Original Article

Localization of *c-mos* mRNA around the animal pole in the zebrafish oocyte with Zor-1/Zorba

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Summary **In oocytes, many maternally supplied products are stored, and these products play important roles in cell cycle regulation and early development. Mos protein, which is coded on the *c-mos* gene, promotes oocyte maturation and is involved in MAP-kinase signaling pathway. In *Xenopus*, maternally supplied *c-mos* mRNA undergoes poly(A) addition, and translational activation *via* CPE (cytoplasmic polyadenylation element) and CPEB (CPE-binding protein). The elongated poly(A) is shortened and the *c-mos* mRNA is degraded during early embryogenesis *via* EDEN (embryo deadenylation element) and EDEN-BP (EDEN-binding protein).**

We cloned the full-length zebrafish *c-mos* gene, which is conserved at the protein coding region in vertebrates. *c-mos* mRNA has two putative CPE sequences in its 3'UTR, which binds to zebrafish CPEB homologous protein, Zor-1. We could not observe EDEN sequence, and could not detect interaction between *c-mos* mRNA and zebrafish EDEN-BP homologous protein, Brul, even though immuno precipitation and RT-PCR experiments suggested that *c-mos* mRNA interacts with Zor-1 *in vivo*. Interestingly, we found *c-mos* mRNA is located in the animal cortex of zebrafish oocyte, where Zor-1 protein exists. Taken together, these results suggest that the animal cortex is the central core of oocyte maturation in zebrafish.

Keywords: CPEB, *c-mos*, CELF/Bruno, RNA localization, oocyte maturation

1. Introduction

In vertebrates, the cell cycle of an oocyte is arrested at the first prophase of meiosis. Oocyte maturation is initiated by maturation promoting factor (MPF) and is stopped in Metaphase II by cytostatic factor (CSF) before fertilization (*1*,*2*). The activated MPF promotes the cell cycle to overcome the Metaphase I check point. It causes germinal vehicle break down (GVBD) (*1*,*2*). Activities of CSF keep the cell cycle in Metaphase II of meiosis (*1*,*2*). These activities are regulated by the quantitative level of these components. MPF is composed of cdc2 and cyclin B. The translation of *cyclin B* mRNA is activated and cyclin B protein begins to increase from the initiation of the GVBD (*3*,*4*). Mos protein, a component of the CSF which acts as a serine/threonine kinase, increases greatly during oocyte maturation, and acts as MAPKKK (mitogen-activated protein kinase kinase kinase) (*3*,*4*). Mos protein and cyclin B protein synthesis are activated at the translation level of these mRNAs during oocyte maturation (*5*,*6*). In *Xenopus*, *cyclin B*1 and *c-mos* mRNAs are maternally supplied and stored with short poly(A) tails (20~50 nt). After the stimulation of oocyte maturation, poly(A) tails of these maternally supplied mRNAs are elongated to 150~200 nt and their translation is activated. After fertilization, these elongated poly(A) tails are shortened, and these mRNAs are degraded rapidly (*7*,*8*).

The polyadenylation and translational activation of *cyclin B1* and *c-mos* mRNA are controlled by *cis*-elements, which are CPEs (cytoplasmic polyadenylation elements, U5AU, U4A2U) and poly(A) addition signals (AWUAAA) of the 3' untranslationed region (UTR)

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in mouse and *Xenopus*. In this process, CPEB (CPE binding protein) binds to the CPE with Maskin protein (*9*). The binding between CPEB and CPEs depends on two RRMs (RNA recognition motifs) and a Zinc finger motif (ZnF) (*10*). The phosophorylation of CPEB raises a conformation arrangement and is forced to assemble with PAP (poly(A) polymerase) *via* binding to CPSF (cleavage and polyadenylation specific factor), which binds to the poly(A) addition signal (*11*). Since PAP carries out the polyadenylation of CPE containing mRNAs, elongated poly(A) containing mRNAs are produced. It is known that these mRNAs form circular structures, based on the interaction between elongated poly(A) and the eIF4F at its 5' cap structure, *via* binding to PABP (poly(A) binding protein). Then, this structure promotes recycling of 40S ribosomal complex, and activates the translation of its mRNA. Deadenylation of poly(A) tails after fertilization has been reported in *Xenopus c-mos* and *Eg5* mRNA. The deadenylation of these mRNAs depends on embryo deadenylation elements (EDENs), which specifically bind to EDEN-binding protein (EDEN-BP) (*12*). EDEN-BP is an RNA recognition motif (RRM) type RNA-binding protein, belonging to CELF/Bruno-like family. Bruno, one of the members of CELF/Bruno-like family, acts as the translational repressor of *oskar* and *gurken* mRNAs in *Drosophila* oocytes (*13*). Another family member, CUGBP1, plays a very important role in myotonic dystrophy *via* splicing regulation (*14*). Besides translational regulators, CPEB and EDEB-BP, it is clear that microRNAs control the translational levels of the target mRNAs in recent studies. For example, miR-430 mediates deadenylation and clearance of maternal mRNA in early embryogenesis in zebrafish (*15*).

