

# Zebrafish cellular nucleic acid-binding protein: gene structure and developmental behaviour

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## Abstract

Here we analyse the structural organisation and expression of the zebrafish cellular nucleic acid-binding protein (zCNBP) gene and protein. The gene is organised in five exons and four introns. A noteworthy feature of the gene is the absence of a predicted promoter region. The coding region encodes a 163-amino acid polypeptide with the highly conserved general structural organisation of seven CCHC Zn knuckle domains and an RGG box between the first and the second Zn knuckles. Although theoretical alternative splicing is possible, only one form of zCNBP is actually detected. This form is able to bind to single-stranded DNA and RNA probes in vitro. The analysis of zCNBP developmental expression shows a high amount of CNBP-mRNA in ovary and during the first developmental stages. CNBP-mRNA levels decrease while early development progresses until the midblastula transition (MBT) stage and increases again thereafter. The protein is localised in the cytoplasm of blastomeres whereas it is mainly nuclear in developmental stages after the MBT. These findings suggest that CNBP is a strikingly conserved single-stranded nucleic acid-binding protein which might interact with maternal mRNA during its storage in the embryo cell cytoplasm. It becomes nuclear once MBT takes place possibly in order to modulate zygotic transcription and/or to associate with newly synthesised transcripts.

*Keywords:* CCHC-motif; Early embryogenesis; Single-stranded nucleic acid-binding protein; Developmental pattern expression; Whole-mount immunohistochemistry

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## 1. Introduction

Cellular nucleic acid-binding protein (CNBP) is a small protein containing seven tandem CCHC-type zinc knuckle domains (C- $\phi$ -X-C-G-X<sub>3</sub>-H-X<sub>4</sub>-C, where  $\phi$ =aromatic amino acid and X=variable amino acid). It exhibits striking sequence similarities with retroviral nucleocapsid proteins (McGrath et al., 2003). CNBP cDNAs have been cloned from mammals (Warden et al., 1994; Flink and Morkin, 1995; Yasuda et al., 1995; Michelotti et

al., 1995; Shimizu et al., 2003), chicken (van Heumen et al., 1997; Ruble and Foster, 1998) and amphibians (Flink et al., 1998; De Dominicis et al., 2000; Armas et al., 2001). CNBPs show a highly conserved structural organisation and amino acid sequence (Armas et al., 2001). Moreover, human (Flink and Morkin, 1995) and mouse (Shimizu et al., 2003) genes show a high level of conservation in their organisation. In human, the existence of two alternatively spliced products, CNBP  $\alpha$  and CNBP  $\beta$ , has been described (Flink and Morkin, 1995). The presence of two isoforms was also reported in *Xenopus laevis* (Flink et al., 1998; De Dominicis et al., 2000), but these are slightly different from those observed in mammals due to different alternative splicing events. In chicken, no alternatively spliced isoforms were found (van Heumen et al., 1997).

CNBP was initially described as a DNA-binding protein acting as a negative transcriptional regulator in the coordinated control of cholesterol metabolism (Rajavashisth et al.,

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*Abbreviations:* CNBP, cellular nucleic acid-binding protein; EST, expressed sequence tag; MBT, midblastula transition; EMSA, electrophoretic mobility shift assay; RACE, rapid amplification of cDNA ends; PBS, phosphate saline buffer; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

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1989), although this has been subsequently ruled out (Warden et al., 1994). As a negative regulator of transcription, CNBP was described controlling the expression of the early promoter-enhancer of the JC virus (Liu et al., 1998) and the  $\beta$ -myosin heavy chain gene (Flink and Morkin, 1995). CNBP was also shown to upregulate the expression of macrophage colony-stimulating factor (CSF-1) in fibroblasts (Konicek et al., 1998) and a reporter gene via the CT element of the *c-Myc* promoter (Michelotti et al., 1995). However, the stronger affinity of CNBP for single-stranded nucleic acids (Pellizzoni et al., 1997), the high homology and functional support of CNBP CCHC Zn knuckles with those present in retroviral RNA-binding proteins (McGrath et al., 2003) and the cytoplasmic location of the protein (Warden et al., 1994; Calcaterra et al., 1999) suggest that it could interact with RNA molecules. Accordingly, CNBP has been reported to interact with the 5' region of intron 1 of the rat *c-Myc* primary transcript (Yasuda et al., 1995), poly(A)<sup>+</sup>RNAs from *Bufo arenarum* oocyte and embryo extracts (Calcaterra et al., 1999) and the 5' untranslated region (5' UTR) of several *Xenopus laevis* ribosomal protein mRNAs (Pellizzoni et al., 1997). This last class of mRNAs is under translational regulation during development, and CNBP was proposed as a protein involved in this regulation (Pellizzoni et al., 1998). Recent work has shown that homozygous mutant mice *CNBP*<sup>-/-</sup> are unable to develop normal forebrain structures (Chen et al., 2003). Consequently, although CNBP's mechanism of action was not clearly addressed, this protein seems to be involved in essential developmental processes.

