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Multiple Sequence Elements and a Maternal mRNA Product Control cdk2 RNA Polyadenylation and Translation during Early *Xenopus* Development

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Cytoplasmic poly(A) elongation is one mechanism that regulates translational recruitment of maternal mRNA in early development. In *Xenopus laevis*, poly(A) elongation is controlled by two *cis* elements in the 3′ untranslated regions of responsive mRNAs: the hexanucleotide AAUAAA and a U-rich structure with the general sequence UUUUUAAU, which is referred to as the cytoplasmic polyadenylation element (CPE). B4 RNA, which contains these sequences, is polyadenylated during oocyte maturation and maintains a poly(A) tail in early embryos. However, cdk2 RNA, which also contains these sequences, is polyadenylated during maturation but deadenylated after fertilization. This suggests that *cis*-acting elements in cdk2 RNA signal the removal of the poly(A) tail at this time. By using poly(A) RNA-injected eggs, we showed that two elements which reside 5′ of the CPE and 3′ of the hexanucleotide act synergistically to promote embryonic deadenylation of this RNA. When an identical RNA lacking a poly(A) tail was injected, these sequences also prevented poly(A) addition. When fused to CAT RNA, the cdk2 3′ untranslated region, which contains these elements, as well as the CPE and the hexanucleotide, promoted poly(A) addition and enhanced chloramphenicol acetyltransferase activity during maturation, as well as repression of these events after fertilization. Incubation of fertilized eggs with cycloheximide prevented the embryonic inhibition of cdk2 RNA polyadenylation but did not affect the robust polyadenylation of B4 RNA. This suggests that a maternal mRNA, whose translation occurs only after fertilization, is necessary for the cdk2 deadenylation or inhibition of RNA polyadenylation. This was further suggested when poly(A)$^+$ RNA isolated from two-cell embryos was injected into oocytes that were then allowed to mature. Such oocytes became deficient for cdk2 RNA polyadenylation but remained proficient for B4 RNA polyadenylation. These data show that CPE function is developmentally regulated by multiple sequences and factors.

One characteristic of oogenesis is the synthesis and storage of cytoplasmic ribonucleoprotein particles. The mRNAs contained within some of these particles enter polysomes during oocyte maturation when the cell resumes meiosis or during early embryogenesis. Conversely, at these same times, other mRNAs that are engaged in protein synthesis are removed from polysomes (5). Although several mechanisms are likely to regulate the translation of these maternal transcripts (25, 33), one that has received particular attention is poly(A) elongation and removal (reviewed in references 15, 26, 38, and 40). By using Northern (RNA) blot analysis, a correlation between polyadenylation and polysomal recruitment was detected for specific transcripts in oocytes, eggs, or embryos of marine invertebrates (27), *Xenopus laevis* (6, 7, 20–22), and mice (11). In a similar vein, RNAs that lose their poly(A) tails were generally found to be removed from polysomes (13, 22, 34). Whether poly(A) tail gain or loss regulated translational recruitment or repression, however, could not be determined from observations of endogenous mRNAs alone. Recent studies with mRNA-injected oocytes and embryos have firmly established this relationship as one of cause and effect. Generally, poly(A) elongation during maturation and embryogenesis induces mRNA recruitment into polysomes (18, 23, 31, 37) while mRNA deadenylation promotes message loss from polysomes (13, 36). Furthermore, when poly(A) removal is prevented, the message remains polysomal (12, 36).

mRNA-injected oocytes have also been useful for identifying the *cis*-acting elements that control polyadenylation during maturation. Two elements, both in the 3′ untranslated regions (UTRs) of responsive mRNAs, were shown to be necessary for this process. The first, AAUAAA, is a highly conserved and nearly ubiquitous hexanucleotide that also is notable for its role in nuclear pre-mRNA cleavage and polyadenylation. The second, which is a more variable U-rich sequence with the general structure UUUUUAAU, is called the cytoplasmic polyadenylation element (CPE) (9, 18, 19, 23). In some respects, the CPE and hexanucleotide also control poly(A) removal during *Xenopus* oocyte maturation. More specifically, the lack of either of these sequences leads to "default" deadenylation (10, 35, 36). In mice, a somewhat different situation occurs, in which a CPE-like element, termed the adenylation control element, is necessary for both poly(A) elongation during maturation and poly(A) removal during oocyte growth (12).

Because the CPE is the "specificity element" for the polyadenylation reaction, considerable effort has been directed towards identifying the factor(s) that interacts with this region (8, 19, 23, 24). However, perhaps because a number of RNAs with somewhat variable CPEs have been used for these studies, different CPE-binding proteins with different binding characteristics have been detected. This is not surprising, given that the timing and extent of poly(A) elongation is message specific. This variability might also mean that sequences other than the CPE and hexanucleotide can influence polyadenylation.

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To investigate this, we have focused on cdk2 RNA (20), which was first identified and referred to as Eg1 by Paris et al. (21). This RNA undergoes poly(A) elongation during oocyte maturation but is deadenylated soon after fertilization (20). Interestingly, cdk2 RNA has a sequence in its 3′ UTR that is precisely the same as that of the CPE in B4 RNA (20, 23). However, B4 RNA is efficiently polyadenylated during oocyte maturation (6) and maintains its poly(A) tail for some time after fertilization (7, 32). Moreover, following injection, the 3′ UTR of B4 RNA is polyadenylated during both oocyte maturation and early embryogenesis (23, 33a; this report). On the basis of these observations, we suspected that cdk2 RNA, like Eg2 RNA (2, 16), might have unique elements that promote postfertilization deadenylation. In this report, we identify these elements, show that they repress translation by deadenylation, and demonstrate that they are regulated by a maternal mRNA product.

MATERIALS AND METHODS

DNA constructs. All DNA manipulations were performed by using standard methods (17). DNA constructs were confirmed by dideoxy sequencing (28). *Escherichia coli* HB101 served as the bacterial host strain for all plasmid constructions. psB4 was constructed by ligating the 37-bp *Hin*dIII-*Xba*I insert of psGb/B4 (23) to *Hin*dIII-*Xba*I-digested pSP64pA. PCRs were carried out in a final volume of 100 μl containing each of the 5′ and 3′ primers at 0.2 to 0.5 mM, 1 ng of linearized template DNA, deoxynucleotide triphosphates at 0.2 mM, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mg of bovine serum albumin, and 2.5 U of *Taq* polymerase (Perkin-Elmer). pscdk2 was constructed by PCR amplification of the 3′ 188 bases of the cdk2 UTR (bases 1492 to 1679 as described in reference 20) by using 5′ PCR primer 1 (5′ GACCGGATC CCTGCAGAAATTAGATAAAGG), 3′ PCR primer 2 (5′ GGAATTCCAATAATAATATTATTTTAATTAACTTCA GG), and *Eco*RI-digested pcdk2. The 5′ and 3′ termini of the PCR product were digested with *Bam*HI and *Eco*RI, respectively, and ligated to *Bam*HI-*Eco*RI-digested pSP64pA [this removes the poly(A) stretch]. Deletion mutants of pscdk2 were made by ligating the following *Bam*HI-*Eco*RI-digested PCR products into the *Bam*HI and *Eco*RI sites of pSP64pA as described above. pscdk2 linearized with *Eco*RI served as the template in these reactions. 5′Δ35 was made with 5′ PCR primer 3 (5′ GACCGGATCCACAACAGACTGCTATTC TG), 5′Δ56 was made with 5′ PCR primer 4 (5′ GACCGG ATCCAAAAAAAAAATATTTTGCACC), and 5′Δ80 was made with 5′ PCR primer 5 (5′ GACCGGATCCTATTTTT GGATGCAACTG). 3′ PCR primer 2 was used to synthesize all of the above-described deletion PCR products. 3′Δ8 was made with 5′ PCR primer 1 and 3′ PCR primer 6 (5′ GGA ATTCCTATTATTTTAATTAACTTCAGG). 5′Δ803′Δ8 was made with 5′ PCR primer 5 and 3′ PCR primer 6.

