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The 36-Kilodalton Embryonic-Type Cytoplasmic Polyadenylation Element-Binding Protein in *Xenopus laevis* Is ElrA, a Member of the ELAV Family of RNA-Binding Proteins

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The translational activation of several maternal mRNAs in *Xenopus laevis* is dependent on cytoplasmic poly(A) elongation. Messages harboring the UUUUUAU-type cytoplasmic polyadenylation element (CPE) in their 3′ untranslated regions (UTRs) undergo polyadenylation and translation during oocyte maturation. This CPE is bound by the protein CPEB, which is essential for polyadenylation. mRNAs that have the poly(U)$_{12–27}$ embryonic-type CPE (eCPE) in their 3′ UTRs undergo polyadenylation and translation during the early cleavage and blastula stages. A 36-kDa eCPE-binding protein in oocytes and embryos has been identified by UV cross-linking. We now report that this 36-kDa protein is ElrA, a member of the ELAV family of RNA-binding proteins. The proteins are identical in size, antibody directed against ElrA immunoprecipitates the 36-kDa protein, and the two proteins have the same RNA binding specificity in vitro. Cl2 and activin receptor mRNAs, both of which contain eCPEs, are detected in immunoprecipitated ElrA-mRNP complexes from eggs and embryos. In addition, this in vivo interaction requires the eCPE. Although a number of experiments failed to define a role for ElrA in cytoplasmic polyadenylation, the expression of a dominant negative ElrA protein in embryos results in an exogastrulation phenotype. The possible functions of ElrA in gastrulation are discussed.

Early development in many animals is programmed by mRNAs inherited by the egg at the time of fertilization. While many of these mRNAs are translationally dormant in oocytes, they become activated in a sequence-specific manner at subsequent developmental periods. Several mechanisms are likely to control the translation of these messages, but one that has been studied extensively in *Xenopus laevis* is poly(A) elongation (31). Some mRNAs, which encode such proteins as c-Mos, cyclin B1, cyclin-dependent kinase 2 (Cdk2), and the early-development-specific histone B4, are polyadenylated and translated during oocyte maturation (27–29, 33, 39). Other mRNAs, however, undergo these processes sometime after fertilization; they encode such proteins as polypeptide chain release factor (Cl1), Cl2 (function unknown), and activin receptor (28, 34–36).

Both groups of mRNAs contain two *cis*-acting elements located in their 3′ untranslated regions (UTRs) that are required for cytoplasmic polyadenylation. One is the virtually ubiquitous hexanucleotide AAUAAA, which is also important for nuclear pre-mRNA cleavage and polyadenylation. The second, called the cytoplasmic polyadenylation element (CPE), is a U-rich sequence located upstream of the hexanucleotide. The exact sequence of CPE differs between the two groups of mRNAs mentioned. The maturation-type CPE is UUUUUAU (consensus), whereas the embryonic CPE (eCPE) is oligo (U)$_{12–27}$ (reviewed in reference 31). At least two mRNAs that are polyadenylated in embryos also contain a third *cis*-acting sequence in the 3′ UTR: the masking element that prevents precocious polyadenylation during maturation (34, 35). Because deletion of the masking element results in premature polyadenylation during oocyte maturation, it is likely that the factors that support embryonic polyadenylation are present and active in maturing oocytes. In studies to identify these factors, UV cross-linking in egg extracts has shown that the 36- and 45-kDa proteins bind to the eCPE (34); however, only the 36-kDa protein is cytoplasmic and, based on parallel competition assays for both UV cross-linking and polyadenylation, appeared to be functionally important for embryonic polyadenylation (36).

The products of *Xenopus* ELGs (embryonic lethal abnormal vision [ELAV]-like genes) are similar to *Drosophila* ELAV and other members of this family in that they contain a short amino-terminal domain followed by two consecutive RNA recognition motifs (RRMs), a linker region, and a carboxyl-terminal RRM (32). There are four ELG proteins in *Xenopus*, named ElrA, ElrB, ElrC, and ElrD, with molecular masses ranging from 36 to 42 kDa (10). The RRMs are highly conserved among ElrB, -C, and -D; the RRMs for ElrA are less well conserved. In addition, ElrA has shorter amino-terminal and linker regions. By Northern analysis, ElrA appears to be expressed ubiquitously, ElrB is expressed in both maternal and brain tissues, whereas ElrC and -D have a brain-specific expression pattern (10). The ElrA sequence and expression pattern are also conserved in mammals (22, 26). Members of the ELAV family interact with an AU-rich element in the 3′ UTRs of several cytokine and other mRNAs and, at least in the case of human cells, have been implicated in the control of cell growth and differentiation, perhaps by influencing the degradation of such messages (1, 7, 17, 21, 22, 24).

In this report, we show that the 36-kDa eCPE-binding protein and ElrA are the same protein. Although ElrA specifically

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interacts with RNAs harboring the eCPE both in vitro and in vivo, an extensive series of experiments failed to demonstrate that it has a function in cytoplasmic polyadenylation. However, overexpression of a truncated ElrA in injected embryos results in abnormal development beginning at the gastrula stage, which suggests that it plays a role in early embryogenesis.

MATERIALS AND METHODS

UV cross-linking and immunoprecipitation. Oocyte and egg extracts were prepared as described by McGrew and Richter (23) except that the oocytes were first manually enucleated. UV cross-linking and immunoprecipitation were carried out as described by Simon et al. (34) and Hake and Richter (12), respectively. Constructs for an eCPE-containing Cl2 3′ UTR (27 U's) and eCPE-lacking Cl2 3′ UTRs (no U's and 9 U's) were described by Simon et al. (34). To generate ElrA antiserum, the ElrA open reading frame was amplified by PCR, inserted into the pET21D vector, and used to transform bacteria. Following induction, ElrA protein was isolated from inclusion bodies and further purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The ElrA band was then excised and injected into rabbits. To generate ElrB antiserum, a PCR fragment that encodes amino acids 161 to 388 of ElrB was inserted into pGEX-KG, and the truncated protein was expressed in induced bacteria and purified on glutathione *S*-transferase (GST)–agarose (Pharmacia) before injection into rabbits. To generate ElrD antiserum, the complete open reading frame of ElrD was PCR amplified and inserted into pET15b, and bacterially expressed protein was purified on His-Bind columns (Novagen) before injection into rabbits. For the UV cross-linking, 4 μl of oocyte or egg extract was supplemented with 40,000 cpm of ^{32}P-labeled RNA and 5 mg of heparin sulfate per ml and irradiated with 254-nm light. The 40-μl mixture was then made to 48 μl with RNase A (1.67 mg/ml). Following an incubation of 20 min at 37°C, protein was immunoprecipitated with anti-ElrA antibody attached to protein A-Sepharose beads. The proteins were then fractionated on an SDS–10% polyacrylamide gel and visualized by phosphorimaging.