In zebrafish oocytes, some maternally supplied products are localized at the cortex of the animal pole or vegetal pole. *zor-1/zorba* mRNA, which is a *CPEB* homologoue in zebrafish, and *cyclin B* and *pabp* mRNAs are also localized at the cortex of the animal pole in grown oocytes (stage III) (*16,17*). Zor-1 protein expression corresponds to that of its mRNA localized at the animal cortex (*16*). Previously, we reported that *zdazl* and *brul* mRNAs are localized at the cortex of the vegetal pole in zebrafish oocytes (*18,19*). Brul protein is a homologous protein of *Xenopus* EDEN-BP and human CUGBP1, and can specifically bind to the EDEN sequence (*20*). *zdazl* mRNA, *brul* mRNA and Brul protein are transported to the distal ends of the cleavage furrows of the 4-cell stage embryo, where the germ plasma is formed (*21,22*). In addition, *vasa*, *nanos1*, *dnd*, and *askopos* mRNAs were also detected in the cleavage furrows (*23-26*).

Although the zebrafish *c-mos* gene has already been reported (*27-29*), we independently cloned a *c-mos* homologous gene in zebrafish. Zebrafish *c-mos* gene encodes the Ser/Thr kinase domain and has CPE-related sequences in its 3'UTR without the EDEN sequence. The *c-mos* mRNA is localized around the animal pole in zebrafish oocytes. Furthermore, our results suggest that *c-mos* mRNA binds to the Zor-1 protein *in vitro* and *in vivo*, but does not bind to a vegetal localized factor, Brul protein. Therefore, the embryonic deadenylation regulated by EDEN-BP may not be essential for *c-mos* mRNA, but the cytoplasmic polyadenylation of *c-mos* mRNA is one of the basic mechanisms in vertebrates.

2. Materials and Methods

2.1. *RT-PCR and cDNA screening*

Two degenerate primers, MOS/DS (CCAGAATTCTTY TGGGCNGARYTNAAY) and MOS/DA (TCTGTCGA CATYTGCCANARNGTDAT), were prepared based on *c-mos* genes in vertebrates. The zebrafish *c-mos* cDNA fragment was amplified by RT-PCR with MOS/DS and MOS/DA primers from total ovarian RNAs (TaKaRa). The digoxigenin (DIG)-labeled fragment was amplified with CM/PS primer (ATTAAGCTTCAAAACATTG TGCGCGTG) and CM/PA primer (CAAGGATCCGC CAAAAGAATAAACGTC). A zebrafish adult cDNA library (a gift from Dr. Grunwald) in the λZAPII vector was screened under high stringency conditions with the DIG-labeled fragment. Excision of positive phagemid sequences was carried out according to the description supplied by Stratagene. Three independent clones contained the zebrafish *c-mos* cDNA. The sequence of *c-mos* gene has been deposited in the DDBJ (#AB032727).

2.2. *Northern blot*

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14 h light/10 h dark cycle. Gonads were manually isolated from mature adult fish. Total RNA was purified from embryos, whole females and males, the dissected ovaries and testes, and the remaining bodies after dissection. For northern blotting of adult and adult tissues, 10 μg of total RNA were electrophoresed as shown in Figure 2A and Figure 2B *lane* 1. For northern blotting of embryonic stages, 20 μg of total RNA were electrophoresed as shown in Figure 2B *lanes* 2-5. The DIG-labeled cDNA fragments were used as probes. For detection, CSPD (Tropix) was used as a chemiluminescent substrate.

2.3. *In situ hybridization*

Whole mount and sections of *in situ* hybridization was performed essentially as described (*19*). The full-length of the zebrafish *c-mos* gene was used as a probe. For *in situ* hybridization of ovarian sections, specimens were embedded in paraffin and cut at thickness of 9 μm. Stages of oocytes were classified as described (*16*).

2.4. *Preparation of GST fusion protein*

Portions of Zor-1 coding sequence were amplified by PCR, and subcloned into pGEX 6p-1 (Amersham). The Zor-1 primer sets are as follows: GS1 (CAGGAATTC GCCATGGCGTTTTCTCTGA) and GAS1 (GCAGTC GACCACATGGACATCCAGG CTC) for pGST-Zor-1, GS1 and GAS2 (GCAGTCGACTAGGCATCCTCCA AGTATG GATC) for pGST-ΔZnF. Each expression plasmid was transformed in *E. coli* DH5α. GST fusion proteins were induced with 0.1 mM IPTG. GST-Zor-1 and GST-ΔZnF proteins were affinity purified and dialyzed with binding buffer (10 mM Hepes-KOH (pH 7.7), 100 mM KCl, 5% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM PAMSF, 5 μg/mL Pepstatin A). Concentrations of these proteins were calibrated using CBBR-250 (Bio-Rad) and densitometry was performed after SDS-PAGE (MacBAS, Fuji).

2.5. *UV-crosslinking experiments*

Annealed oligo nucleotides corresponding to the *Xenopus* B4-type CPE and the mutant CPE (*7*) were cloned into pSP64 (Promega). Portions of annealed oligonucleotides of the zebrafish *c-mos* 3'UTR; zm-CPE1 (AAATTTTTTATGCAAAATGTTTAATTAA AT GT), zm-CPE2 (AAATGTTCGTGTTTTTGTTT TATTGTGAAGCT) and zm-nc (ATA ACAATTGTT TAATATTGTAAATGTTCGTGT), were cloned into pSP73 (Promega). *In vitro* transcription was carried out as described previously, in the presence of [α-^{32}P] UTP (*30*). UV-crosslinking experiments were performed essentially the same as described (*31*). RNA probes were incubated with the fusion proteins in binding buffer containing 0.5 μg/μL of yeast tRNA at 23°C for 20 min. After UV-irradiation and RNaseA-treatment, the reaction mixture was applied onto SDS-PAGE. Images of binding products were analyzed using a BAS2000 Image Analyzer (Fuji).