The substitution mutants were cloned exactly as were the deletion mutants described above. Under standard PCR conditions, mutant 5′ PCR primers were used to replace wild-type cdk2 sequences from base positions 41 to 188 (for coordinates, see Fig. 3) with a *Kpn*I site, either CCGGTACCCC or CCG GTACCC, at positions 41 to 50 (5′sub1), 51 to 60 (5′sub2), 61 to 70 (5′sub3), 71 to 80 (5′sub4), 81 to 90 (5′sub5), and 91 to 98 (5′sub6). Mutant 3′ PCR primers were used to substitute wild-type bases with cytosines at positions 175 to 179 (3′sub1), 180 to 182 (3′sub2), 183 to 185 (3′sub3), and 186 to 188 (3′sub4) in PCR products that extend from cdk2 base positions 1 through 188.

p5′scdk2/sB4 was created by PCR amplification of bases 1 to 98 of pscdk2 (see Fig. 1) by using *Eco*RI-linearized pscdk2, 5′ PCR primer 7 (5′ CCCGAATTCCAAGCTTGCTGCAGAA ATTAGATAAAGG), and 3′ PCR primer 8 (5′ GCGCGC GCAAGCTTCAGTTGCATCCAAAAATAAAAGG). This product was digested with *Hin*dIII and ligated to psB4 linearized at the *Hin*dIII site, which is the 5′ boundary of the sB4 sequence. p5′scdk2/sB4/3′ scdk2 was constructed by fusing the 14 bases downstream of the cdk2 hexanucleotide to the 3′ side of the B4 hexanucleotide in p5′scdk2/sB4. This was done by PCR amplification of p5′scdk2/sB4 linearized with *Xba*I by using 5′ PCR primer 1 and 3′ PCR primer 9 (5′ GGAATTCC AATAATAATATTATTTTATTTATAGAATTAAACAT TAAAAACC). psB4/3′scdk2 was constructed by *Hin*dIII digestion and religation of p5′scdk2/sB4/3′scdk2 to remove the 5′scdk2 sequence.

Chimeric chloramphenicol acetyltransferase (CAT) constructs were produced as follows. The PCR product of sGb/B4, identical to sB4 except for additional 5′ globin sequences (24), was amplified with the 5′ primer (5′ CGGGATCCGCAGAA GCTCAGAATAAACGC 3′) and the 3′ primer (5′ GGATTC ACTTTATTTATAGAATTAAAC 3′). This product, as well as PCR products scdk2 and 5′scdk2/sB4/3′scdk2 (described above), were digested with *Eco*RI, treated with the Klenow fragment to produce 3′ blunt ends, and digested with *Bam*HI. These products were ligated into the *Bam*HI and *Sma*I sites of pG4-CAT (a gift from L. Browder) located downstream of the CAT-coding sequence.

RNA synthesis and oocyte and embryo injection. DNA templates were linearized with *Eco*RI, except for psB4 and p5′scdk2/sB4, which were linearized with *Xba*I, and all CAT constructs, which were linearized with *Kpn*I and Klenow treated to prevent formation of unwanted RNA products (29). Capped [^{32}P]UTP-labelled transcripts were synthesized by using SP6 or T7 RNA polymerase for CAT constructs essentially as previously described (18). Some RNAs were also polyadenylated in vitro by incubating about 5 ng of radiolabelled RNA in a 20-μl volume containing 50 mM Tris-HCl (pH 8), 0.2 M NaCl, 10 mM $MgCl_2$, 100 μM ATP, 0.05 U of *E. coli* poly(A) polymerase (Pharmacia), and 20 U of rRNasin for 10 min at 37°C. Manually defolliculated stage VI oocytes were injected with approximately 40 nl of radiolabelled RNA (about 10^2 cpm/pg/nl) and cultured in 1× Barth's medium with or without progesterone (1 μg/ml) for up to 6 h, the approximate time of maturation. In some experiments, stage VI oocytes were injected with about 40 nl of deionized water or embryonic poly(A)$^+$ RNA (1 ng/nl) isolated from 1-h embryos as previously described (3) by using PolyATract (Promega) or a Mini Ribosep Ultra RNA kit (Collaborative Biomedical Products) in accordance with the instructions provided. At 16 h later, they were additionally injected with 40 nl of radiolabelled RNA. After 1 h, progesterone was added to some of the oocytes. All oocytes were then incubated for 6 h.

Eggs shed from females stimulated with 1,000 U of human chorionic gonadotropin (Sigma) were fertilized in vitro in accordance with reference 31. They were dejellied in 2% cysteine (pH 7.8) and then placed in 5% Ficoll in 0.1× Barth's medium, where they were injected with about 40 nl of radiolabelled RNA and incubated for 1.5 to 3 h. In some experiments, cycloheximide (CHX; 100 μg/ml) was added either to eggs at the start of fertilization or to 2-h embryos. In both cases, 0.5-h embryos were injected with about 40 nl of radiolabelled RNA and further incubated for 3 h.

RNA from four or five injected oocytes and embryos per sample was extracted by homogenization in 3% *p*-aminosalicylate as previously described (18). Differences in recovery of RNA during extraction sometimes resulted in unequal loading

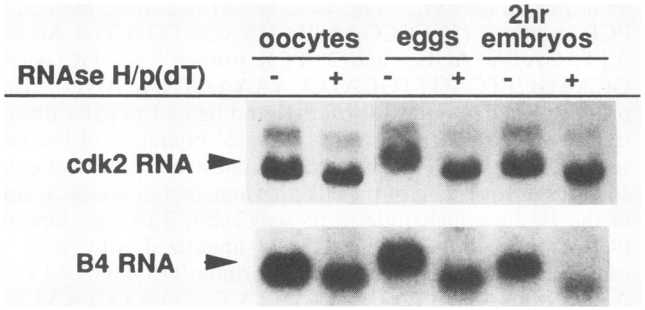

FIG. 1. Analysis of changes in mRNA poly(A) tail length in early development. Northern blots of total RNA untreated (−) or treated (+) with RNase H and poly(dT) [p(dT)] from stage VI oocytes, unfertilized eggs, and 2-h embryos were probed with radiolabelled cdk2 and B4 cDNAs.

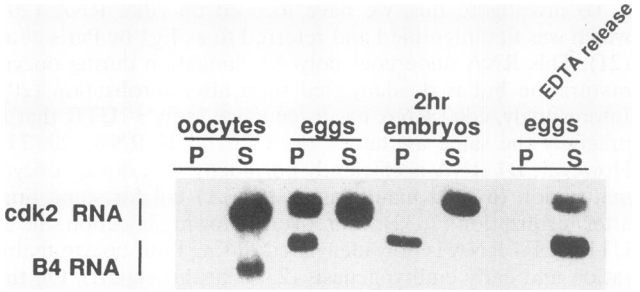

FIG. 2. Polysome recruitment of cdk2 and B4 mRNAs in development. Polysomes (P) and postpolysomal supernatants (S) were prepared from oocytes, eggs, and 2-h embryos by ultracentrifugation, and the RNA extracted from each fraction was Northern blotted and probed with labelled cdk2 and B4 cDNAs. Parallel centrifugation of an egg homogenate was performed in the presence of EDTA, an agent known to dissociate polysomes.

of samples. Radiolabelled RNAs were analyzed by electrophoresis on 5% polyacrylamide–8 M urea gels and autoradiography. Quantification of polyadenylated RNA was done by scanning densitometry of autoradiographs and Sigmaplot.

Polysome isolation, RNA extraction, and Northern analysis. Polysome isolation was performed by centrifugation of oocyte, egg, and embryo homogenates through sucrose cushions, and RNA in the supernatants and pellets was extracted as previously described (39). For Northern analysis, equal amounts of total RNA isolated as described in reference 39 were electrophoresed in 1% agarose–3.7% formaldehyde gels, blotted onto nylon, UV cross-linked, and baked. For RNA from polysome and supernatant fractions, five cell equivalents per lane was electrophoresed. Hybridization was done in the presence of denatured salmon sperm DNA (200 μg/ml) as previously described (4). The blots were probed with gel-purified fragments labelled by random priming. Two cdk2 probes were used. One probe was made from the 1.7-bp *Eco*RI fragment of pEg_{241}, which includes the coding region and 3′ UTR of cdk2/Eg1. This probe hybridizes to the 1.7-nucleotide cdk2 mRNA, as well as several higher-molecular-weight RNAs, as previously reported (20). The second probe was derived from the 0.6-bp *Dra*I-*Eco*RI fragment of pEg_{241}, which hybridizes only to the 3′ UTR of cdk2 RNA. The B4 hybridization probe was derived from the 0.5-bp *Pvu*II-*Acc*I fragment of pB4.0 (32). RNAs with known molecular weights were used as markers.