Analysis of ElrA expression during early embryo development. The preparation of oocytes and fertilized eggs was carried out as described by Hollinger and Gorton (16), and embryo staging was done as specified by Nieuwkoop and Faber (25). Total protein from oocytes, eggs, and embryos of different developmental stages was extracted in XBK buffer (50 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM K-HEPES [pH 7.6], 50 mM sucrose) plus protease inhibitors (10 μg each of leupeptin, chymostatin, and pepstatin per ml), denatured in SDS sample buffer, and resolved by SDS-PAGE (10% gel). Immunoblots were probed with anti-ElrA antibody, and the resulting antigen-antibody complexes were detected by chemiluminescence (ECL kit; Dupont).

Purification of His-ElrA and gel retardation assay. The coding region of ElrA was inserted in frame behind a His tag at the *Bam*HI site in the vector pPET15b (Novagen). His-ElrA was expressed in bacteria following induction with isopropylthiogalactopyranoside and purified by affinity chromatography over a His-Bind metal chelating resin (Novagen) as instructed by the manufacturer. Eighty nanograms of purified His-ElrA was first mixed with radiolabeled RNA probe in 10 μl of reaction mixture consisting of XBK buffer, 10 mM dithiothreitol, 0.5 μg of tRNA, 1 μg of bovine serum albumin, 1 M urea (to aid in protein solubility), and 2.5% glycerol and then incubated at room temperature for 20 min. Five micrograms of heparin was then added, and the mixture was incubated for an additional 10 min. The protein-RNA complexes were resolved by PAGE on a 5% polyacrylamide gel containing 1 M urea and visualized by phosphorimaging.

Immunoprecipitation, RT-PCR, and RNase protection assays. Immunoprecipitation of protein-RNA complexes from cells was performed basically as described by Steitz (41) and Levine et al. (21). Twenty oocytes or embryos were homogenized in 500 μl of SM buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM dithiothreitol, 1 U of RNasin, 1 μl of vanadyl ribonucleoside complex), incubated with protein A–Sepharose CL-4B beads for at least an hour, and centrifuged, and the supernatant was added to fresh antibody-bound protein A-Sepharose beads. The RNA that was extracted from the immunoprecipitated RNA-protein complexes was subjected to reverse transcription (RT)-PCR or RNase protection as described by Simon et al. (36) or Gebauer et al. (8), respectively. The specific primers used were 5′ CCAAACTACTTTTGTCACTGT GACAG 3′ and 5′ AATTCGGAAAGCAATTTC 3′ for Cl2, 5′ CCAAGCTTG GCAAAGCATTTCATTTCAG 3′ and 5′ CGGGATCCCGGAGGGAGGTT AAAGTCTGC 3′ for ActR, 5′ CAGATTGGTGCTGGATATGC 3′ and 5′ ACTGCCTTGATGACTCCTAG 3′ for elongation factor 1α (EF-1α), 5′ AAG ACTGCAAGACGTCATGC 3′ and 5′ GTGCTGAACATGAGATAACG 3′ for ribosomal protein L1a (Rpl1a), 5′ ATGCTGAGCAATACTACTGC 3′ and 5′ TCTGCATCTGCACTATCTGC 3′ for α-tubulin (α-Tub), and 5′ GACCGGAT CCCTGCAGAAATTAGATAAAGG 3′ and 5′ GGAATTCCTATTATTTTAA TTAACTTCAGG 3′ for Cdk2.

Preparation of embryos for histology. Two His-tagged truncated ElrA constructs were made by PCR using pPET15b-ElrA as a template. For R1R2, T7 primer and 5′ GCCAGATCTGGCAGCAAATTTTACTGTAATAGG 3′ were used. The resulting fragment was double digested with *Bam*HI and *Bgl*II and ligated into *Bam*HI site of pPET15b. For R3, 5′ GACGGATCCGGGTTGGTG CATATTTGTCTAC 3′ and T7 terminator primer were used. The resulting fragment was digested with *Bam*HI and ligated into the *Bam*HI site of pPET15b. Capped RNAs were in vitro synthesized by using a T7 message machine (Ambion) after templates were linearized with *Cla*I. Then 1 ng of RNA was injected into each fertilized egg.

Embryos injected with RNA (two different preparations) were collected at different stages and fixed, embedded in paraffin, sectioned, and stained as described by Kelly et al. (18). Whole embryos were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde. After washing and dehydration, they were embedded in paraffin and cut into 10-μm-thick sections. After the paraffin was removed, the tissues were rehydrated and stained with hematoxylin and eosin Y.

Construction and purification of GST fusion protein. Because His-tagged mutant ElrA proteins made in bacteria were insoluble, GST fusions were also constructed. To construct GST fusion proteins, *Xho*I-*Bip*I (blunt ended with Klenow enzyme) fragments of His-tagged constructs were isolated and inserted into *Xho*I and *Hin*dIII (blunt ended with Klenow enzyme) sites of vector pGEX-KG. The fusion proteins were overexpressed in bacteria and were further purified by affinity chromatography on glutathione-agarose as described by Smith and Johnson (37).