2.6. *IP- RT-PCR*

Whole ovaries from adults were harvested and homogenized in ice-cold extract lysis buffer (15 mM Hepes-KOH (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 5% glycerol, 0.1% NP-40, 0.1% Triton X-100, 1 mM DTT, 0.2 mM PASMF, 1 mM EDTA). After pelleting yolk and debris, the supernatants were combined with RNase inhibitor (Promega).

The anti-Zor-1/Zorba antibody and the pre-immune serum were a gift from Dr. Bally-Cuif (*16*). The anti-Brul was prepared essentially as described previously (*21*). The pre-immune serum, Zor-1- and Brul-antibody were each separately mixed with Protein G Agarose (GE). 200 μg of oocyte extract was added to each of above mentioned solutions. Gels were washed five times with washing buffer (10 mM Hepes-KOH (pH 7.9), 400 mM KCl, 5% glycerol, 1 mM DTT, 0.2 mM PAMSF). Immunoprecipitated RNA was purified by phenol/chloroform extraction followed by ethanol precipitation. We carried out the reverse transcription reaction and the polymerase chain reaction (PCR) with an RT-PCR kit (TaKaRa) and specific primers of zebrafish *c-mos*; CM/PS and CM/PA, eIF 4A; eIF4A5' (TAAGGATCCGATTTACGAC GTGTACCG) and eIF4A3' (ATTGAATTCCAAACGTCTGTGGAG ATC) and dazl mRNA; dazl5' (GTAGGATCCATG GTTCAGGGGGTTCAG) and dazl3' (GTCCTCGA GCTACATAAGGGTTAGCAAAG). The RT-PCR products were electrophoresed in 6% native polyacrylamide gels. After staining with SYBR Green I (Molecular Probe), the images of the products were analyzed using an FM-BIO II (HITACHI) bioimager.

3. Results

3.1. *Cloning of zebrafish c-mos gene*

We designed degenerate primers that contain well conserved regions of *c-mos* gene from several species. To isolate the zebrafish *c-mos* homologus gene, we carried out RT-PCR experiments and cDNA screening from the zebrafish whole adult cDNA library. Three independent clones were obtained and sequenced (Figure 1). Three clones are essentially identical, and are similar to *Xenopus c-mos* gene. Northern blot analysis showed that the *c-mos* mRNA was highly expressed as a single approximately 1.3 kb product (Figure 2A). This size matched the *c-mos* cDNA which we cloned. This suggested that the *c-mos* cDNA contained almost the full length of the zebrafish *c-mos* gene. The zebrafish *c-mos* gene encodes the Ser/Thr kinase domain and contains the 50 nt of the poly(A) tail, the polyadenylation signal (AUUAAA) and two putative CPE sequences (Figure 1). The polyadenylation signal and putative CPEs are located within 50 nt of the 3'UTR. We could not detect EDEN-conserved sequence in the zebrafish *c-mos* gene.

3.2. *Expression and localization pattern of c-mos*

To examine expression patterns of *c-mos* gene, northern blot analysis was performed in adult fish at first. The *c-mos* mRNA was detected in the RNAs of females and ovaries, but was not detected in the RNAs of somatic organs of female or male fish (Figure 2A). This result shows that the *c-mos* mRNA was highly and specifically expressed in ovaries.

To examine when the *c-mos* gene was expressed in zebrafish oogenesis, we carried out whole mount *in situ* hybridization. The *c-mos* mRNA was expressed during early oogenesis, in stage Ia and Ib oocytes (data not shown). Before stage III, the *c-mos* transcripts were detected throughout the oocyte (Figure 3A). Interestingly, *c-mos* mRNA was localized in one part