CAT assays. In vitro-synthesized CAT RNAs were suspended in water (0.1 mg/ml), and about 40 nl was injected per oocyte or embryo. CAT assays were performed essentially as described in reference 31 and quantified in a PhosphorImager (Molecular Dynamics) or by scanning densitometry and Sigmaplot.

RESULTS

By Northern blot analysis, we examined the poly(A) metabolism of B4 mRNA, which encodes a histone-like protein (32), and cdk2 (Eg1) mRNA, which encodes a cyclin-dependent kinase (20), in stage VI oocytes, unfertilized eggs, and 2-h embryos (Fig. 1). Both transcripts underwent a size increase of between 100 and 200 bases during maturation, as evidenced by their larger size in eggs relative to oocytes. In 2-h embryos, cdk2 RNA returned to the size observed in oocytes while B4 RNA migrated at a rate similar to that in eggs. To demonstrate that these changes in migration rate were due to alterations in poly(A) tail length, a portion of each sample was treated with RNase H in the presence of poly(dT) (Fig. 1). This treatment eliminated the differences in mobility among the respective RNAs at the three developmental stages. Thus, both B4 and cdk2 RNAs have a short poly(A) tail of less than 100 bases in oocytes which is elongated to up to 200 bases in eggs. In early embryos, the poly(A) tail of cdk2 RNA is shortened to less than 100 bases while that of B4 is maintained.

To assess whether poly(A) metabolism correlates with translation, we determined whether cdk2 and B4 RNAs in oocytes, eggs, and embryos partitioned with polysomes following their isolation by ultracentrifugation. The Northern blot shown in Fig. 2 demonstrates that negligible amounts of both RNAs were present on polysomes in oocytes and greater than 90% was present in the supernatant (compare polysomal pellet [P] and supernatant [S] lanes). However, in eggs, up to 50% of cdk2 and greater than 90% of B4 RNA shifted from the supernatant to the polysomal pellet. This redistribution of B4 and cdk2 RNAs is coincident with their polyadenylation in eggs (Fig. 1). By 2 h postfertilization, when most of the cdk2 RNA was poly(A) shortened (Fig. 1), it partitioned into the supernatant. B4 RNA, which retains its poly(A) tail at this stage (Fig. 1), remained in the polysomal pellet. As a control, EDTA treatment of egg homogenates released both RNAs into the supernatant. Thus, for these RNAs, we confirmed that polyadenylation correlates with translational recruitment whereas deadenylation correlates with translational repression (20, 22).

Analysis of sequences that regulate deadenylation. We suspected that sequences regulating cdk2 RNA deadenylation would reside in the 3′ UTR of this message. Accordingly, the 3′ UTRs of cdk2 and, as a control, B4 mRNAs were subcloned and designated scdk2 and sB4 RNAs. The salient sequences are shown in Fig. 3. These RNAs contain identical CPEs (UUUUUAAU) and very similar polyadenylation hexanucleotides. Disruption of either of these elements prevents polyadenylation during meiotic maturation (23, 24; data not shown). However, scdk2 RNA contains two other CPE-like sequences (Fig. 3), at least one of which has some activity (data not shown).

On the basis of preliminary experiments, we thought that cdk2 RNA deadenylation in embryos might be related to a possible lack of poly(A) elongation activity specific for this RNA. To assess this, we synthesized this RNA, as well as B4 RNA, in two forms. One form contained a poly(A) tail of about 75 residues so that we could monitor deadenylation. The second form lacked a poly(A) tail, which we thought could help

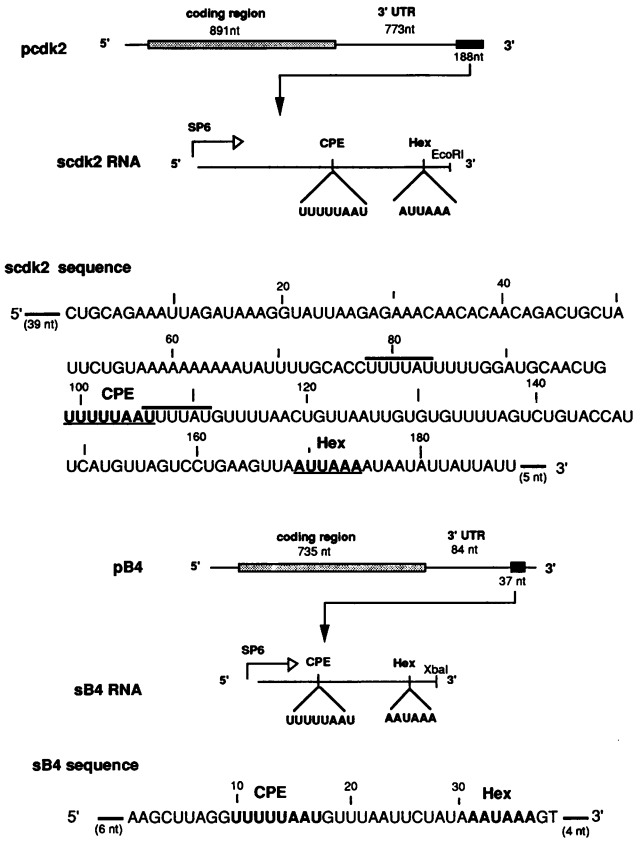

FIG. 3. Partial sequences and salient features of scdk2 and sB4 RNAs. A 188-nucleotide (nt) region of the cdk2 3′ UTR was amplified by PCR and cloned into pSP64pA. Following DNA linearization at the *Eco*RI site, an scdk2 runoff transcript was produced that contained an additional 39 nucleotides of the polylinker (in parentheses) at the 5′ end and 5 nucleotides at the 3′ end. Thirty-seven nucleotides of the B4 3′ UTR were cloned into pSP64pA. Following DNA digestion with *Xba*I, an sB4 runoff RNA was produced that contained 6 nucleotides of the polylinker at the 5′ end and 4 nucleotides at the 3′ end. For both RNAs, the CPE and polyadenylation hexanucleotide (Hex) are in boldface. Other U-rich sequences similar, but not identical, to the CPE (UUUUUAAU) are overlined.

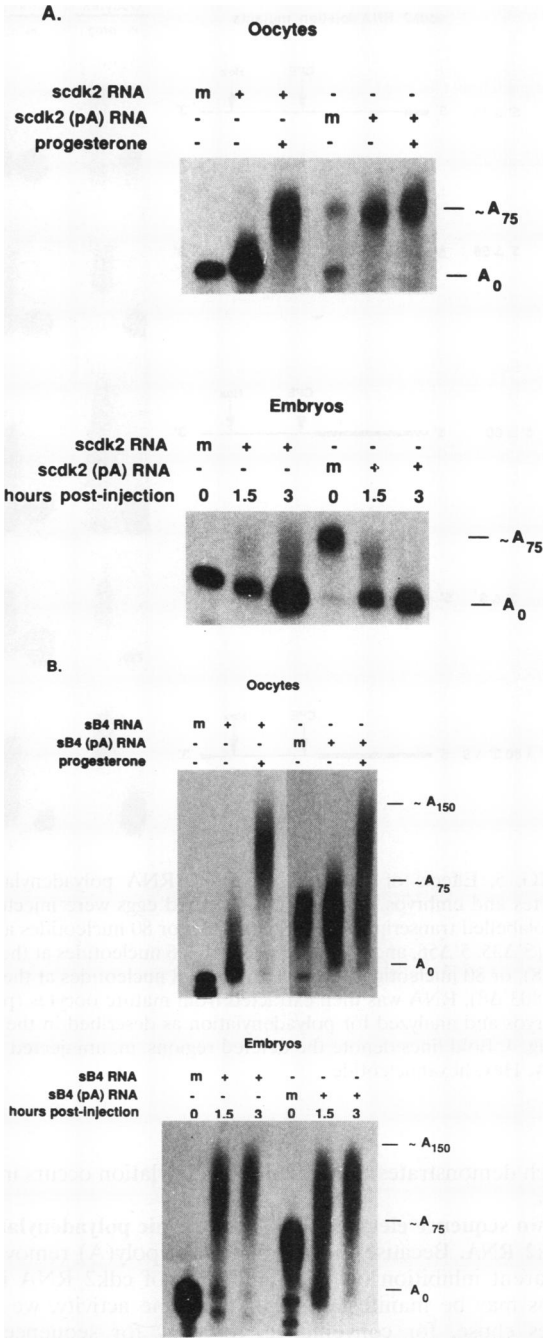

FIG. 4. Analysis of scdk2 and sB4 RNA polyadenylation following oocyte and egg injection. (A) Poly(A)$^-$ and poly(A)$^+$ scdk2 RNAs were injected into oocytes, some of which were induced to mature with progesterone (top), and embryos, which were allowed to develop for 1.5 and 3 h (bottom). RNA was then extracted and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. (B) The same as panel A, except that sB4 RNA was analyzed. m, noninjected marker RNA. The approximate number of adenylate residues gained or lost is indicated to the right of each panel.