RESULTS

In previous experiments, we identified two *Xenopus* proteins with molecular sizes of 36 and 45 kDa that were UV cross-linked to the eCPEs of Cl1, Cl2, and ActR mRNAs (35, 36). However, only the 36-kDa protein was cytoplasmic and therefore seemed the most likely one to be involved in mRNA polyadenylation in embryos. Members of the ELG family encode proteins that bind U-rich sequences in cytokine mRNAs similar to the eCPE and that range in size from 36 to 42 kDa (1, 4, 21, 22). In *Xenopus*, several ELGs are expressed in the oocyte and early embryo (10). To assess whether the 36- or 45-kDa proteins might be encoded by a member of this family, we immunoprecipitated UV cross-linked RNA-protein complexes with antisera specific for different members of this family (Fig. 1). Among the antisera tested, only ElrA antiserum (lane 5) immunoprecipitated a cross-linked protein from egg extracts. However, the mobility of this cross-linked band did not correspond precisely to either the 36- or 45-kDa protein; instead, the band migrated somewhat heterogeneously between these two sizes. Although it is not unusual to see a slight mobility change of a protein after immunoprecipitation (13), we still did not know which of the two proteins the anti-ElrA antibody recognized. However, because the remaining supernatant following ElrA immunoprecipitation seemed to be somewhat depleted of the 36-kDa protein (lane 9), we thought that it was the most likely one to have reacted with the antibody. In addition, Western analysis of egg protein revealed that only ElrA migrated with a size of 36 kDa; ElrB migrated with a size of about 43 kDa, whereas ElrD was not detected. Anti-ElrD antibody, however, is active because it reacts with extract prepared from overexpressing bacteria (11).

To determine whether the 36-kDa UV cross-linked protein was the one that reacted with anti-ElrA antibody, we took advantage of our previous observation that only the 36-kDa protein is cytoplasmic (36). Thus, extracts were prepared from enucleated oocytes and used for UV cross-linking as described above. As shown in Fig. 1B, the UV-cross-linked protein was immunoprecipitated from eggs and enucleated oocytes (lanes 5 and 6), demonstrating that anti-ElrA antibody recognized the 36-kDa eCPEB. Thus, these initial experiments are consistent with the possibility that ElrA and the 36-kDa eCPEB are the same protein.

The *Drosophila* ELAV protein is a nuclear protein in postmitotic neurons, yet the 36-kDa protein is cytoplasmic. To confirm that ElrA is cytoplasmic, *Xenopus* oocytes were fractionated into nuclear and cytoplasmic compartments and probed on a Western blot with ElrA antiserum. Figure 1C shows that ElrA was exclusively cytoplasmic. Similar results were obtained

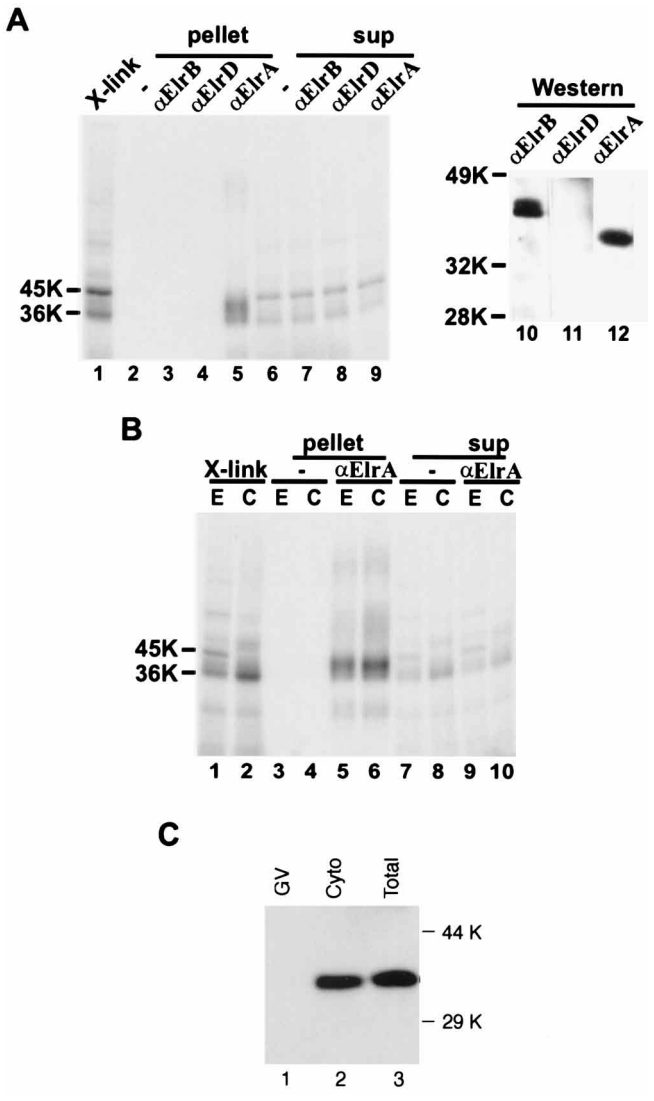

FIG. 1. Identification of ElrA as the 36-kDa eCPE-binding protein. (A) Radiolabeled Cl2 3′ UTR was UV cross-linked with egg extract protein (lane 1) and subjected to immunoprecipitation with protein A-Sepharose only (lanes 2 and 6) or protein A-Sepharose complexed with anti-ElrB (lanes 3 and 7), anti-ElrD (lanes 4 and 8), and anti-ElrA (lanes 5 and 9) antibodies. Cross-linked proteins in the immunoprecipitate pellet (lanes 2 to 5) and 1/20 supernatant (sup; lanes 6 to 9) are shown along with Western blots of egg protein probed with anti-ElrB (lane 10), anti-ElrD (lane 11), and anti-ElrA (lane 12) antibodies. (B) Radiolabeled Cl2 3′ UTR was UV cross-linked with either egg extract protein (E; lane 1) or enucleated oocyte cytoplasmic protein (C; lane 2) and subjected to immunoprecipitation with protein A-Sepharose only (lanes 3, 4, 7, and 8) or protein A-Sepharose complexed with an anti-ElrA antibody (lanes 5, 6, 9, and 10). As in panel A, cross-linked proteins in the immunoprecipitate pellet (lanes 3 to 6) and 1/20 supernatant (lanes 7 to 10) are shown. (C). Oocytes were manually dissected into nuclear (GV; lane 1) and cytoplasmic (Cyto; lane 2) compartments and probed on a Western blot with anti-ElrA. Total oocyte protein was also analyzed (lane 3).

when radiolabeled ElrA was injected into oocytes and then fractionated into nuclear and cytoplasmic compartments (11).