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AAATTAGCGACCAGCTCTCCGATTTCACAGTTATCATGCGCCATGCCCTCACCAATCCCC      60
                                                  M  P  S  P  I  P
GTCACCCGACTTTTGCCAAAGGATTTCGGCCTCGAGTTTGGCGCATGCAGCAGCCCGCTG     120
V  T  R  L  L  P  K  D  F  G  L  E  F  G  A  C  S  S  P  L
ACCAAAACTGCCAGTGGATCTACCCTGCGCGTGCCCACAAACAAGTTTCATGGTAAAGTC     180
T  K  T  A  S  G  S  T  L  R  V  P  T  N  K  F  H  G  K  V
GCACACAGGCTCTGGTCCTCCGTGATCCACTGGCGCGAGCTGCAGGCTCTGGAGCCCATA     240
A  H  R  L  W  S  S  V  I  H  W  R  E  L  Q  A  L  E  P  I
GGCAGCGGTGGATTCGGTACGGTGTTCAGAGGCACATACTTCGGCGAGACTGTCGCTGTG     300
G  S  G  G  F  G  T  V  F  R  G  T  Y  F  G  E  T  V  A  V
AAAAAGGTCAAGTGTGTGAAAAACAAACTGGCATCGAGGCAAAGTTTCTGGGCGGAACTC     360
K  K  V  K  C  V  K  N  K  L  A  S  R  Q  S  F  W  A  E  L
AACGCCGCGCACCTGCACCATCAAAACATTGTGCGCGTGCTCGCGGCCACCACGTGCACT     420
N  A  A  H  L  H  H  Q  N  I  V  R  V  L  A  A  T  T  C  T
CCTGCGCATCTCAACACCAAAGACAACATCGGGACGATCGTAATGGAGTTCGCAGGCAAT     480
P  A  H  L  N  T  K  D  N  I  G  T  I  V  M  E  F  A  G  N
ATAAATCTACAGAAGCTCATTTATGGGCTCACAGACTTGCTTCCTGTGGAGAAGTGTATA     540
I  N  L  Q  K  L  I  Y  G  L  T  D  L  L  P  V  E  K  C  I
AAGTATTCAATAGACATCGCGCGCGCCCTCCAGCACCTGCACGCGCACGGCGTAGTGCAC     600
K  Y  S  I  D  I  A  R  A  L  Q  H  L  H  A  H  G  V  V  H
CTGGATTTAAAACCAGCCAATGTCTTGTTGTCAGAACAGGGTGTTTGTAAAATCGCAGAT     660
L  D  L  K  P  A  N  V  L  L  S  E  Q  G  V  C  K  I  A  D
TTTGGGTGCTCGTTTAAAATATCCAGCACAAGTGACACCGTGACGCACATGAATGAAATC     720
F  G  C  S  F  K  I  S  S  T  S  D  T  V  T  H  M  N  E  I
GGCGGCACGTTTACGCACCGGGCGCCCGAGCTGCTGAAAGGTGAGGAAGTGTCGCCGCGC     780
G  G  T  F  T  H  R  A  P  E  L  L  K  G  E  E  V  S  P  R
GTGGACGTTTATTCTTTTGGCATAACGTTGTGGCAGCTGCTCACCCGAGAGCCGCCCTAT     840
V  D  V  Y  S  F  G  I  T  L  W  Q  L  L  T  R  E  P  P  Y
GAGGGAGACAGACAGTATATCCTGTACGCTGTTGTGGGGTATAACCTGCGCCCTTTGACC     900
E  G  D  R  Q  Y  I  L  Y  A  V  V  G  Y  N  L  R  P  L  T
AGCAGGAATGTTTTTACCCAGTTTTTTATTGGACAGAATTGTCAAAAACTGATCAGCCGG     960
S  R  N  V  F  T  Q  F  F  I  G  Q  N  C  Q  K  L  I  S  R
TGTTGGGACGGCGACCCCAGCATCCGACCGACCGCAGATAAGTTTGTCGACGAACTTTCA    1020
C  W  D  G  D  P  S  I  R  P  T  A  D  K  F  V  D  E  L  S
GTTTTACTGTAAAATTGAATCTCCGCGTTTGCAAAAAAAAAAAAAAAAAAAAATCTTCAAT    1080
V  L  L  .
GAAACGTTTTTTTATAACAATTGTTTAATATTGTAAATGTTCGTGTTTTTGTTTTATTGT    1140

GAAGCTAAATTTTTTTATGCAAAAATGTTTAATTAAATGTTTCCTTGTGTTTAAAAAAAAA    1200

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                       1241
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Figure 1. Nucleotide and amino acid sequences of the zebrafish *c-mos* gene. The nucleotide sequence is indicated in black. The amino acid sequence is indicated with color and shows the open reading frame. The underlined section shows annealing positions of the degenerate primers. The yellow highlights indicate putative CPE (cytoplasmic polyadenylation element) sequences. The orange highlight indicates the polyadenylation signal.

