visible or at the latest at 6 h after injection. RNA was extracted and analyzed on a 5% gel and then by autoradiography. Symbols are as noted in the legend to Fig. 1.

However, injected RNA Cl1Δ287-468, which lacked sequences between the CPE and the hexanucleotide, was polyadenylated during oocyte maturation, suggesting that sequences downstream of the CPE are involved in the abrogation of polyadenylation of this RNA during oocyte maturation. Therefore, both Cl1 and Cl2 RNAs contain a masking element.

Proteins that interact with the Cl1 and Cl2 CPEs. To examine the factors that might be involved in cytoplasmic polyadenylation during early embryogenesis, we have sought to identify the proteins that interact with the CPEs of Cl1 and Cl2 RNAs. To achieve this, we chose to perform UV cross-linking experiments with *Xenopus* egg extracts. Radiolabeled Cl1 and Cl2 RNAs were added to extracts, incubated in the presence of heparin to minimize nonspecific RNA-protein interactions, and irradiated with UV light. The proteins that became radioactive by label transfer were then analyzed on an SDS–10% polyacrylamide gel and then by autoradiography. Figure 4A shows that although several proteins cross-linked to the two RNAs, two prominent proteins with molecular sizes of 36 and 45 kDa bound to both. To investigate whether these two proteins bind to the poly(U) CPE, we performed a cross-linking experiment using Cl1 RNAs with 18 (Cl1-3'UTR and Cl1Δ1-179) or 0 (Cl1Δ1-219) uridine residues in their CPEs. Only the RNAs containing the poly(U) CPE were able to interact with the 36- and 45-kDa proteins (Fig. 4B), suggesting that these proteins cross-link specifically to the CPE.

To examine further the binding specificities of the 36- and 45-kDa proteins, we performed cross-linking experiments with Cl2 RNAs that contained progressively fewer U residues in the CPEs (Fig. 4B and C). The Cl2 3'UTR RNA containing 27 (wild type) or 12 U residues in the CPE was cross-linked very strongly to the 36- and 45-kDa proteins. An RNA containing a CPE with 10 U residues still cross-linked to the proteins, though to a lesser extent. An RNA containing a CPE with only nine U residues, however, cross-linked only the 45-kDa protein; an RNA whose entire CPE was deleted was not cross-linked to either protein. These results correlate very well with the cytoplasmic polyadenylation of these different RNAs in injected *Xenopus* embryos (33). Cl2 RNA containing a CPE with 12 U residues was polyadenylated as extensively as wild-type RNA (27 U). A Cl2 RNA containing a CPE with only 10 U residues was polyadenylated, albeit to a reduced amount, and a Cl2 RNA containing only 9 U residues in its CPE was not polyadenylated at all. Thus, the extent of Cl2 RNA polyadenylation in vivo closely correlates with the extent of binding of the 36-kDa protein to the CPE in vitro. Given this finding, we speculate that the 36-kDa protein and possibly the 45-kDa protein could function in cytoplasmic polyadenylation.

Specificity of binding of the 36- and 45-kDa proteins. To further examine the specificity of binding of the 36- and 45-kDa proteins to the CPE, we performed competition experiments by adding increasing amounts of unlabeled sense and

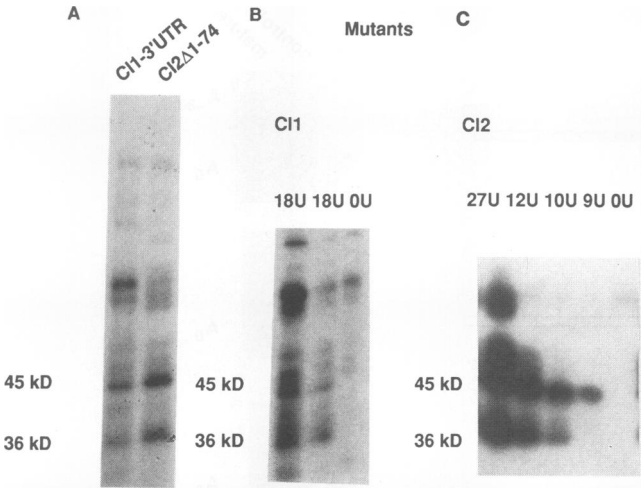

FIG. 4. Analysis of the proteins that cross-link to Cl1 and Cl2 RNAs in *Xenopus* egg extracts. (A) *Xenopus* egg extracts were primed with [^{32}P]UTP-labeled Cl1 and Cl2 RNAs, supplemented with heparin, and irradiated with UV light. The RNA was then digested with RNase A, and the proteins that became radioactive by label transfer were analyzed on an SDS–10% polyacrylamide gel and then by autoradiography. (B and C) Similar UV cross-linking experiments with Cl1 and Cl2 mutant RNAs. (B) Cl1 RNAs, 18 U residues in the CPE (Cl1-3′UTR), 18 U's (Cl1Δ1-179), and no U (Cl1Δ1-219); (C) Cl2 RNAs, 27 U residues in the CPE (Cl2Δ1-454), 12 U's (Cl2Δ1-470,488-504), 10 U's (Cl2Δ1-470,486-504), 9 U's (Cl2Δ1-470,485-504), and no U (Cl2Δ1-504).