Cytoplasmic polyadenylation of maternal Cl2, Cl1, and ActR mRNAs occurs in *Xenopus* embryos from 3 to 9 h after fertilization. Although Northern blotting has previously shown that ElrA mRNA is present during that time (10), we wanted to ascertain whether ElrA protein was also present. A Western blot shows that ElrA protein was present at roughly equal levels in oocytes, eggs, and embryos at least up to the gastrula stage (Fig. 2). The mobility of ElrA from egg extracts (lanes E) was slightly lower than those from other development stages, which may indicate a modification such as phosphorylation. The observation that ElrA is cytoplasmic and is present in oocytes and embryos is consistent with the idea that it may be involved in cytoplasmic polyadenylation during embryo development.

ElrA specifically recognizes the eCPE in vitro. To assess whether ElrA DNA does indeed encode an eCPE-binding protein, His-tagged ElrA protein was expressed in bacteria and used in a gel retardation assay (Fig. 3A). Bacterially expressed His-ElrA shifted the mobility of Cl2 3′ UTR that contained an eCPE (lane 2) but not one that lacked an eCPE (lane 4). To demonstrate that the binding of ElrA to the eCPE was specific, we also performed a competition UV cross-linking experiment (Fig. 3B). Radiolabeled eCPE-containing Cl2 3′ UTR plus increasing amounts of unlabeled Cl2 3′ UTR either containing or lacking an eCPE (no U's and nine U's) was mixed with His-ElrA and then subjected to UV cross-linking. While there was no competition for binding of His-ElrA with Cl2 3′ UTR lacking an eCPE (no U's [lanes 6 to 9] and nine U's [lanes 11 to 13]), there was clear competition for binding with Cl2 3′ UTR containing an eCPE (lanes 2 to 5). Thus, ElrA has a strong binding preference for eCPE-containing RNA.

We also performed binding experiments using homoribopolymers as competitors. Figure 3C shows that only poly(U) (lanes 2 to 4), not poly(A) (lanes 5 to 7) or poly(C) (lanes 8 to 10), competed with Cl2 RNA for ElrA binding. This result further demonstrated that ElrA binds directly to poly(U).

Other members of the ELAV family, such as Hel-N1 (the human homolog of ElrB) and HuD (the human homolog of ElrD), are known to bind to AU-rich elements (4, 6). To rule out the possibility that ElrA binds to Cl2 RNA simply because of its affinity to AU-rich sequences, we performed a gel retardation assay using the histone B4 3′ UTR (Fig. 3A, lanes 5 to 8), which contains an AU-rich sequence composed of a maturation-type CPE. The result shows that ElrA did not alter the mobility of the B4 3′ UTR irrespective of whether it contained an AU-rich CPE. These results indicate that ElrA encodes an eCPE-binding protein and that the binding of ElrA to its target sequence is selective and sequence specific.

ElrA binds eCPE-containing mRNA in vivo. We next wanted to determine whether ElrA interacts with the eCPE in vivo. To do so, we conducted immunoprecipitation experiments in which the RNA component of specific mRNPs can be assessed. Protein-RNA complexes from oocytes or 6-h embryos were immunoselected with an anti-ElrA antibody and then subjected to RNA extraction and RT-PCR using primers specific for Cl2 and ActR mRNAs (Fig. 4). As expected, Cl2 and ActR mRNAs were both detected in ElrA-RNA complexes in oocytes and embryos (lanes 1 and 3). However, EF-1α, Rpl1a, α-Tub, and Cdk2 mRNAs, all of which contain no eCPE, were not immunoselected with the antibody.

To test whether the in vivo interaction of ElrA and mRNA was eCPE dependent, we injected a Cl2 3′ UTR containing or

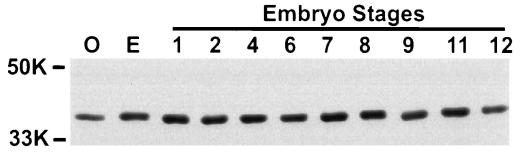

FIG. 2. ElrA expression during early embryo development. About one oocyte or embryo equivalent was applied to each lane of a Western blot probed with an anti-ElrA antibody. Oocyte (O) and egg (E) proteins were also analyzed.