Little is known about fish *CNBP*. Hence, considering the advantages of zebrafish as an experimental model to study the developmental biology of vertebrates, we have studied the zebrafish CNBP in order to address its biological function. Here, we report the molecular cloning of the coding sequence and the 3' UTR, the organisation of the gene and the temporal and spatial expression pattern of zebrafish CNBP (*zCNBP*). The extreme conservation of the structure and the expression pattern observed among vertebrates suggests an important role for CNBP in the control of cellular and developmental processes.

## 2. Experimental procedures

### 2.1. Biological material

Adult zebrafish were raised and maintained on a 14/10 h light/dark cycle at 28.5 °C and were bred in marbled tanks as described (Westerfield, 1995). Embryos were staged according to Kimmel et al. (1995).

### 2.2. Cloning and sequencing of *zCNBP* cDNA

CNBP cDNA coding region was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using total

RNA from adult fish purified by a spin or vacuum (SV) total RNA isolation system (Promega; Madison, WI, USA). Oligodeoxynucleotides used were: Sense, 5' - TCCCCCGGGTCCGCAGCTTTAGAATGGACA-3'; anti-sense, 5' -GTGACAGAGGAAAAGAAGCTTCGCA-GACGC-3'. 3' -RACE (Frohman et al., 1988) was carried out by two nested PCR reactions. Oligonucleotides used for the first reactions were: sense, 5' -GGTGAGCAGGGACACAT-CGCCAGAG-3'; antisense, 5' -GAGGACTCGAGCT-CAAGCTTTTTTTTTTTTTTTTTTTT-3'. Oligonucleotides used for the nested reaction were: sense, 5' -CGAAG-GATCCGAGGATGCGTGCTACAACACTG-3'; antisense, 5' -GAGGACTCGAGCTCAAGC-3'. The amplification products were inserted into the pGEM-T Easy vector for sequencing.

### 2.3. Nucleic acid sequence analysis, databases homology searches and sequence alignment

cDNAs were sequenced by the dideoxy chain termination method using the TaqTrack Sequencing System (Promega). Database homology searches were performed using the BLASTN and BLASTP programs (Altschul et al., 1997). Sequence alignments and identity and similarity indexes calculations were carried out using the software package GeneDoc ver. 2.6. ([www.psc.edu/biomed/genedoc](http://www.psc.edu/biomed/genedoc)). Complete mRNA and gene organisation analysis were obtained from the [www.ensembl.org](http://www.ensembl.org) website.

### 2.4. mRNA expression analysis

Total RNA was isolated from *Danio rerio* tissues and from embryos at different developmental stages (150–200 embryos/developmental stage) by SV total RNA isolation system (Promega). First strand cDNA was prepared essentially as described elsewhere (Armas et al., 2001). Tissue CNBP and  $\beta$ -actin cDNAs were amplified by PCR (40 cycles) using specific primers: CNBP primers as described in Section 2.2.  $\beta$ -actin primers were: sense, 5' -CGGAACCGCTCATTGCC-3'; antisense, 5' -ACCCA-CACTGTGCCCATCTA-3'. Products were resolved in 2% agarose gel electrophoresis. Amplification products were visualised by Ethidium Bromide staining and were analysed using Image Pro Plus software. Embryo RNAs were subjected to semiquantitative RT-PCR following reported protocols (Marone et al., 2001) and 1  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dATP was added to the CNBP PCR reaction mixture. Products were separated by 8% polyacrylamide electrophoresis and were dried and exposed to X-ray films (Kodak BioMax Ms film) for 24 h. Radioactive bands were cut out from the gel and were measured using a Wallac Scintillation counter.  $\beta$ -actin bands were visualised by Ethidium Bromide staining and analysed using the Image Pro Plus software (Media Cybernetics; Silver Spring, MD, USA). RT-PCRs were carried out in triplicate for each sample.