us examine poly(A) addition. The RNAs were synthesized in vitro in the presence of [^{32}P]UTP and injected into fully grown *Xenopus* oocytes, some of which were incubated with progesterone to induce maturation, and into fertilized eggs. Following incubation for various times, the RNA was extracted and analyzed by electrophoresis in 5% denaturing polyacrylamide gels and autoradiography (Fig. 4). Nonadenylated scdk2 RNA was efficiently polyadenylated during oocyte maturation (Fig. 4A, top) but only slightly adenylated in embryos; we regard this as background adenylation (Fig. 4A, bottom). In contrast, adenylated scdk2 RNA was not further polyadenylated during maturation (top) but was efficiently deadenylated in embryos (bottom). This result demonstrates that the 3′ UTR of cdk2 RNA contains sequences that promote embryonic deadenylation and prevent apparent poly(A) addition.

Figure 4B shows results of identical experiments with sB4 RNA. As expected, nonadenylated B4 RNA was efficiently polyadenylated during both oocyte maturation and embryogenesis (compare top and bottom). In contrast to cdk2 RNA, some adenylated B4 RNA was further polyadenylated during maturation (top), as well as in embryos (bottom). Thus, cdk2 and B4 RNAs are under different types of polyadenylation control in embryos. We also found that sB4 RNA that does not contain a CPE is deadenylated in embryos (data not shown),

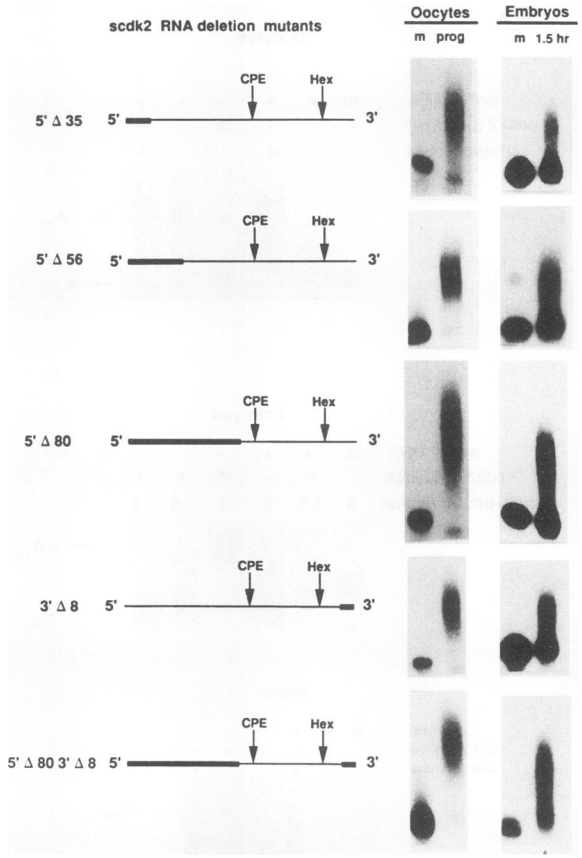

FIG. 5. Effects of deletions on scdk2 RNA polyadenylation in oocytes and embryos. Oocytes and fertilized eggs were injected with radiolabelled transcripts that lacked 35, 56, or 80 nucleotides at the 5′ end (5′Δ35, 5′Δ56, and 5′Δ80, respectively), 8 nucleotides at the 3′ end (3′Δ8), or 80 nucleotides at the 5′ end and 8 nucleotides at the 3′ end (5′Δ803′Δ8). RNA was then extracted from mature oocytes (prog) or embryos and analyzed for polyadenylation as described in the legend to Fig. 4. Bold lines denote the deleted regions. m, uninjected marker RNA. Hex, hexanucleotide.

which demonstrates that default deadenylation occurs in these cells.

Two sequence elements block embryonic polyadenylation of scdk2 RNA. Because the stimulation of poly(A) removal and apparent inhibition of polyadenylation of cdk2 RNA in embryos may be manifestations of the same activity, we sometimes chose, for convenience, to assay for sequences that inhibit scdk2 RNA polyadenylation. We created 5′ and 3′ deletion mutants with no alteration in the CPE or hexanucleotide. Removal of 35, 56, and 80 bases from the 5′ end of scdk2 (5′Δ35, 5′Δ56, and 5′Δ80, respectively) had no effect on maturation-specific polyadenylation (Fig. 5, oocytes). In all cases, >95% of the recovered RNA was polyadenylated in progesterone-treated oocytes. However, in 1.5-h embryos, these same 5′ deletions partially reversed the inhibition of adenylation (Fig. 5, embryos). The smallest deletion mutant, 5′Δ35, was about 20% adenylated, while the larger deletions, 5′Δ56 and 5′Δ80, were nearly 40% adenylated compared with wild-type scdk2 RNA, which was <5% polyadenylated (Fig. 4A). [Percent adenylated here refers to those transcripts that receive poly(A) tails irrespective of the number of adenylate residues added.] These results demonstrate that sequences within an 80-base domain upstream of the cdk2 CPE are partly responsible for blocking embryonic polyadenylation.

Because full adenylation was not achieved by these 5′ scdk2 deletions in embryos, additional inhibitory sequences were still present. Surprisingly, removal of the last eight bases at the 3′ end of scdk2 RNA resulted in about 40% adenylation in embryos (Fig. 5, 3′Δ8). Furthermore, a double-deletion mutant whose 5′ and 3′ sequences were removed was adenylated by nearly 80% in embryos (Fig. 5, 5′Δ803′Δ8). We conclude that sequences both 5′ of the CPE and 3′ of the polyadenylation hexanucleotide are necessary to efficiently block polyadenylation in early embryos.

By constructing 5′ and 3′ substitution mutations, we assessed whether a more discrete inhibitory sequence was located within the broad regions flanking the CPE and hexanucleotide. An 8- to 10-base *Kpn*I site was substituted sequentially for the 58 bases upstream of the CPE (Fig. 6A). As expected, these RNAs were fully adenylated in mature oocytes. In embryos, however, the substitutions resulted in less than 20% polyadenylation in all cases. Thus, no small, short element in the 5′ region prevents embryonic polyadenylation of scdk2 RNA.

A similar approach was taken to examine the 14-base region 3′ of the hexanucleotide. Cytosine substitutions for every three to five bases were introduced, and the resulting RNAs were analyzed for polyadenylation (Fig. 6B). As with the 5′ substitutions, these RNAs were fully adenylated in mature oocytes. However, polyadenylation of two mutants, 3′sub1 and 3′sub4, was less efficiently inhibited in embryos than was that of the wild type, indicating that the mutated sequences in those RNAs are important for efficient inhibition.