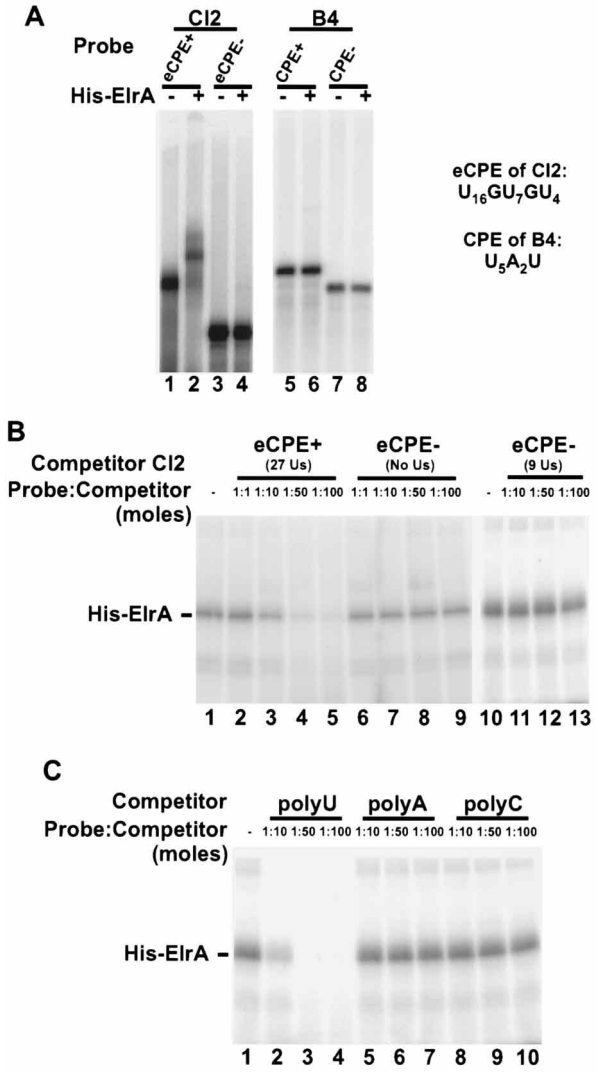

FIG. 3. In vitro interaction between ElrA and eCPE. (A) Gel mobility shift assay. Eighty nanograms of bacterially expressed His-ElrA was mixed with radiolabeled Cl2 3′ UTR that either contained (lane 2) or lacked (lane 4) an eCPE or with radiolabeled B4 3′ UTR that either contained (lane 6) or lacked (lane 8) a maturation-type CPE. No protein was added in the odd-numbered lanes. The sequences for the eCPE of Cl2 and the CPE of B4 RNAs are shown. (B) UV cross-linking competition assay. Eighty nanograms of His-ElrA was UV cross-linked with radiolabeled Cl2 3′ UTR (lanes 1 and 10) in the presence of an excess amount of unlabeled Cl2 3′ UTR that either contained (lanes 2 to 5) or lacked (no U's [lanes 6 to 9] and nine U's [lanes 11 to 13]) an eCPE. The molar ratios of the RNAs are indicated. (C) UV cross-linking competition assay. Eighty nanograms of His-ElrA was UV cross-linked with radiolabeled Cl2 3′ UTR (lane 1) in the presence of an excess amount of unlabeled poly(U) (lanes 2 to 4), poly(A) (lanes 5 to 7), or poly(C) (lanes 8 to 10).

lacking the eCPE into oocytes or fertilized eggs. After a 3-h incubation, the RNA-protein complexes were immunoselected with an anti-ElrA antibody; this was followed by RNA extraction and detection of Cl2 RNA by RNase protection (Fig. 5A). The results show that only the eCPE-containing Cl2 RNA could be detected in the immunoselected RNA-ElrA complexes from either oocytes or embryos (lanes 6 and 12). A similar result was obtained when the ActR 3′ UTR was injected (Fig. 5B). That is, only the RNA containing the eCPE could be immunoselected with the anti-ElrA antibody (lane 5). These results indicate that ElrA binds in vivo at least two eCPE-containing mRNAs through the eCPE.

ElrA in early development. Although ElrA binds the eCPE in vivo, we could not assign a function for the protein in cytoplasmic polyadenylation, primarily because eCPE-mediated polyadenylation in extracts is very weak (see Discussion). However, we have examined whether ElrA could play a role in development by the expression of a potential dominant negative form of the protein. As stated earlier, ElrA is comprised of three RRMs plus a hinge region (Fig. 6). This arrangement results in two separate RNA-binding domains, one composed of RRMs 1 and 2 and the other consisting of RRM 3. With a human homolog, HuD (ElrD), RRMs 1 and 2 are responsible for binding to the AU-rich RNA sequences (4), although another study with Hel-N1 (ElrB) shows that RRM 3 is responsible for specific RNA binding (21). To assess which domain in ElrA binds the eCPE, we have expressed ElrA RRMs 1 and 2, as well as RRM 3, as GST-tagged fusion proteins in bacteria. When used in RNA gel shift assays, only GST-RRM 1+2 bound RNA in an eCPE-dependent manner (Fig. 6; compare lanes 2 to 4 with lanes 6 to 8). Presumably, RRM 3 either binds to another RNA sequence or may be used in protein-protein interactions. We noted, however, that in this experiment, GST–wild-type ElrA bound some eCPE-lacking RNA, which we believe was due to the large amount of expressed protein added to the gel shift reaction.

Next, we injected mRNAs encoding wild-type ElrA, ElrA-RRM 1+2, and ElrA-RRM 3 into fertilized eggs and examined the morphology of developing embryos at two subsequent times. When assessed in whole mount, only embryos derived from eggs injected with ElrA-RRM 1+2 exhibited a "mushroom cap" appearance when their noninjected siblings had reached stage 13 (compare Fig. 7a and b). When noninjected controls were stage 30 tadpoles (Fig. 7c), the embryos that had received ElrA-RRM 1+2 mRNA were somewhat amorphous in appearance (Fig. 7d).

An examination of tissue sections of ElrA-RRM 1+2 mRNA-injected embryos reveals that they were normal up to

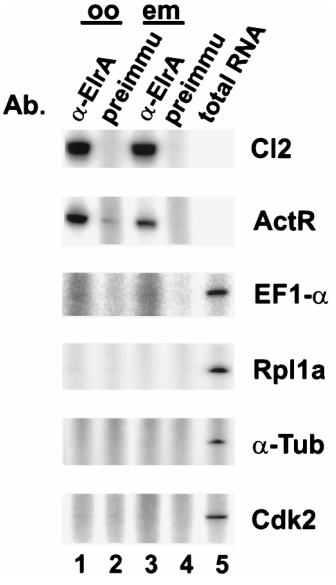

FIG. 4. In vivo interaction of ElrA and eCPE-containing RNA. Cell extracts from about 10 oocytes (oo) or embryos (em) were immunoprecipitated with either an anti-ElrA antibody (Ab.) (lanes 1 and 3) or preimmune serum (lanes 2 and 4), and the RNA that was coimmunoprecipitated was detected by RT-PCR. Two eCPE-containing RNAs (Cl2 and ActR) and four eCPE-lacking RNAs (EF1-α, Rpl1a, α-Tub, and Cdk2) were analyzed.