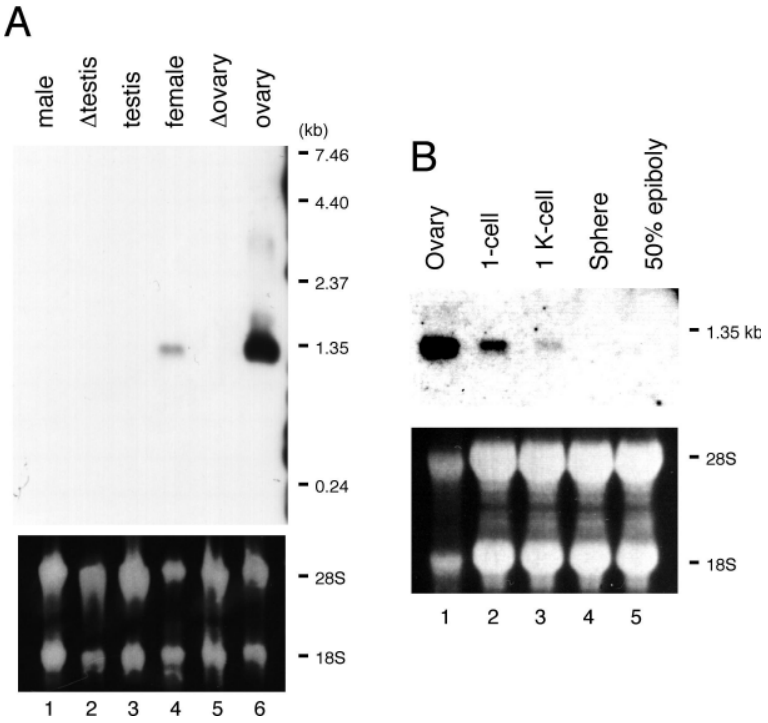

Figure 2. Northern blot analysis for zebrafish *c-mos* mRNA. The upper panels show *c-mos* mRNA. As a control, the lower panels show ribosomal RNAs. The RNA size markers are indicated on the right side of the panels. (A) The expression analysis of *c-mos* mRNA in adult zebrafish. 10 μg of total RNA from adult male, Δtestis, testis, adult female fish, Δovary and ovary are applied in each lane. Δtestis and Δovary indicate somatic tissues from adult fish, not including testis or ovary, respectively. (B) The expression analysis during early embryogenesis. Ten μg of total RNA from the ovary and 20 μg of total RNA from the embryo are applied to each lane.

of stage III oocytes (Figures 3B and C). To clarify where *c-mos* mRNA was localized, we performed *in situ* hybridization on ovary sections. The micropyle, indicated as the future animal pole, was observed in oocytes after stage III and *c-mos* mRNA was detected in the cortex around the micropyle (Figure 3D). These results show that the zebrafish *c-mos* mRNA is localized in the cortex of the future animal pole in oocytes after stage III. In a previous study, Bally-Cuif *et al*. (*16*) reported that *zor-1/zorba* mRNA and Zor-1/Zorba protein was localized around the future animal pole in zebrafish oocytes. To investigate whether the region of *c-mos* mRNA localization is the same as that of *zor-1* mRNA, we performed *in situ* hybridization on continuous sections. We observed that the *c-mos* and *zor-1* mRNAs were co-localized approximately in the animal cortex of stage III oocytes (Figures 3D and E). These results show that *c-mos* mRNA is one of the animal pole localized factors in zebrafish oocytes (*16*).

After fertilization, the *c-mos* mRNA was detectable in the blastomere of 1-cell stage embryo (Figure 3F). This mRNA completely disappeared between 1k-cell stage and sphere stage (Figures 3G and H). Northern blot analysis showed that the *c-mos* mRNA decreased in the maternal stage embryos, and was not observed in the RNA of sphere stage embryo (Figures 2B). From