Table 2.

Identity indexes from the alignment of the nucleotide sequences of 5' UTR and 3' UTR from *zCNBP*-mRNA and *CNBP*-mRNAs from other organisms

|                          | 5'UTR              |                     |                     |                          |                      |                       | 3'UTR              |                     |                     |                          |                      |                       |
|--------------------------|--------------------|---------------------|---------------------|--------------------------|----------------------|-----------------------|--------------------|---------------------|---------------------|--------------------------|----------------------|-----------------------|
|                          | <i>Danio rerio</i> | <i>Homo sapiens</i> | <i>Mus musculus</i> | <i>Rattus norvegicus</i> | <i>Gallus gallus</i> | <i>Xenopus laevis</i> | <i>Danio rerio</i> | <i>Homo sapiens</i> | <i>Mus musculus</i> | <i>Rattus norvegicus</i> | <i>Gallus gallus</i> | <i>Xenopus laevis</i> |
| <i>Danio rerio</i>       | 100                |                     |                     |                          |                      |                       | 100                |                     |                     |                          |                      |                       |
| <i>Homo sapiens</i>      | 25                 | 100                 |                     |                          |                      |                       | 80                 | 100                 |                     |                          |                      |                       |
| <i>Mus musculus</i>      | 28                 | 66                  | 100                 |                          |                      |                       | 81                 | 97                  | 100                 |                          |                      |                       |
| <i>Rattus norvegicus</i> | 28                 | 64                  | 98                  | 100                      |                      |                       | 81                 | 97                  | 100                 | 100                      |                      |                       |
| <i>Gallus gallus</i>     | 38                 | 37                  | 33                  | 31                       | 100                  |                       | 79                 | 97                  | 96                  | 96                       | 100                  |                       |
| <i>Xenopus laevis</i>    | 23                 | 26                  | 29                  | 29                       | 22                   | 100                   | 75                 | 87                  | 88                  | 88                       | 88                   | 100                   |

Identity %

trout EST databases show that this Histidine residue is absolutely conserved in these species. The structural and biological consequences of this substitution are unknown but its conservation among these fish suggests that it could be characteristic of teleosts. Further analysis showed the existence of a nuclear localisation signal PKKEREQ between amino acids 76 and 83 and the existence of putative phosphorylation sites (Fig. 1-A). Neither organellar nor

cellular membrane localisation signals were detected, and transmembrane segments are not evident by this analytical method.

### 3.2. Analysis of the zebrafish *CNBP* gene organisation

Comparative studies of *CNBP* gene structure among different vertebrates has not been carried out because only