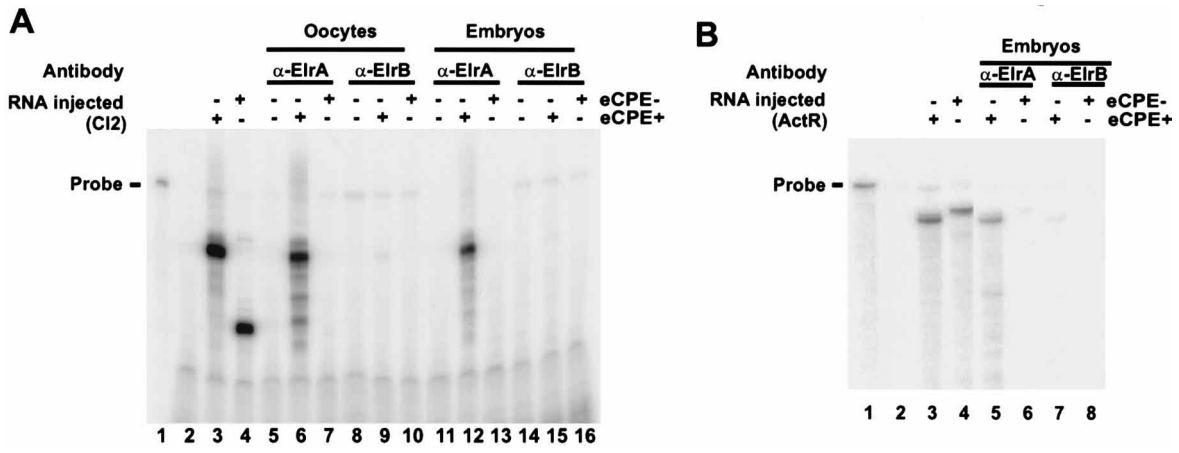

FIG. 5. In vivo interaction of ElrA and RNA requires an eCPE. (A) The 3′ UTR of Cl2 either containing (lanes 6, 9, 12, and 15) or lacking (lanes 7, 10, 13, and 16) an eCPE was injected into oocytes or fertilized eggs. Cell extracts were then prepared from these oocytes and resulting embryos, as well as from uninjected oocytes and eggs (lanes 5, 8, 11, and 14), which were used for mRNP immunoprecipitation. RNA was extracted from material immunoprecipitated with either an anti-ElrA or anti-ElrB antibody and was analyzed by RNase protection. Lane 1, probe alone; lane 2, probe digested with RNase A; lane 3, protected product from in vitro-synthesized RNA that contained an eCPE; lane 4, protected product from in vitro-synthesized RNA that lacked an eCPE. (B) RNase protection of injected ActR 3′ UTR coimmunoprecipitated with an anti-ElrA antibody. Lanes 1 to 4 are the same as in panel A except that only fertilized eggs were injected.

stage 9 (compare Fig. 8a and d), but by stage 11, they began to show signs of abnormal gastrulation (compare Fig. 8b and e). By stage 13, the injected embryos exhibited a clear exogastrulation phenotype (compare Fig. 8c and f).

Using this exogastrulation phenotype as a measure, we compared the morphologies of embryos injected with mRNA encoding wild-type and truncated ElrA proteins (Table 1). While wild-type ElrA mRNA injection elicited no phenotype, and ElrA-RRM 3 mRNA injection induced exogastrulation in only 3% of embryos, ElrA-RRM 1+2 mRNA injection resulted in exogastrulation 70% of the time. Thus, ElrA-RRM 1+2 probably acts as a dominant negative mutation by binding mRNA, which suggests that ElrA has an important function during *Xenopus* gastrulation.

DISCUSSION

Although cytoplasmic polyadenylation of maternal mRNA is widespread among metazoans, an analysis of the one known factor that regulates this process has come exclusively from studies of *Xenopus* and the mouse. During oocyte maturation in both species, the 62-kDa CPE-binding protein (CPEB) interacts with the UUUUUAU-type CPE (9, 12) and at least in the frog (the only system in which it has been tested), it is essential for cytoplasmic polyadenylation. This conclusion is based on two observations. First, immunodepletion of CPEB from egg extracts destroys their ability to polyadenylate exogenous RNA. However, supplementation of this depleted extract with CPEB synthesized either in vitro or in bacteria restores polyadenylation (12, 14). Second, the injection of a CPEB antibody into oocytes prevents polyadenylation in vivo (40). CPEB, which is also conserved among several invertebrates (3, 20, 43), is phosphorylated during maturation (12, 30), although the extent to which this is important for activity of the protein is unclear (5). The specificity of the interaction of CPEB for RNA resides in three parts of the protein: the two RRMs and a cysteine-histidine region that coordinates a metal ion (15).

Because CPEB is destroyed late in maturation and is almost undetectable in the early cleavage stages (12), it seemed quite unlikely to regulate polyadenylation in the embryo. Moreover, UV cross-linking experiments revealed that two smaller proteins with sizes of 36 and 45 kDa interacted with the poly(U)$_{12–27}$ eCPE. In this report, we show that the 36-kDa species, the only one that is cytoplasmic, is ElrA (10). The two proteins have the same molecular size, antibody directed

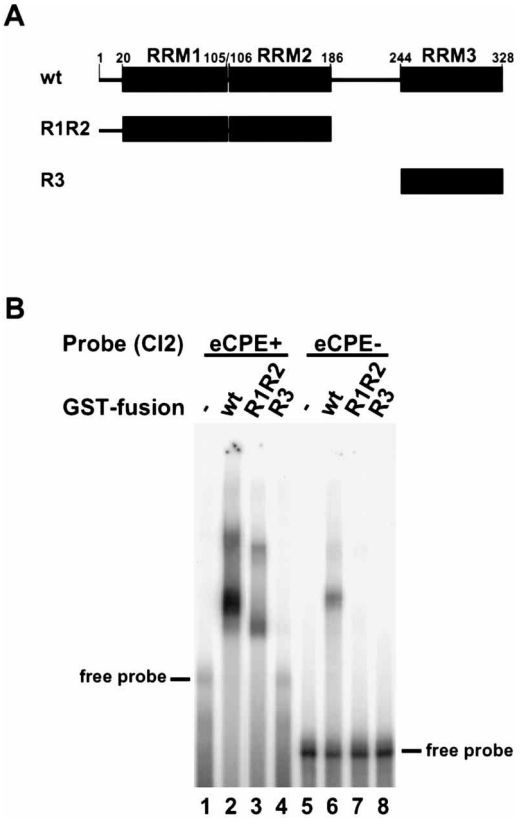

FIG. 6. RNA binding of truncated ElrA proteins. (A) Diagram of GST-tagged fusion proteins made in bacteria. The locations of RRMs are indicated. wt, wild type. (B) Gel shift analysis of ElrA proteins. Wild-type, ElrA-RRM 1+2, and ElrA-RRM 3 proteins were used in a gel shift assay with Cl2 RNA either containing (lanes 2 to 4, respectively) or lacking (lanes 6 to 8, respectively) an eCPE. Lanes 1 and 5 show free probe without protein.