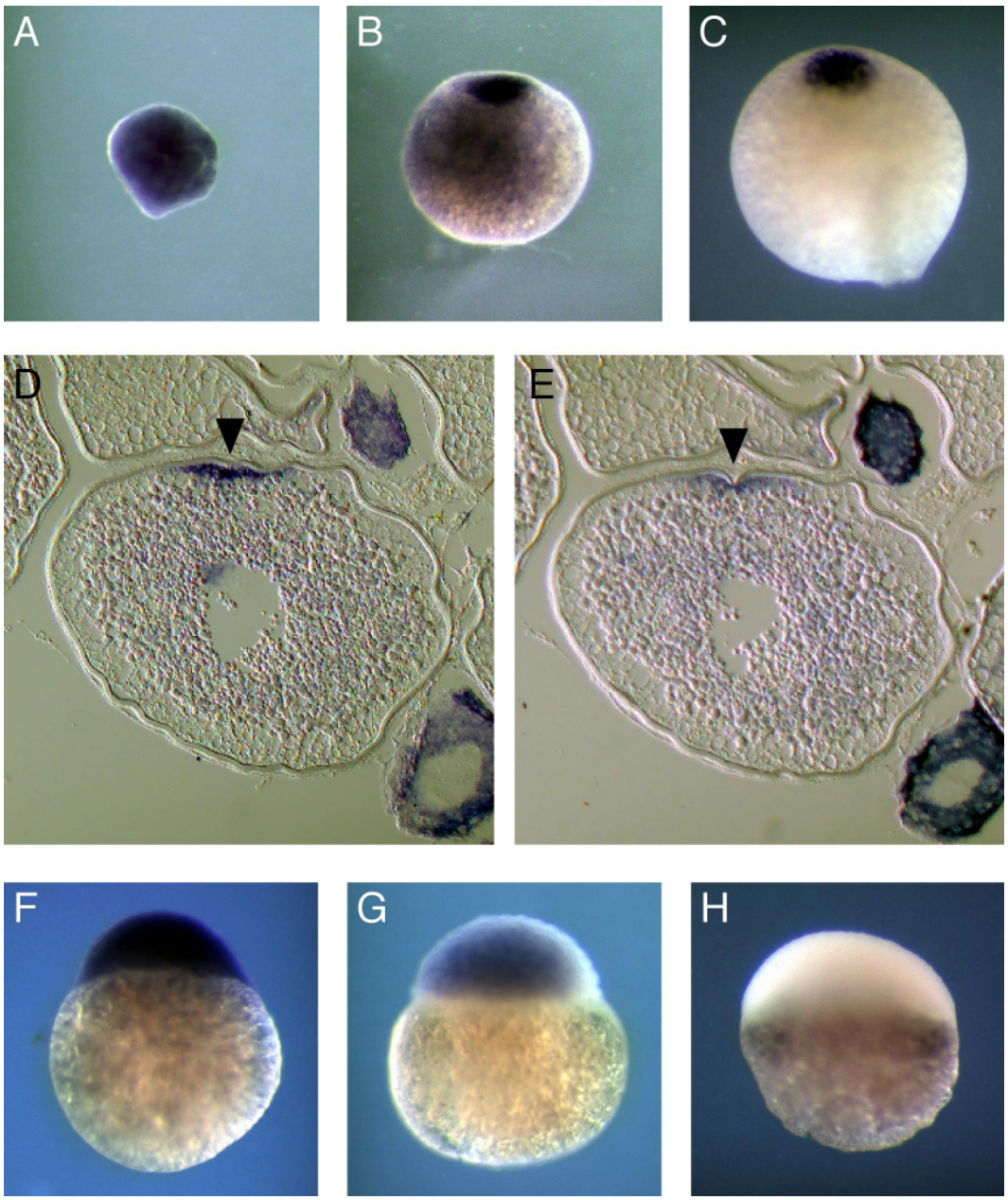

Figure 3. *In situ* hybridization of *c-mos* mRNA in zebrafish oogenesis and early embryogenesis. (A-C) whole mount *in situ* hybridization of *c-mos* mRNA during oogenesis. The expression patterns of *c-mos* mRNAs at stage II oocyte (A), early stage III oocyte (B) and stage III oocyte (C) are shown. The serial vertical sections are used for *in situ* hybridization to analyze the expression of *c-mos* mRNA (D) and *zor-1* mRNA (E) as a control. The arrowheads indicate the micropyles, which indicate the animal pole of zebrafish oocytes. (F-H) whole mount *in situ* hybridization of *c-mos* mRNA during embryogenesis. The expression patterns of *c-mos* mRNAs in the 1-cell stage embryo (F), the 1k-cell stage embryo (G) and the sphere stage embryo (H) are shown.

these results, it is suggested that *c-mos* mRNA was rapidly destroyed during early embryogenesis, like *Xenopus c-mos* and *cyclin B1* mRNAs.

3.3. *Binding between the CPEs of c-mos mRNA and Zor-1 protein in vitro*

Zor-1 protein, which is encoded by the animal pole localized *zor-1* mRNA, was localized around the animal pole of oocytes along with *cyclin B*, *pabp*, *Vg1* and *c-mos* mRNAs (*16*, Figure 3D). Since the *zor-1* gene is a highly conserved CPEB homologus gene in zebrafish (*16*), we analyzed whether the zebrafish Zor-1 specifically bound to the CPE using UV-crosslinking experiments. We found that the Zor-1 bound to the CPE of *Xenopus cyclin B1* mRNA, but did not bind to a mutated RNA of the CPE (Figure 3C, *lanes* 1 and 2). This showed that Zor-1 bound to the CPE specifically. In *Xenopus*, it has been shown that the ZnF of CPEB is important for the CPE-binding in a manner dependent on Zinc ion (*10*). The lack of a ZnF in Zor-1 disrupted the CPE-binding (Figures 4B and 4C). The lack of Zn^{2+}