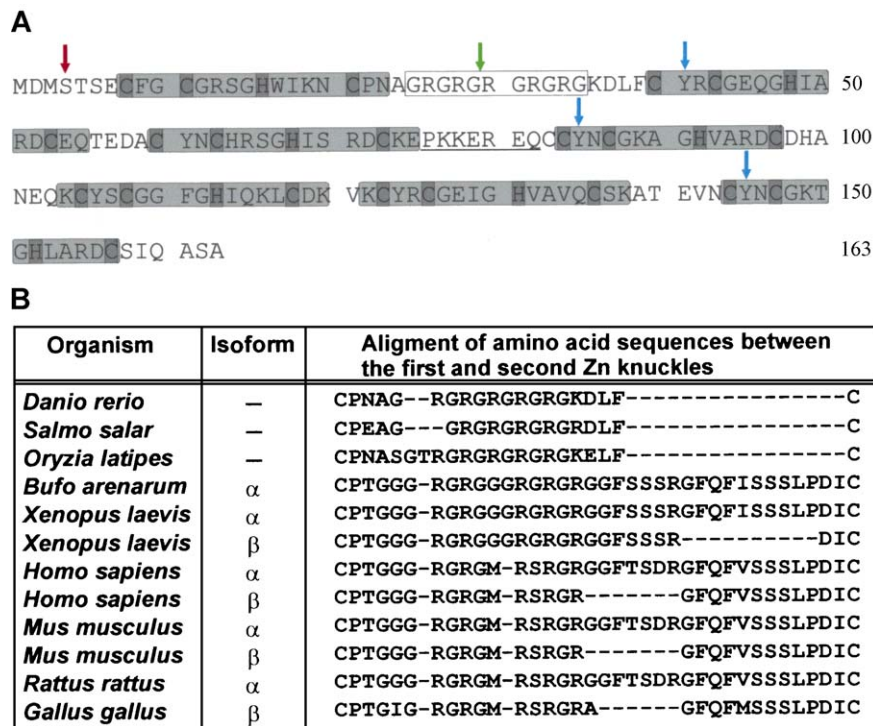


Fig. 1. *zCNBP* amino acid sequence analysis. (A) The amino acid residues involved in CCHC motifs are indicated in light grey boxes. Amino acids that participate in coordination of the Zn atom are boxed in dark grey. The RGG box is boxed and the putative nuclear localisation signal is underlined. Red and blue arrows show the putative phosphorylation sites for casein kinase II and tyrosine kinase, respectively. The green arrow shows a possible site of proteolysis. (B) Alignment of amino acid sequences from different vertebrates showing the linker peptide between the first and the second Zn knuckles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mammalian genes have been characterised (Flink and Morikin, 1995; Shimizu et al., 2003). We looked for the complete zebrafish CNBP gene in the [www.ensembl.org](http://www.ensembl.org) website. It is located entirely within contig *ctg30270.2*. Considering that most of the zebrafish genome is represented in the genomic databases and that the CNBP gene was found in a single contig, it is highly probable that, in zebrafish, CNBP gene is present in one copy per haploid genome, as in most vertebrates (Ruble and Foster, 1998; Shimizu et al., 2003). The CNBP gene is organised in five exons, of which the last four contain coding sequences. Except for exon 1, that contains the major part of the variable 5' UTR, most exon sizes are similar to those described for mammals. Analysing intron features, intron 1 is considerably smaller than its mammalian counterpart; intron 2 is slightly variable among human, mouse and fish; and introns 3 and 4 are much larger in zebrafish compared to mammals (Table 3). As it was observed in mouse (Shimizu et al., 2003), in the zebrafish CNBP gene upstream sequences, there is neither a TATA box nor CAAT box near the putative transcription start site. The conservation of these features among quite evolutionarily distant animals suggests that CNBP expression is developmentally regulated in vertebrates (Smale and Baltimore, 1989).

### 3.3. Searching for zCNBP isoforms

The [www.ensembl.org](http://www.ensembl.org) website shows the existence of three theoretical zCNBP-mRNA products as a consequence of the alternative splicing of exon 5 and exon 3. Alternative splicing events could yield different mRNA and polypeptide sizes of 1449 b and 161 amino acids, 884 b and 161 amino acids, and 378 b and 126 amino acids, respectively. Moreover, although the zebrafish second intron does not show predicted alternative splicing, this is the intron involved in CNBP pre-mRNA alternative splicing observed in mammals and amphibians. To clone a zebrafish CNBP cDNA, we used oligonucleotides which produce PCR products spanning the complete coding region. This cDNA shows a unique *Bst*XI restriction site which yields two digestion products of about 215 bp and 315 bp. Therefore, different restriction patterns may be obtained if different zCNBP isoforms were possible. Seven clones containing the coding region were treated with *Bst*XI, and the digestion products were analysed by polyacrylamide gels (Fig. 2-A). All of the clones showed identical