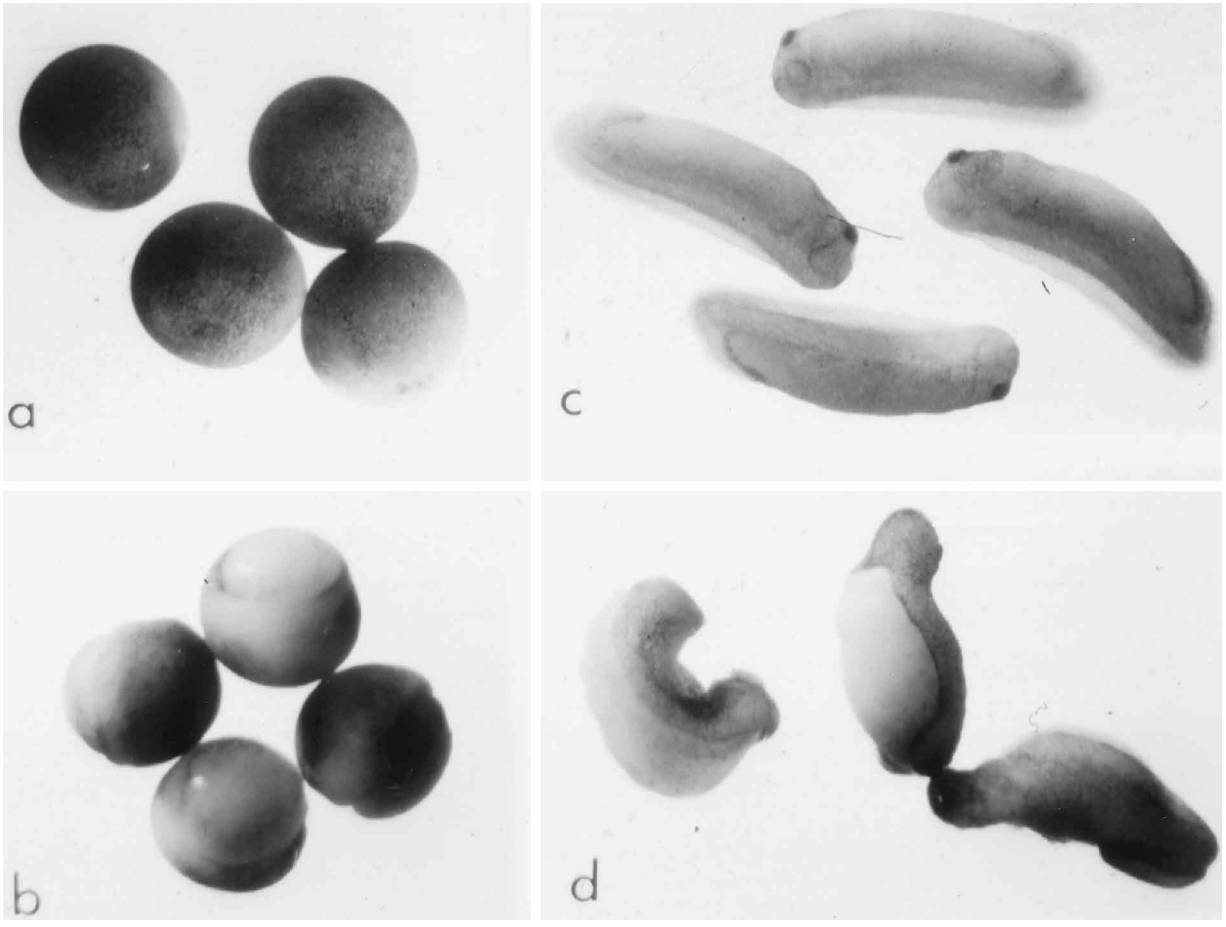

FIG. 7. Morphology of mRNA-injected embryos. Noninjected (a and c) and ElrA-RRM 1+2 mRNA-injected (b and d) eggs were examined in whole mount when the embryos reached stage 13 (a and b) or stage 30 (c and d).

against ElrA immunoprecipitates the 36-kDa protein, and the two proteins show the same binding specificity for the eCPE both in vitro and in vivo.

Using the analysis of CPEB activity in cytoplasmic polyadenylation during maturation as a paradigm, we have attempted several experiments to assess the possible function of ElrA in embryonic polyadenylation. First, although egg extracts faithfully and vigorously polyadenylate RNAs carrying the maturation-type CPE, they only weakly polyadenylate RNAs with an embryonic-type CPE. This is the case with embryo extracts as well and occurs irrespective of whether the RNA also contains a masking element (44). Because of this, we could not assess the effects of ElrA immunodepletion on polyadenylation. Although we do not know why embryonic polyadenylation is so poor, it is not due to the degradation of ElrA (44). A second assay that we used successfully in the examination of CPEB activity was antibody injection. In that case, we were able to affinity purify antibody that still (presumably) bound the protein avidly in vivo. With ElrA, we could not purify antibody with a sufficiently high affinity and/or titer to perform a similar experiment (44). Third, we injected truncated ElrA proteins made in bacteria into fertilized eggs with the idea that they might block polyadenylation in vivo. This did not occur, which could be due to a number of possibilities, including aberrant folding of the protein or perhaps a requirement that it be in excess over endogenous ElrA beyond what we could inject. Fourth, although injected mRNA encoding a truncated ElrA protein induced a gastrulation-defective phenotype, it did not inhibit polyadenylation of endogenous as well as injected Cl2 or activin receptor mRNAs (44) (see below). However, because the abnormal phenotype was observed only beginning at stage 11, polyadenylation, at least of our test RNAs, had already taken place. Thus, while our data do not support a role for ElrA in cytoplasmic polyadenylation, neither do they disprove it. Indeed, given that ElrA binds the eCPE in vivo, one may still surmise that it is involved in this process.