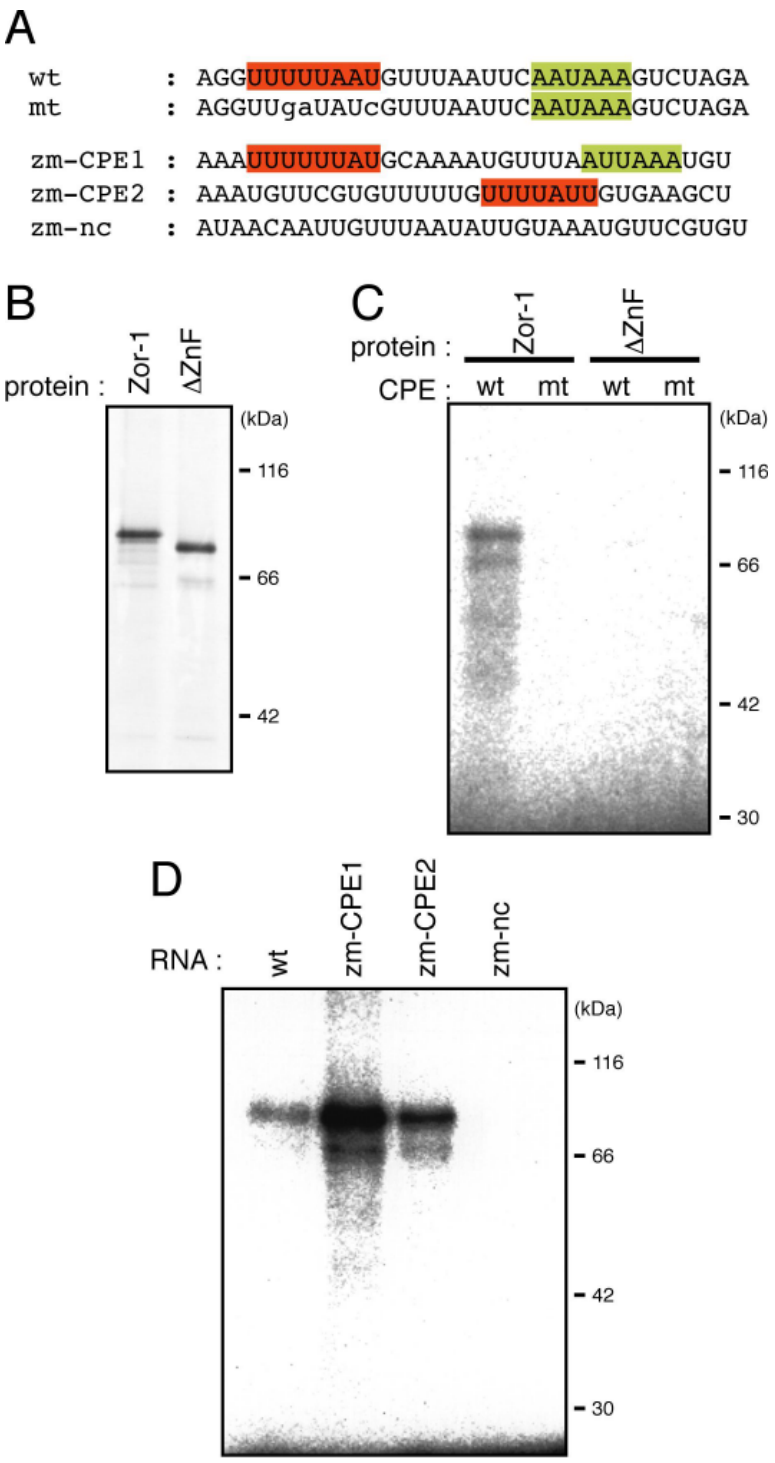

Figure 4. UV-crosslinking experiments between zebrafish *c-mos* and Zor-1 protein. ^{32}P labeled RNA sequences are shown (A). The first line is the CPE sequence of *Xenopus cyclin B1*, and its mutant is in the second line. The other lines indicate two putative CPE sequences, and a control sequence in zebrafish *c-mos* mRNA. As described in Figure 1, the CPE sequences and polyadenylational signals are highlighted in orange (CPE) and yellow (poly(A) signal). Zebrafish CPE binding protein, Zor-1, and its deletion mutant proteins of zinc finger domain (ΔZnF) are prepared as GST-fusion proteins, and analyzed in SDS PAGE with CYBR staining (B). UV-crosslinking experiments were carried out using Zor-1 proteins with the CPE RNA of *cyclin B1* (C) and with the putative CPE sequences of *c-mos* (D).

ion in the binding reaction also reduced the binding activity of Zor-1 with the CPE (data not shown). These results mean that the similarities between the zebrafish Zor-1 and the *Xenopus* CPEB are not only amino acid identities but also CPE-binding.

As we described above, zebrafish *c-mos* mRNA has two putative CPE sequences (Figure 1). Therefore, we checked whether these CPE sequences were bound to Zor-1 protein. We found that Zor-1 protein bound strongly to the CPE1 and CPE2 sequences, but not to the non-CPE sequence in zebrafish *c-mos* mRNA (Figure 4D). The binding of Zor-1 with CPE1 or CPE2 of zebrafish *c-mos* mRNA seems to be much stronger than that of Zor-1 with the *Xenopus* CPE.

3.4. *The interaction between the c-mos mRNA and Zor-1 protein in vivo*

We detected the binding activity of Zor-1 protein to the CPEs of zebrafish *c-mos in vitro*. Our results and previous studies have shown that maternally supplied *c-mos* mRNA and Zor-1 protein exist in the animal cortex of zebrafish oocytes (*16*). Therefore, we tested whether Zor-1 protein interacted with *c-mos* mRNA *in vivo* using IP-RT-PCR (immuno precipitation and RT-PCR). The band of *c-mos* mRNA was detected in co-precipitated RNA by Zor-1, but not in RNA of the pre-immune serum (Figure 5). We tested *eIF4A* mRNA as a negative control. Actually, we could not detect any PCR products from *eIF4A* mRNA. These results suggested that *c-mos* mRNA and Zor-1 protein were associated in zebrafish oocytes.

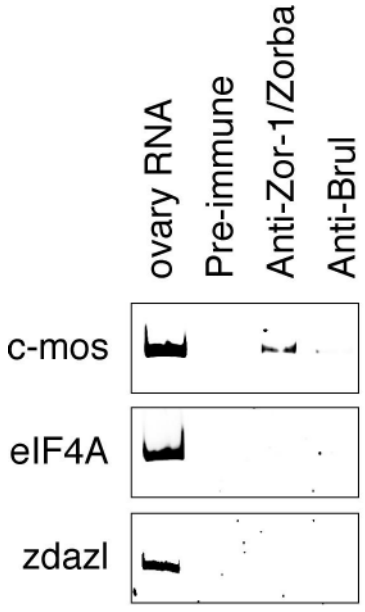

Figure 5. Immunoprecipitation and RT-PCR experiment. Anti-Zor-1/Zorba, anti-Brul and pre-immune serums were each separately mixed with protein G resin and prepared as Antibody conjugated columns. Oocyte extracts were applied to these columns. The RNAs co-precipitated with either Zor-1 or Brul protein, were purified and used for the RT-PCR. The left lane shows the PCR products from *c-mos*, *dazl* and *eIF4A* mRNAs, as controls which were amplified from 200 µg of oocyte extracts. The PCR products of co-purified RNA were analyzed in 6% native acrylamide gel, stained with SYBR Green I, and imaged with the FM BIO II (Hitachi).