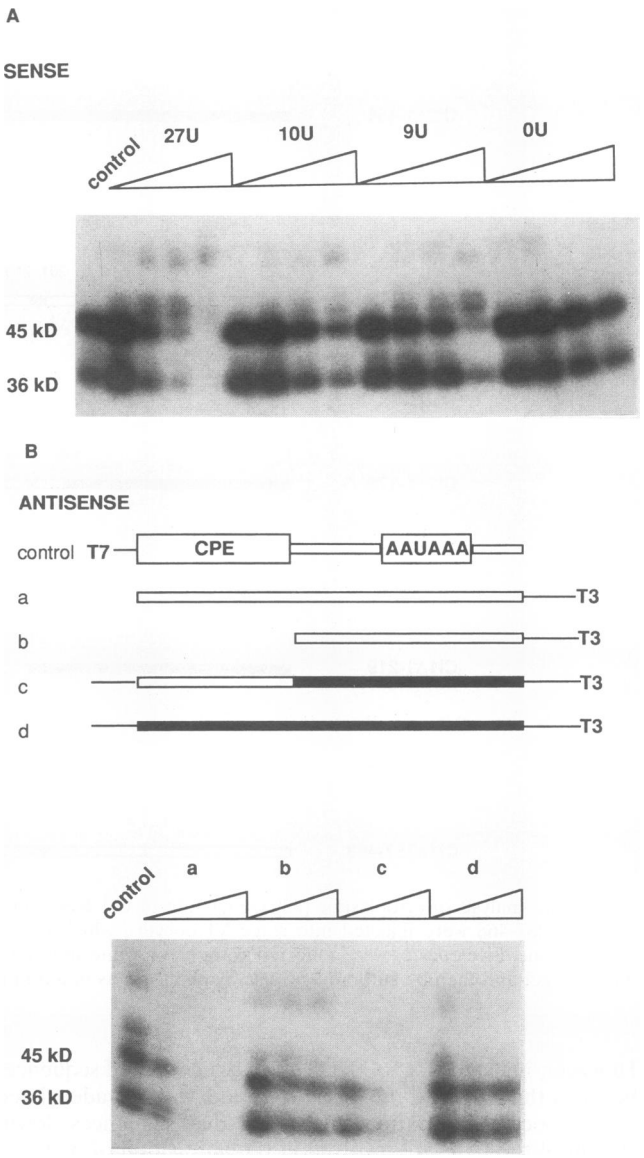

FIG. 5. Analysis of the binding specificities of the 36- and 45-kDa proteins to the CPE of Cl2 RNA. Radiolabeled Cl2Δ1-454 RNA was mixed with unlabeled sense and antisense RNAs as indicated, added to *Xenopus* egg extracts, and irradiated with UV light. The resulting radioactive proteins were analyzed on an SDS–10% polyacrylamide gel and then by autoradiography. (A) Competition experiments with sense RNA. Increasing amounts (10, 100, 200, and 500 ng) of unlabeled Cl2 RNA containing 27 (wild-type RNA, Cl2Δ1-454), 10 (Cl2Δ1-470,486-504), 9 (Cl2Δ1-470,485-504), and no (Cl2Δ1-504) U residues in the CPE were added to 10 ng of radiolabeled Cl2Δ1-454 RNA. (B) Competition experiments with antisense RNA. Increasing amounts (100, 200, and 500 ng) of unlabeled Cl2 antisense RNA were mixed with 10 ng of radiolabeled Cl2Δ1-454 RNA (156 bases, including vector sequence), incubated at 85°C for 5 min, and then cooled to room temperature before addition to *Xenopus* egg extracts. Antisense RNA a contained the wild-type 3′ end of Cl2 RNA encoding the CPE and the hexanucleotide region (Cl2Δ1-454, 171 bases), antisense RNA b encoded the hexanucleotide and sequences between the elements (Cl2Δ1-504, 121 bases), antisense RNA c encoded the CPE sequence only (Cl2Δ1-454,515-559, 150 bases), and antisense RNA d encoded nonspecific sequences (i.e., polylinker, Cl2Δ1-454,480-559, 105 bases). Open box, sequences present; solid box, sequences deleted; control, radiolabeled Cl2Δ1-454 RNA only incubated with *Xenopus* egg extract.