The 5′ and 3′ polyadenylation-inhibitory elements act synergistically. To determine whether the 5′ and 3′ polyadenylation-inhibitory elements of scdk2 RNA are sufficient for blocking of embryonic polyadenylation, we constructed several chimeric RNAs composed of B4 and cdk2 sequences and tested their relative polyadenylation efficiencies in embryos. The first chimeric RNA contained the entire region upstream of the scdk2 CPE (98 bases) fused 5′ to sB4 RNA (Fig. 7, 5′scdk2/sB4). Unexpectedly, this RNA was polyadenylated very efficiently, >95%, demonstrating that the 5′scdk2 sequence is not sufficient to inhibit embryonic adenylation. However, fusion of the 3′ inhibitory element to the 3′ end of sB4 RNA decreased polyadenylation efficiency in embryos by about 40% (Fig. 7, sB4/3′scdk2). Fusion of both 5′ and 3′ inhibitory elements to sB4 RNA completely prevented embryonic polyadenylation (Fig. 7, 5′scdk2/sB4/3′scdk2). In comparable experiments, replacement of the B4 hexanucleotide (AAUAAA) with the cdk2 hexanucleotide (AUUAAA) in these chimeric constructs had no significant effect on polyadenylation (data not shown). Also, substitution of the 5′ or 3′ scdk2 RNA sequences with *Xenopus* β-globin and a polylinker did not prevent sB4 RNA polyadenylation in embryos (data not shown).

To ascertain whether the 5′ and 3′ cdk2 polyadenylation-inhibitory sequences also mediate deadenylation, we added an approximately 75-nucleotide poly(A) tail to 5′scdk2/sB4/3′scdk2 RNA and injected it into oocytes and embryos (Fig. 8). Although the poly(A) tail of this RNA was not further elongated during maturation (top), it was efficiently removed during embryogenesis (bottom). As a corollary, this RNA, when injected as a nonadenylated transcript, acquired a poly(A) tail during maturation (top) but was unable to do so during embryogenesis (bottom). Thus, the 5′ and 3′ scdk2 sequences that block polyadenylation in embryos also promote deadenylation at this time. These results also show that the cdk2 RNA sequences between the CPE and hexanucleotide

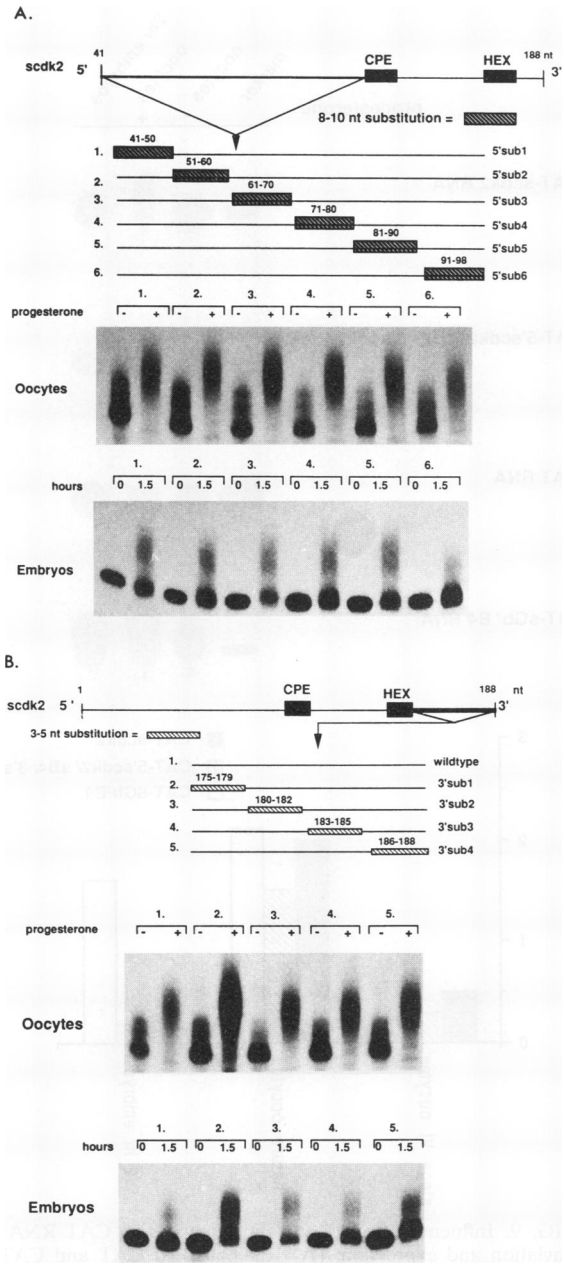

FIG. 6. Nucleotide substitution analysis of the 5′ and 3′ polyadenylation-inhibitory regions. (A) Oocytes and fertilized eggs were injected with radiolabelled in vitro transcripts containing cdk2 sequences 41 to 188. These transcripts have substitutions in the region upstream of the CPE (bases 41 to 98) at the positions indicated. The 5′subl-5 RNAs contain the 10-base substitution CCGGTACCCC, and the 5′sub6 RNA contains the 8-base substitution CGGTACCC. (B) Oocytes and fertilized eggs were injected with radiolabelled in vitro transcripts containing cdk2 sequences 1 to 188. These transcripts have poly(C) substitutions in the region downstream of the hexanucleotide (HEX) (175 to 188) at the positions indicated. In both parts, injected oocytes were incubated with or without progesterone for 6 h and fertilized eggs were incubated for 0 or 1.5 h. RNA was then extracted, and polyadenylation was analyzed as described in the legend to Fig. 4. nt, nucleotide.

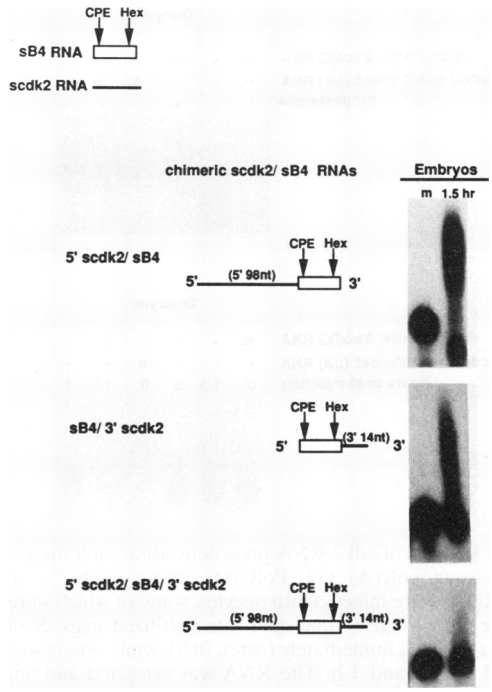

FIG. 7. Embryonic polyadenylation inhibition of chimeric scdk2/sB4 transcripts. Chimeric RNAs composed of 5′ scdk2 sequences 1 to 98 fused to sB4 RNA (5′scdk2/sB4), 3′ scdk2 sequences 175 to 188 fused to sB4 RNA (sB4/3′scdk2), and both of these sequences fused to sB4 RNA (5′scdk2/sB4/3′scdk2) were injected into fertilized eggs. RNA was extracted at 1.5 h and analyzed as described in the legend to Fig. 4. Hex, hexanucleotide. nt, nucleotides. m, uninjected marker RNA.

have no detectable effect on deadenylation because where this region was removed (5′scdk2/sB4/3′scdk2 RNA), deadenylation was not prevented nor was polyadenylation promoted in embryos (Fig. 8). Furthermore, the spacing between the two polyadenylation-inhibitory sequences is flexible to the degree that it can vary from 36 bases (5′scdk2/sB4/3′scdk2 RNA) to as many as 76 bases (scdk2 RNA).