The injection of mRNA encoding ElrA RRMs 1 and 2 consistently produced an exogastrulation phenotype (Table 1). Because these two RRMs are the regions that interact with the eCPE (Fig. 6), we believe that they bound an mRNA(s) in vivo and inhibited its expression. The exogastrulation phenotype implies a defect in cell adhesion. In *Drosophila*, mutations in *elav* affect the accumulation of a neuron-specific form of neuroglian, a cell adhesion molecule (19). While we do not know which mRNA(s) was thus affected, we have examined two cell adhesion molecules that are thought to be involved in gastrulation, fibronectin and integrin β1 (38). Both RNAs contain a putative eCPE in their 3′ UTRs, and fibronectin mRNA is detected in ElrA coimmunoprecipitation experiments (44). However, Western blots indicate that both fibronectin and integrin β1 levels were unaffected by ElrA-RRM 1+2 mRNA injection (44). Thus, the aberrant expression of other RNAs is probably the cause of exogastrulation. We should also note that an exogastrulation phenotype can be induced nonspecifi-

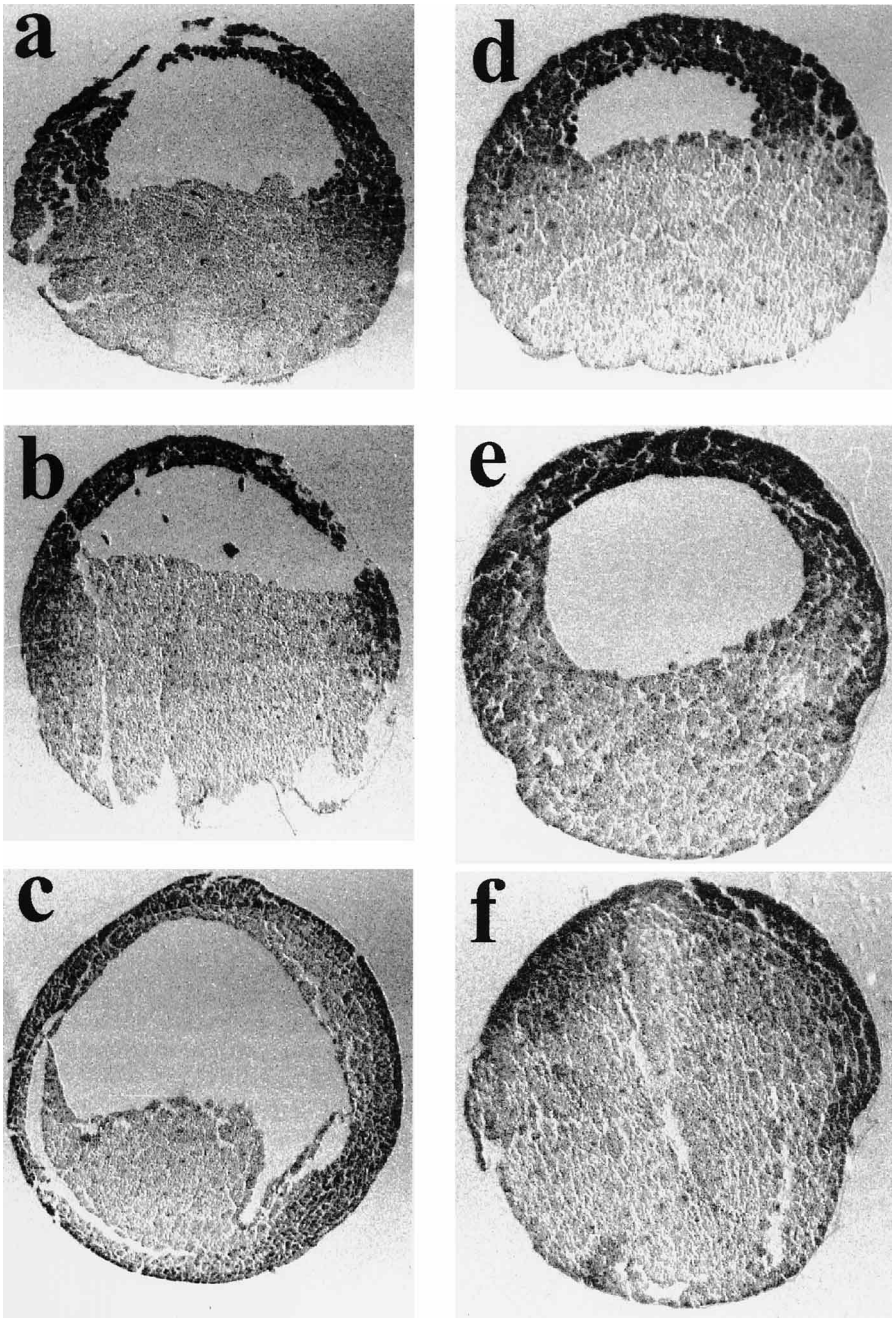

FIG. 8. Analysis of sectioned material from mRNA-injected embryos. Noninjected (a to c) and ElrA-RRM 1+2 mRNA-injected (d to f) eggs were examined when the embryos had reached stages 9 (a and d), 11 (b and e), and 13 (c and f). The embryos were paraffin embedded, sectioned, and stained with hematoxylin and eosin Y.

cally by RNA injection (31). However, in this study (Table 1), only transcripts encoding RRMs 1 and 2 did so, which strongly indicates that this was a specific response.

An additional consideration of the activity of ElrA should include RNA degradation. Members of this protein family do interact with the AUUUA degradation sequence (2, 4, 24). However, we should point out that the minimum number of uridine residues that can promote cytoplasmic polyadenylation in embryos is 12, which strongly correlates with the number of uridines required for binding of the 36-kDa protein/ElrA (34, 35). Thus, if ElrA has a function related to mRNA degradation in early development, it would probably have to do so via this

TABLE 1. Assessment of exogastrulation following mRNA injection

Agent injected	No. of embryos injected[a]	No. of exogastrulae[b]	% Exogastrulation
None	128	1	0.8
Water	83	0	0
RNA[c]			
Wild-type ElrA	74	0	0
ElrA-RRM 1+2	139	98	70.5
ElrA-RRM 3	65	2	3.1

[a] Only those embryos that survived at least 4 h after injection were counted.
[b] Embryos were examined at stage 13.
[c] RNA concentration was 0.1 μg/μl; about 10 nl was injected per egg.

sequence, which has not previously been identified with this activity.

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