antisense RNAs with radioactively labeled Cl2Δ1-454 RNA to egg extract, which was then subjected to UV irradiation. Figure 5A shows that Cl2Δ1-454 RNA, which contains a CPE with 27 U residues, competed very well with itself, but competitor RNAs containing only 10 or 9 U residues show substantially reduced competition for binding. No competition for binding of the 36- and 45-kDa proteins was observed with an RNA containing no CPE. Similar competition studies were carried out with an antisense RNA that covered the 105 3′-terminal sequences of Cl2 RNA including the CPE and the hexanucleotide (Fig. 5B, RNA a) and truncated versions of this RNA (Fig. 5B, RNAs b to d). The addition of the entire antisense RNA (RNA a, Cl2Δ1-454, 171 bases) as well as one that was complementary only to the CPE (RNA c, Cl2Δ1-454,515-559, 150 bases), prevented binding of the 36- and 45-kDa proteins. However, RNAs that did not contain regions that were complementary to the CPE (RNAs b and d, Cl2Δ1-504 [121 bases] and Cl2Δ1-454,480-559 [105 bases]) did not prevent binding of the two proteins. Thus, these experiments demonstrate that the binding of the 36- and 45-kDa proteins to the CPE is specific.

As we have shown, the 36- and 45-kDa proteins bind to the CPEs of both Cl1 and Cl2 RNAs. To examine whether the proteins bind to these CPEs with the same avidity, we performed a competition experiment with the two RNAs (Fig. 6A). First, Cl2Δ1-74 RNA containing a CPE with 27 uridine residues was radiolabeled and mixed with increasing amounts of the same unlabeled RNA or unlabeled Cl1-3′UTR RNA (CPE with 18 uridine residues) and then added to an egg extract for UV cross-linking. As before, Cl2Δ1-74 competed with itself for binding. However, Cl1-3′UTR did not compete well even at the highest level of competitor RNA (50-fold molar excess). In a reciprocal experiment, Cl1-3′UTR was radiolabeled and mixed with unlabeled Cl1-3′UTR and Cl2Δ1-74 RNAs. While Cl1-3′UTR RNA competed with itself

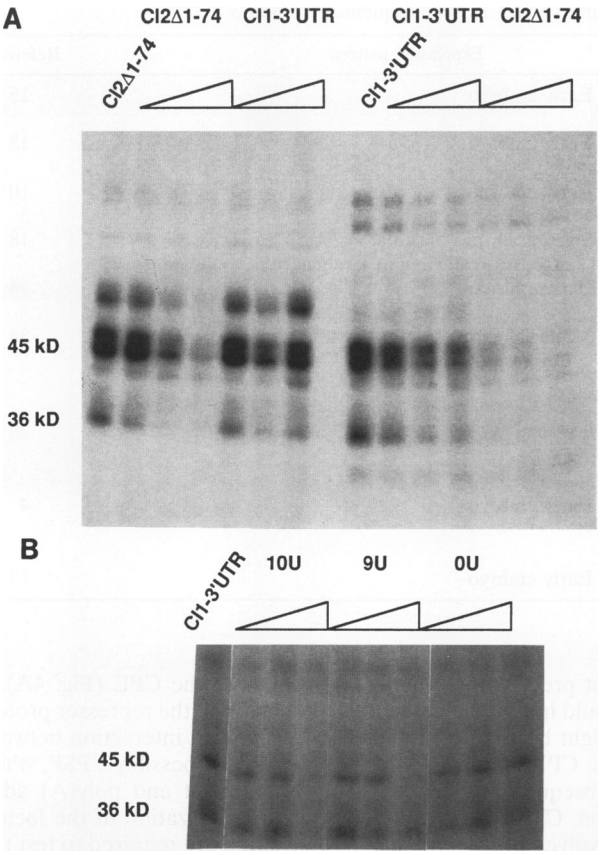

FIG. 6. (A) Competition analysis between Cl1 and Cl2 RNAs for binding of the 36- and 45-kDa proteins. Ten nanograms of radiolabeled Cl2Δ1-74 RNA or Cl1-3'UTR RNA was mixed with 100, 200, and 500 ng of unlabeled Cl2Δ1-74 or Cl1-3'UTR; added to *Xenopus* egg extracts; and irradiated with UV light. The resulting radioactive proteins were analyzed on an SDS–10% polyacrylamide gel and then by autoradiography. (B) Competition analysis among Cl1-3'UTR and RNAs containing 10, 9, and no U residues. Ten nanograms of radiolabeled Cl1-3'UTR was mixed with 25, 50, and 125 ng of unlabeled Cl2Δ1-470,486-504 (10 U's), Cl2Δ1-470,485-504 (9 U's), and Cl2Δ1-504 (no U's); added to *Xenopus* extracts; and irradiated with UV light. The samples were analyzed as described for panel A.

for binding to the 36- and 45-kDa proteins, Cl2Δ1-74 RNA was an even better competitor.