Polyadenylation control of translation. Polysome analysis of endogenous cdk2 RNA (Fig. 2) showed that poly(A) addition and removal (Fig. 1) correlate with translational activation and repression, respectively. To determine whether poly(A) metabolism could be causative for these changes in translation, we created two chimeric RNAs by fusing the scdk2 sequence and the 5′scdk2/sB4/3′scdk2 sequence to the CAT-coding region. Radiolabelled in vitro transcripts were injected into oocytes and embryos and analyzed by denaturing gel electrophoresis and autoradiography. As expected, both of these RNAs underwent size changes characteristic of polyadenylation during maturation but were not adenylated in 2-h embryos (Fig. 9A). A chimeric RNA in which the sGb/B4 sequence, which contains no scdk2 polyadenylation-inhibitory sequences, was placed downstream of CAT-coding sequences was injected as a positive control. As expected, CAT-sGb/B4 RNA underwent a shift in size characteristic of polyadenylation in mature oocytes and in embryos (Fig. 9A). With CAT activity derived from injected CAT RNA as an internal control, Fig. 9B shows that although the chimeric RNAs were translated at relatively low levels in oocytes, they rose significantly during maturation. However, in 2-h embryos, the relative CAT activities of the two CAT-scdk2 chimeras fell to levels equivalent to those in

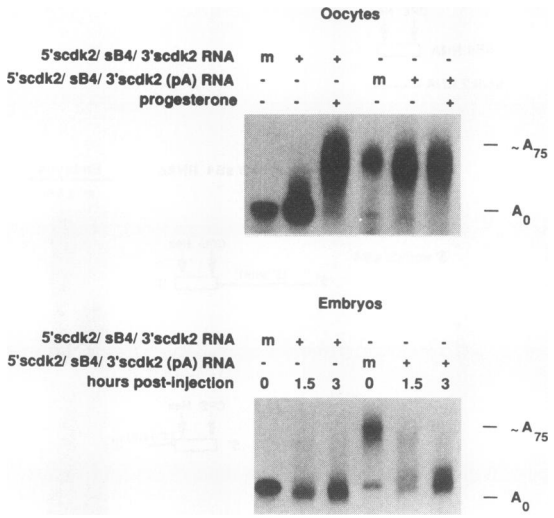

FIG. 8. Effects of cdk2 RNA polyadenylation-inhibiting sequences on an existing poly(A) tail. Poly(A)$^-$ and poly(A)$^+$ 5′scdk2/sB4/3′scdk2 RNAs were injected into oocytes, some of which were induced to mature with progesterone, and into fertilized eggs. Some of the fertilized eggs were immediately frozen (0 h), while others were further incubated for 1.5 and 3 h. The RNA was extracted and analyzed as described in the legend to Fig. 4. The number of adenylate residues added or removed from the transcripts is indicated on the right.

immature oocytes while, in contrast, that of CAT-sGb/B4 was approximately four times higher, equivalent to its activity in mature oocytes. These results, together with those in Fig. 9A, show that the 3′ UTR of cdk2 RNA controls translational stimulation and inhibition by modulating poly(A) tail length in early *Xenopus* development.

Regulation by *trans*-acting factors. In a series of UV cross-linking experiments, we attempted to identify proteins involved with the specific inhibition of cdk2 RNA polyadenylation in embryos. By using our mutant RNAs, however, we could identify no protein likely to be involved with this process (see Discussion). Consequently, we used a different approach to identify factors that might be involved. This was suggested by the studies of McGrew and Richter (19), who noted that under certain circumstances, CHX treatment could affect polyadenylation of G10 RNA. We reasoned that if CHX could also modulate cdk2 RNA polyadenylation, then we might be able to clone factors using an RNA expression assay.

In a preliminary experiment, we assessed whether CHX treatment had any effect on endogenous cdk2 RNA polyadenylation. The drug was added to the culture medium either at the time of fertilization or 1.5 h later. Northern analysis of total RNA from unfertilized eggs and 3-h embryos showed that deadenylation of this RNA required protein synthesis soon after fertilization, while the polyadenylation of B4 RNA remained unaffected (data not shown).

Is de novo synthesis of a factor also required for blocking of cdk2 RNA polyadenylation in embryos? Eggs were incubated with CHX at the time of fertilization or 2 h later. In both cases, scdk2 RNA was injected into 0.5-h embryos and extracted 3 h later (Fig. 10). When CHX was added at the time of fertilization, about 70% of scdk2 RNA was adenylated, whereas <10% of the RNA was adenylated in untreated embryos (Fig. 10, lanes 1 to 4). However, when CHX was added 2 h after fertilization, scdk2 RNA remained unadenylated (Fig. 10, compare lanes 2 and 5). CHX had no significant effect on the

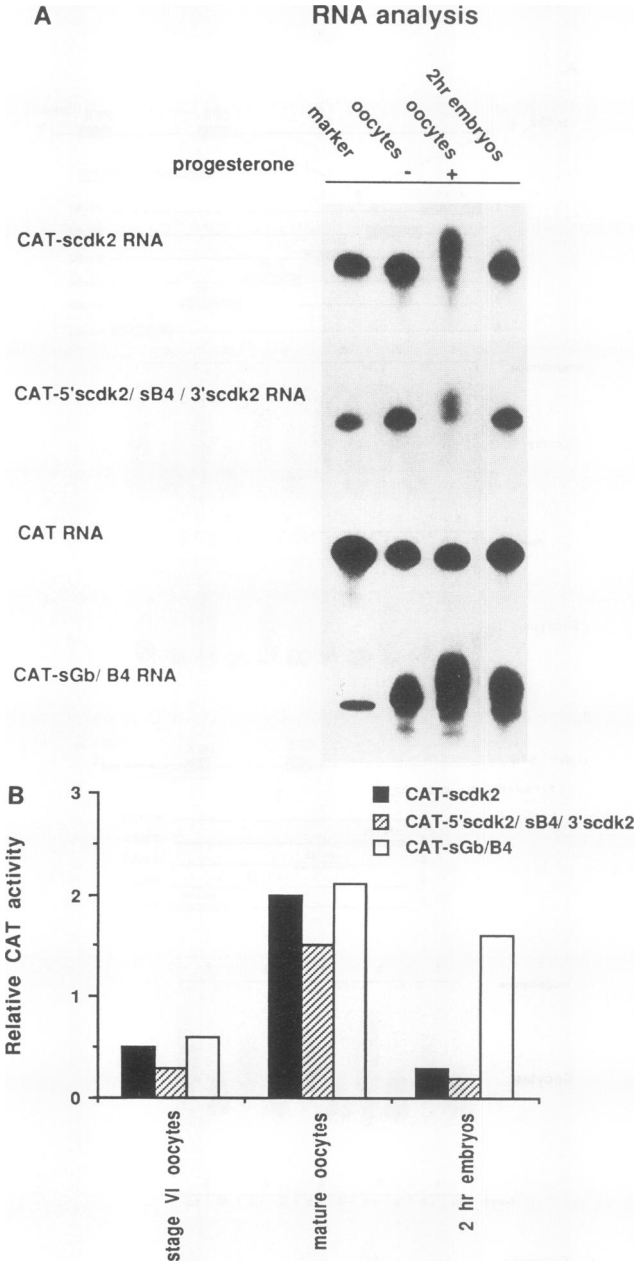

FIG. 9. Influence of scdk2 and B4 sequences on CAT RNA polyadenylation and expression. (A) Radiolabelled CAT and CAT chimeric RNAs were synthesized in vitro and injected into oocytes, some of which were induced to mature with progesterone, and into fertilized eggs. RNAs were extracted from oocytes at maturation and 2-h embryos and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The marker is uninjected RNA. (B) Unlabelled CAT and chimeric CAT RNAs were injected into oocytes and eggs as described for panel A. Cell extracts were prepared at the times indicated, and CAT assays were performed. CAT activity is expressed as the amount derived from the CAT chimeric RNAs relative to CAT RNA.

polyadenylation of sB4 RNA during embryogenesis, irrespective of the time at which it was added (Fig. 10, lanes 6 to 10). These data demonstrate that inhibition of scdk2 RNA polyadenylation, like stimulation of cdk2 RNA deadenylation, requires protein synthesis soon after fertilization.