Although *Xenopus c-mos* mRNA has the EDEN sequence, which can be bound to EDEN-BP, zebrafish *c-mos* mRNA doesn't have any EDEN-like sequence. Furthermore, *brul* mRNA, zebrafish EDEN-BP homologous gene transcript, is mainly localized at the vegetal cortex in the oocyte. Brul protein is detected in the cleavage furrows in 4-cell stage embryo (*22*), but the localization of Brul protein in oocytes is unclear. Indeed, this was consistent with our result that we could not detect any interaction between *c-mos* mRNA and Brul protein in the IP RT-PCR assay (Figure 5).

4. Discussion

We have identified the full-length of *c-mos* gene whose transcript was specifically expressed in ovary. We found that *c-mos* mRNA, which was detected throughout oocyte stage Ia and Ib, was localized in the animal cortex in the oocyte after stage III. Since *c-mos* mRNA conserved two putative CPE sequences in its 3'UTR, we assume that it is involved in the cytoplasmic polyadenylation event. Actually, we showed the animal pole localized CPEB homologue, Zor-1 protein interacting with *c-mos* mRNA *in vivo* and *in vitro*. These results suggested that the spatial regulation of translational control was very closely related to oocyte maturation in zebrafish.

After fertilization, *c-mos* mRNA was completely degraded during early embryogenesis. However, the zebrafish *c-mos* gene lacks any EDEN-like sequence (Figure 1). No interaction between *c-mos* mRNA and Brul protein was detected by the IP-RT-PCR experiment (Figure 5). Furthermore, *c-mos* mRNA is localized at the animal cortex of oocytes, though *brul* mRNA is mainly localized at the vegetal cortex (*19*). These results suggest that there was no remaining mechanism for the embryonic deadenylation of *c-mos* mRNA, in contradiction to reported *Xenopus* results (*12*). However, Northern blot analysis showed that maternally supplied *c-mos* mRNA was rapidly degraded in early embryogenesis (Figure 2B). Therefore, we need to consider other mechanisms for the degradation. It became clear that microRNAs are strongly affected by RNA metabolism and its translational repression. For instance, miR-430 plays a very important role in the degradation of maternally supplied mRNAs in somatic cells during early embryogenesis (*15*). There is so far no report that miRNAs regulate *c-mos* mRNA in vertebrates. Although, we preliminarily searched for the miR-430 binding site in 3'UTR of zebrafish *c-mos* mRNA, we could not detect any binding site.

On the other hand, zebrafish *c-mos* mRNA has two CPE sequences in its 3'UTR (Figure 1). We found that Zor-1 protein interacted with zebrafish *c-mos*

mRNA *in vivo* and *in vitro* (Figures 4D and 5). *c-mos* mRNA was localized at the animal cortex of zebrafish oocytes (Figure 3). Similarly, it was reported that *zor-1* mRNA was localized at the animal cortex (*16*). Taken together, it is likely that the machinery for cytoplasmic polyadenylation during oocyte maturation is conserved in zebrafish and *Xenopus*. Many of the factors, involved in the cytoplasmic polyadenylation event, are located at the animal cortex in zebrafish oocytes. We assume that the animal cortex is a central location where oocyte maturation, including mRNA metabolism of *c-mos* mRNA takes place. However, it remains to be clarified whether or not *c-mos* mRNA undergoes cytoplasmic polyadenylation during oocyte maturation in zebrafish. Recently, Zhan and Sheets reported that zebrafish *cyclin B1* mRNA undergoes cytoplasmic polyadenylation *via* the CPE sequence, and was translationally activated in oocyte maturation (*32*).

One of the vegetally localized mRNAs, *zdazl*, reached the vegetal cortex *via* the METRO-like pathway (*33*). Although *vasa* and *nanos1* mRNAs, which are localized in the cleavage furrow and are essential for germ cell formation are not localized at the vegetal cortex, these mRNAs temporally join the pathway (*33*). Another vegetally localized mRNA, *brul* mRNA, reached the vegetal cortex *via* a late pathway known in *Xenopus* Vg1 mRNA localization (*19,34*). These METRO-like pathway-related mRNAs and vegetally localized mRNAs are finally localized at the distal ends of cleavage furrows in the 4-cell stage embryo, where zebrafish germ plasmas are located (*21*). However, many of the animally localized products are involved in cell cycle regulation during oogenesis as well as early embryogenesis. Since maternally supplied products stored in the oocytes are essential for metabolism and development of the zebrafish embryos, the oocytes are much larger than usual somatic cells. It is reasonable for oocytes and eggs to carry out a cellular event in a specific area, such as in the animal cortex. However, it is unclear how the animally localized mRNAs are transported and anchored to the animal cortex in the zebrafish oocyte. It has been reported that the vegetally localized mRNAs have multiple localization signals (*33*). Since the 3'UTR of zebrafish *c-mos* mRNA is quite short (~150 nt), this mRNA may become a good model for analyzing the mechanisms of animal pole localization in the zebrafish oocyte.

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