In a final competition experiment, we have examined the ability of Cl1 RNA to bind the 36- and 45-kDa proteins in the presence of Cl2 RNAs whose CPEs have different numbers of uridine residues. As shown in Fig. 6B, a Cl2 RNA with no CPE did not compete for the binding of either protein. When the CPE had 9 uridine residues, it competed somewhat for the binding of the 45-kDa protein, and when the CPE contained 10 uridine residues, it competed for the binding of both proteins. Therefore, these data, together with those presented in Fig. 4 and 5, show that the 36- and 45-kDa proteins bind most tightly to the Cl1 and Cl2 CPEs when they contain 10 or more uridine residues.

DISCUSSION

In an earlier study, Simon et al. (33) showed that a maternal mRNA, Cl2, required a poly(U) CPE and AAUAAA hexanucleotide to undergo cytoplasmic poly(A) elongation, and subsequent translational activation, in early *Xenopus* embryogenesis. In this study, we have demonstrated that an additional maternal mRNA, Cl1, also requires a poly(U) CPE for embryonic polyadenylation. These results suggest that the poly(U) CPE may be a general regulatory element for those RNAs that undergo cytoplasmic polyadenylation in embryos. However, only two Cl-type RNAs were identified by Paris et al. (24) in their original differential hybridization using poly(A)$^+$ RNA from eggs and cleavage-stage embryos. To identify additional maternal transcripts whose expression in early embryos may be regulated by CPE-dependent polyadenylation, we screened the GenEMBL database, a sample from which is presented in Table 1. RNAs encoding such proteins as the activin receptor, noggin, and fibronectin all contain long U stretches in their 3'UTRs, which we hypothesize could function as CPEs.

Developmental regulation of cytoplasmic polyadenylation. Although Cl1 and Cl2 RNAs have nearly identical CPEs, they are polyadenylated at different times during embryogenesis (33) (Fig. 1). At least one parameter responsible for this is the position of the CPE relative to the hexanucleotide. For example, Cl2 RNA contains a CPE that resides 55 nucleotides upstream of the 3' end and has a distance of 31 nucleotides between the two *cis* elements. This RNA is polyadenylated by about 50% 3 h after injection. When the distance between the *cis* elements was reduced to 10 nucleotides, the RNA was polyadenylated by 100% 1.5 h after injection, which was the earliest time tested (33) (Fig. 2). Cl1 RNA, whose CPE is 271 bases upstream of the most distal hexanucleotide, is polyadenylated by about 50% at 9 h after injection. A deletion of the sequence between the *cis* elements, which brings them within 14 bases of one another, results in 100% polyadenylation at 1.5 h (RNA Cl1Δ235-505AUUAAA; Fig. 2). In addition to timing, the length of the poly(A) tail that the RNAs acquire also is affected by the number of nucleotides between the two *cis* elements. That is, the 80-base poly(A) tail acquired by Cl1 RNA is increased to about 120 bases in Cl1Δ235-505AUUAAA. Thus, the timing and extent of embryonic polyadenylation are defined not by an additional element but instead by the proximity of the two *cis* elements. In this respect, embryonic polyadenylation differs from that which occurs during oocyte maturation. Although maternal mRNAs are polyadenylated at different times and to different extents during oocyte maturation, this might be due rather to slight differences in the maturation-type CPE and not to the distance between the CPE and the hexanucleotide (21, 26, 32). Indeed, one report shows directly that RNAs containing the *cis* elements in proximity do not gain a longer poly(A) tail during maturation as might be predicted from the results obtained with Cl1 and Cl2 RNAs during embryogenesis (26).