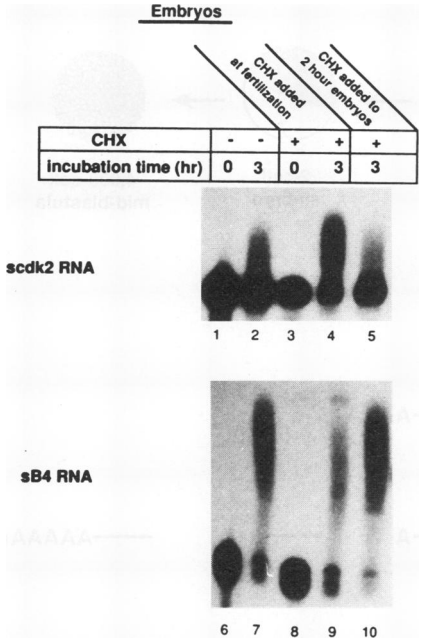

FIG. 10. Dependence on translation of a maternal mRNA for inhibition of scdk2 RNA polyadenylation. Fertilized eggs were injected with radiolabelled scdk2 and sB4 RNAs and incubated for the times indicated in the absence (lanes 1, 2, 6, and 7) or presence (lanes 3 to 5 and 8 to 10) of CHX. CHX was added to eggs with sperm during in vitro fertilization and was present throughout the subsequent incubation period (lanes 3, 4, 8, and 9) or added to 2-h embryos (lanes 5 and 10). In both cases, RNA was injected into 0.5-h embryos. Total incubation time was 3 h from the point of RNA injection. After incubation, the RNA was extracted and analyzed for polyadenylation as described in the legend to Fig. 4.

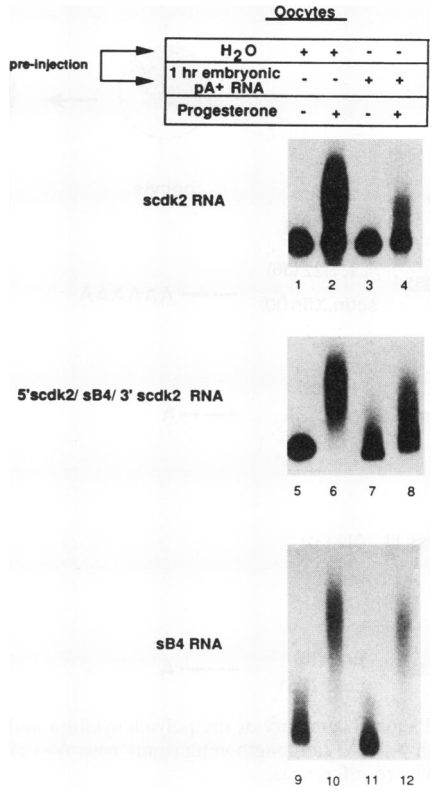

FIG. 11. Embryo poly(A)$^+$ (pA$^+$) RNA inhibits scdk2 RNA polyadenylation when injected into oocytes. Stage VI oocytes were either preinjected with deionized water or 1-h embryo poly(A)$^+$ RNA. After 16 h, radiolabelled scdk2 RNA (lanes 1 to 4), 5'scdk2/sB4/3'scdk2 RNA (lanes 5 to 8), or sB4 RNA (lanes 9 to 12) was injected. Progesterone was added to some oocytes after 1 h, and RNA was recovered from all samples 6 h later and analyzed for polyadenylation as described in the legend to Fig. 4. Note that the apparent difference in poly(A) tail length of B4 RNA in lanes 10 and 12 is due to "smiling" during electrophoresis and that in fact the poly(A) tail lengths are identical.

One hypothesis that emerged from the experiments described here is that a maternal mRNA, which is translationally dormant in oocytes, is "unmasked" after fertilization and encodes a protein necessary for scdk2 RNA deadenylation (or inhibition of adenylation). To analyze this further, poly(A)$^+$ RNA was isolated from 1-h embryos and injected into oocytes. Following 16 h of incubation to allow new protein synthesis, the oocytes were also injected with scdk2 RNA, chimeric 5'scdk2/sB4/3'scdk2 RNA, and sB4 RNA and incubated with or without progesterone. If the injected mRNA encoding the inhibitory protein was successfully translated in oocytes, then maturation-induced polyadenylation of the wild-type and chimeric scdk2 RNAs should be reduced relative to that of the controls (i.e., water-injected oocytes). Figure 11 shows that this indeed was the case. As expected, scdk2 RNA was polyadenylated in mature oocytes preinjected with water; however, virtually none was polyadenylated in mature oocytes preinjected with embryonic poly(A)$^+$ RNA (Fig. 11, lanes 1 to 4). Similarly, the chimeric RNA that contained the 5' and 3' inhibitory sequences (5'scdk2/sB4/3'scdk2) was prevented from undergoing full polyadenylation during maturation in oocytes preinjected with embryonic poly(A)$^+$ RNA but not in those preinjected with water (lanes 5 to 8). Injection of tRNA also had no effect on polyadenylation (data not shown). Polyadenylation of sB4 RNA during maturation, however, was unaffected by the preinjected material (lanes 9 to 12). [The slightly shorter poly(A) length of sB4 RNA in lane 12 is an electrophoresis artifact. Note that both of the samples in lanes 11 and 12 migrated slightly faster than the samples in lanes 9 and 10.] These results demonstrate that embryos contain a poly(A)$^+$ mRNA that encodes a protein required for blocking of cdk2 RNA polyadenylation.

DISCUSSION

A number of maternal mRNAs that are translationally controlled by changes in poly(A) tail length at different times of early *Xenopus* development have been examined (for references, see Fig. 12). What has emerged from these studies is a complex pattern of polyadenylation and deadenylation (summarized in Fig. 12). Some of the mRNAs examined in detail, such as G10, B4, and D7, are polyadenylated and translated during oocyte maturation. Polyadenylation of these RNAs requires a CPE with the general structure UUUUUAAU (9, 18, 19, 23). For other RNAs, such as those of the "Cl (cleavage) class" that are polyadenylated after fertilization (21), the CPE is dodecauridine (31). For a third class of RNAs, which includes those encoding ribosomal proteins L1 and S22, it is the absence of a CPE that causes default deadenylation during oocyte maturation (10, 36).

Members of a fourth RNA class, designated Eg (21, 22), are polyadenylated during oocyte maturation and deadenylated

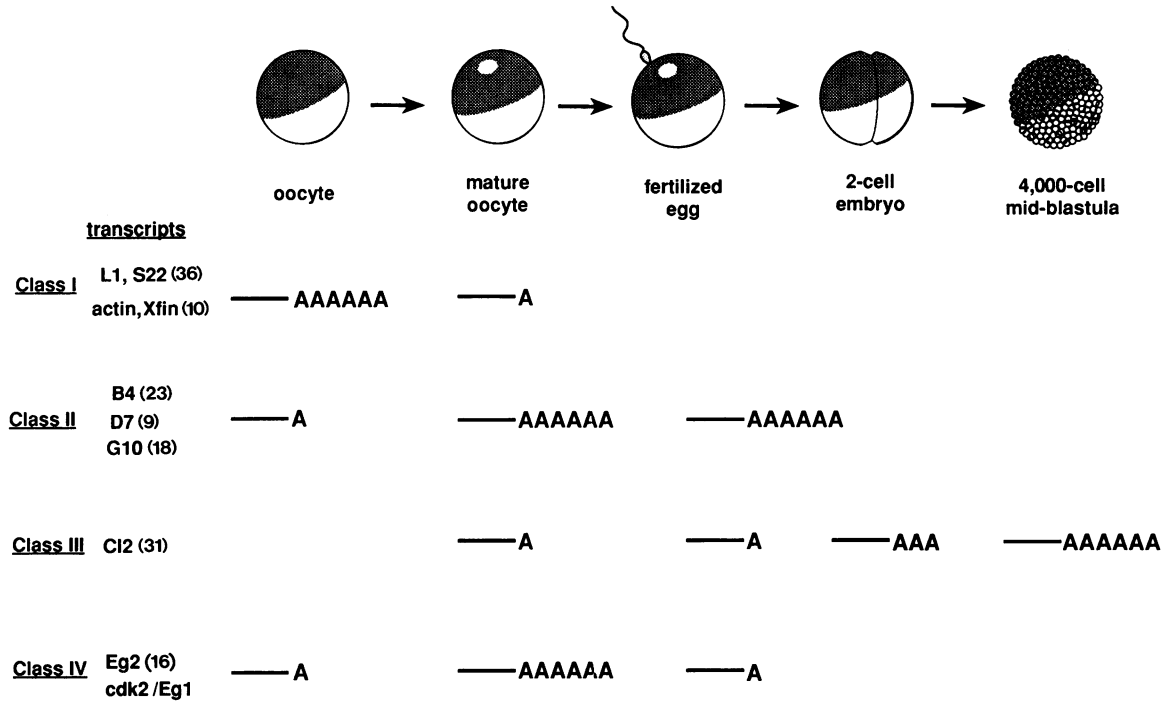

FIG. 12. Pictorial summary of the polyadenylation and deadenylation of some RNAs during early *Xenopus* development. The developmental stage at which poly(A) tail lengthening (numerous A's) or shortening (one A) occurs is indicated above the transcripts (solid bar). The numbers in parentheses are references.

soon after fertilization. cdk2 RNA (formerly Eg1 [20]), which is a member of this class, initially struck us as somewhat paradoxical because it has a CPE identical to that of B4 RNA, yet the latter transcript is polyadenylated during oocyte maturation and maintains a poly(A) tail for some time thereafter (6, 7). In the present study, we found that cdk2 RNA contains additional sequences that specifically promote deadenylation after fertilization. However, these same sequences also prevent poly(A) addition to this RNA when it is injected into fertilized eggs. Therefore, if cdk2 sequences promote deadenylation, is the deadenylated transcript repressed from further adenylation? Or does inhibition of poly(A) addition cause deadenylation, perhaps by default, as in CPE-negative RNAs during maturation (10, 36)? Unfortunately, efforts to separate the two activities have not been successful. For example, for the cdk2 and chimeric cdk2 poly(A)$^+$ RNAs that we tested in injected embryos, promotion of deadenylation is always observed in tandem with the apparent inhibition of poly(A) addition when the same poly(A)$^-$ transcripts are injected. Thus, while these alternatives are quite different mechanistically, the result is the same, namely, a deadenylated and nontranslating cdk2 RNA (see below).

Identification of polyadenylation-regulatory elements. By extensive mutational analysis, we identified two cdk2 sequence elements that reside 5′ of the CPE and 3′ of the hexanucleotide which, when fused to B4 RNA, promote deadenylation and inhibit poly(A) addition in early embryos. Somewhat surprisingly, however, extensive mutagenesis of the approximately 100-base 5′ region had no effect on the inhibition of poly(A) addition. Perhaps related to this, some inhibition of embryonic poly(A) addition also occurred when the 5′ cdk2 sequence was replaced with globin sequences (data not shown). One possible explanation for this is that RNA length, but not a specific sequence, is important for the inhibition. The 14-base 3′ sequence, on the other hand, partially blocks polyadenylation when fused to B4 RNA and also shows sensitivity to mutation. In one model suggested by these observations, a factor prevents polyadenylation in embryos by binding to the 3′ element and this interaction is stabilized by sequences 5′ of the CPE. Such a factor could recruit a deadenylating enzyme; alternatively, it could promote deadenylation by blocking stable association of the polyadenylation apparatus to the 3′ end of the RNA.

Legagneux et al. (16) also have examined sequences that promote embryonic deadenylation of an Eg-type RNA. Those investigators fused various regions of Eg2 RNA to CAT-coding sequences and injected the chimeric RNA, which was constructed to contain a poly(A) tail, into two-cell embryos. Examination of poly(A) tail length 1 and 3 h later showed that a distal 410-base region, but not a proximal 497-base region, of the Eg2 3′ UTR promoted embryonic deadenylation. In a recent, more detailed study, a 17-nucleotide sequence within the distal 410-nucleotide region was shown to be required for Eg2 RNA embryonic deadenylation (2). Deletion of this sequence from a globin Eg2-410 chimeric RNA stimulated polyadenylation in embryos. Unlike the two cdk2 RNA sequences that act synergistically to promote deadenylation, the Eg2 deadenylation motif is a discrete contiguous sequence that acts alone (i.e., deletion of the sequence promotes polyadenylation in embryos). However, the Eg2 deadenylation sequence does not function when transferred to another RNA, in contrast to the scdk2 RNA sequences. In addition, the deadenylation sequences of cdk2 and Eg2 show no significant similarity.

To identify factors that interact with the cdk2 RNA sequences mentioned above, we used oocyte, egg, and embryo extracts in a series of UV cross-linking experiments. We thought it likely that an approximately 54-kDa protein that

cross-linked to cdk2 RNA in all extracts (data not shown) was probably the same protein(s) as the p53/p55 doublet that Legagneux et al. (16) showed cross-linked to Eg2 and Eg5 RNAs, which both undergo embryonic deadenylation. However, when the chimeric sB4/scdk2 RNA was used as a substrate in cross-linking reactions, no protein in the 54-kDa range was detected although this RNA was as efficiently deadenylated as scdk2 RNA (data not shown). Further studies of the interaction between p53/p55 and Eg2 RNA by Bouvet et al. (2) demonstrated that the 17-nucleotide region is required for cross-linking of the protein doublet in embryo extracts, especially the first three bases, UGU, which are also required for efficient deadenylation of this RNA. On the basis of these data, p53/p55 was inferred to be involved in embryonic deadenylation of Eg2 RNA, although there is no direct evidence of this. Because p53/p55 does not cross-link to both of the cdk2 RNA deadenylation substrates, and because cdk2 and Eg2 RNAs do not share similar *cis*-acting deadenylation elements, we conclude that the embryonic deadenylation mechanism of these two RNAs is at least partially message specific.

We have shown that protein synthesis in embryos is required for deadenylation of endogenous cdk2 RNA, as well as polyadenylation inhibition of injected scdk2 RNA. This differs from the deadenylation activity in mature oocytes, which does not require de novo protein synthesis (35). Because essentially no transcription occurs between maturation and the midblastula stage, we surmised that a maternal mRNA which encodes a protein necessary for cdk2 RNA deadenylation [or prevention of cdk2 RNA poly(A) addition] is unmasked and translated soon after fertilization. This notion is supported by the observation that poly(A)$^+$ RNA isolated from two-cell stage embryos and injected into oocytes specifically repressed scdk2 RNA polyadenylation during maturation. Although the identity of the protein encoded by the effector RNA is unknown, it could be an RNA-binding protein or an enzyme that modifies the activity of an existing RNA-binding protein, such as a kinase or phosphatase. One possible target for such an activity is the 58-kDa protein that cross-links B4 RNA in a CPE-dependent manner (24), as well as cdk2 RNA (data not shown), and is phosphorylated in polyadenylation-competent oocyte and egg extracts (24). However, by immunoprecipitating protein-RNA complexes, we did not detect any changes in cross-linking of p58 to cdk2 and B4 RNAs or in its phosphorylation state in egg and early-embryo extracts. Our isolation of an RNA fraction that specifically inhibits cdk2 RNA polyadenylation when injected into oocytes that then undergo maturation will enable us to clone a cDNA for this protein by RNA expression.

Translational control by polyadenylation. For several of the mRNAs described in Fig. 12, mRNA injection studies have shown that poly(A) elongation induces translational recruitment and that poly(A) removal induces translational repression. The data presented in this study firmly agree with these observations. When attached to CAT-coding sequences, the cdk2 3′ UTR stimulates poly(A) addition and translational recruitment during maturation. In addition, it promotes deadenylation and translational silencing after fertilization. The notion that 3′ UTRs may contain multiple elements under developmental control is also supported by a recent study of c-*mos* and cyclin mRNAs in which the 3′ UTRs were shown to determine both the timing and extent of translation unique to each message in a poly(A)-dependent fashion (30).

How poly(A) metabolism leads to polysomal association is unclear. For example, although a "mature-length" poly(A) tail is needed for translational recruitment of some RNAs (23, 37), ongoing poly(A) addition is necessary for others (18, 31). In addition, the requirements to keep an RNA on polysomes may be different from those for its initial recruitment. This was suggested by experiments in which an mRNA that normally is translated in oocytes but deadenylated and removed from polysomes during maturation (1) can be stabilized there if the RNA is constructed to contain a CPE (36). In this case, it may be that the CPE maintains poly(A) tail length by promoting a forward reaction, i.e., poly(A) addition, at the expense of a reverse reaction, i.e., poly(A) removal (38). Thus, poly(A) retention by this dynamic equilibrium somehow stabilizes an mRNA in polysomes.

Despite significant advances in the understanding of cytoplasmic poly(A) addition and removal, how these events control translation needs much further clarification (14). As factors involved with the polyadenylation process are purified, however, it is likely that the molecular mechanisms of translational recruitment, maintenance, and repression will be revealed.

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