In addition to the CPE and hexanucleotide, both Cl1 and Cl2 RNAs have a third element that prevents precocious polyadenylation during oocyte maturation. For Cl2 RNA, a 468-base sequence which includes the CPE prevents polyadenylation during maturation but not embryogenesis. The Cl1 RNA masking element contains at most 326 bases, although the contribution of the CPE to the inhibitory function of this sequence has not been determined. Surprisingly, a comparison of the two masking elements reveals no similarities in sequence or secondary structure. Moreover, neither the Cl1 nor the Cl2 masking element shows any sequence similarity to the 3'UTRs of the RNAs shown in Table 1. Thus, it is unclear how the divergent sequences, and possibly structures, of the Cl1 and Cl2 masking elements prevent premature polyadenylation in maturing oocytes.

Detection of CPE-binding proteins. With the eventual goal of cloning and characterizing factors that regulate embryonic polyadenylation, we have focused on proteins that interact with

TABLE 1. Maternal mRNAs in *X. laevis* that contain embryonic CPE-like sequences in their 3′UTRs

RNA	Putative CPE (nucleotide no.)	Expression pattern	Reference
Activin receptor XAR7	U_{14} (2023–2036) U_{14} (2078–2082)	Early embryo	15
Activin receptor XAR1	$U_3AU_{10}CU_2$ (1817–1823)	Early embryo	13
ATPase-β3	U_{17} (921–937)	Early embryo	10
Fibronectin	U_2GU_{11} (7903–7916) $U_4AU_4AU_9$ (8136–8154)	Maternal expression Starting at mid-blastula; zygotic expression starting at late gastrula	5 18
LaminB	U_{12} (3048–3059) $U_{10}GU_{10}$ (3151–3171)	Early embryo	14
Noggin	U_{10} (1475–1484) U_9 (1539–1547) U_4AU_{10} (1760–1774)	Maternal expression at blastula; zygotic expression at gastrula	34
TGF-β 2	U_{11} (2648–2658) $UGU_{10}GU_2$ (2883–2896) U_{11} (3238–3248)	?	28
Xotch	$U_{12}GU_{10}$ (8412–8434) U_{25} (8665–8689) $U_8G_2U_3GU_3$ (9120–9136)	Early embryo	4
Xwnt11	U_2GUGU_{10} (2155–2169)	Early embryo	17

the CPE. Two proteins, with sizes of 36 and 45 kDa, specifically photo-cross-link to the CPEs of Cl1 and Cl2 RNAs in egg extracts. Of the two, the 36-kDa protein may be the most important. This is based on the observation that a mutant Cl2 RNA whose CPE contains 10 uridines, but not 9 uridines, is polyadenylated in embryos following injection into fertilized eggs (33). Similarly, the 36-kDa protein photo-cross-links to a Cl2 RNA that contains a CPE with 10, but not 9, uridine residues (Fig. 4). Thus, there is a close correlation between polyadenylation in vivo and the binding of the 36-kDa protein in vitro. Irrespective of whether the 36-kDa protein and/or the 45-kDa protein is involved in embryonic polyadenylation, we note that both are present in eggs and embryos (data not shown). This is an expected result because injected Cl1 and Cl2 RNAs can be polyadenylated in both cell types, depending on whether their masking elements are present as discussed above. It should be noted, however, that neither Cl1 nor Cl2 RNA cross-links to CPEB, a 62-kDa protein that binds to the UUUUUAU CPE and regulates polyadenylation during oocyte maturation (12, 27). Conversely, an RNA containing the UUUUUAU CPE, B4, is not bound by the 36- and 45-kDa proteins. Thus, we propose that different factors are involved in cytoplasmic poly(A) elongation during oocyte maturation and early embryogenesis.

Because the 36- and 45-kDa proteins recognize the poly(U) CPE but not the hexanucleotide, different factors may bind to the hexanucleotide. Recently, it has been shown that cleavage and polyadenylation specificity factor (CPSF), a complex of at least three proteins that recognizes AAUAAA and that is known to be important for nuclear polyadenylation (reviewed in reference 30), may be involved with cytoplasmic polyadenylation as well (3). One might hypothesize that CPE-binding proteins are the specificity factors that dictate which RNAs are polyadenylated at specific times during development and that CPSF recruits a poly(A) polymerase (PAP) to extend the poly(A) tail.

In one model for the regulation of poly(A) elongation of Cl1 and Cl2 RNAs in *Xenopus* embryos, cytoplasmic polyadenylation is prevented in oocytes by a repressor protein(s) that binds to the masking element. Such a protein could recognize a structure or sequence. Although the repressor probably does not prevent the binding of proteins to the CPE (Fig. 4A), it could inhibit their activity. In the embryo, the repressor protein might be destroyed and thereby allow an interaction between the CPE-binding proteins (p36/p45) and possibly CPSF, which subsequently facilitates PAP recruitment and poly(A) addition. Clearly, the isolation and characterization of the factors involved in embryonic polyadenylation are required to test this model.

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