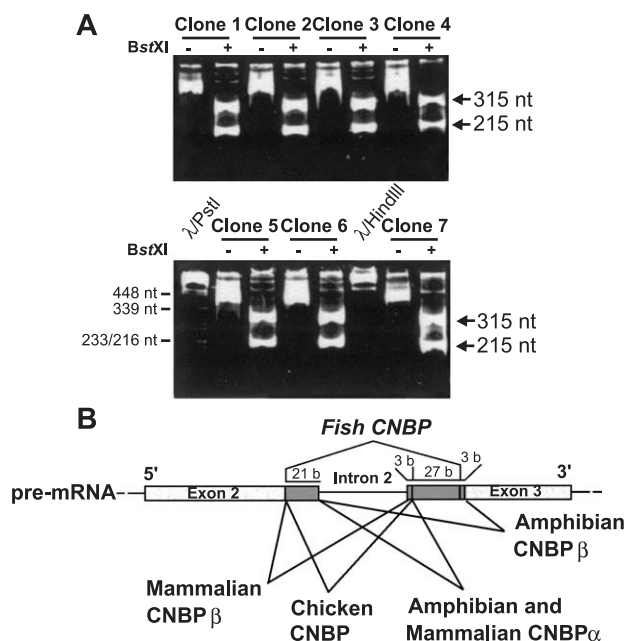


Fig. 2. Analysis of possible zCNBP isoforms generation. (A) *Bst*XI restriction analysis of PCR products from seven different CNBP clones. DNAs were analysed in 8% polyacrylamide gel electrophoresis and visualised by Ethidium Bromide staining. Both nontreated [(-), left side] and digested [(+), right side] products from each clone were loaded onto the gel. Molecular markers used are indicated. (B) Graphic representation of CNBP pre-mRNA splicing events among vertebrates. For each organism, the precise borders of the exons were deduced from the alignment of mRNAs and genomic sequences. The comparison between splice patterns among vertebrates was obtained from the alignment of their exons. Two different splicing patterns exist at the 3' end of exon 2, and at the 5' end of exon 3 there are four different splicing patterns.

restriction patterns, suggesting that alternative splicing affecting the coding region did not occur during primary transcript processing. To confirm this, we carried out BLASTN searches into the zebrafish and medaka EST databases. The alignment of the 21 ESTs found confirmed the existence of only one kind of transcript equivalent to the one analysed in this work. A unique isoform was also reported in *Gallus gallus*, but in this case, it is more similar to the mammalian  $\beta$  isoform (van Heumen et al., 1997). The presence of two isoforms in mammals and in amphibians and only one in fish and in chicken, together with the existence of different acceptor and donor splice sites used during CNBP RNA maturation in mammals (Flink and

Table 3  
Exon and intron numbers, and length (nt) comparison between different CNBP genes

| Organism                              | Exon 1                             | Intron 1                              | Exon 2                             | Intron 2                          | Exon 3                           | Intron 3                           | Exon 4                             | Intron 4                           | Exon 5                              |
|---------------------------------------|------------------------------------|---------------------------------------|------------------------------------|-----------------------------------|----------------------------------|------------------------------------|------------------------------------|------------------------------------|-------------------------------------|
| <i>Homo sapiens</i>                   | 835 <sup>a</sup> /81 <sup>b</sup>  | 3356 <sup>a</sup> /12004 <sup>b</sup> | 138 <sup>a</sup> /138 <sup>b</sup> | 181 <sup>a</sup> /95 <sup>b</sup> | 93 <sup>a</sup> /93 <sup>b</sup> | 169 <sup>a</sup> /168 <sup>b</sup> | 198 <sup>a</sup> /199 <sup>b</sup> | 507 <sup>a</sup> /508 <sup>b</sup> | 977 <sup>a</sup> /982 <sup>b</sup>  |
| <i>Rattus norvegicus</i> <sup>b</sup> | 151                                | 5847                                  | 138                                | 99                                | 93                               | 155                                | 199                                | 1112                               | 1061                                |
| <i>Mus musculus</i>                   | 216 <sup>c</sup> /167 <sup>b</sup> | 5025 <sup>c</sup> /5110 <sup>b</sup>  | 138 <sup>c</sup> /138 <sup>b</sup> | 98 <sup>c</sup> /98 <sup>b</sup>  | 93 <sup>c</sup> /93 <sup>b</sup> | 141 <sup>c</sup> /144 <sup>b</sup> | 202 <sup>c</sup> /199 <sup>b</sup> | 978 <sup>c</sup> /965 <sup>b</sup> | 981 <sup>c</sup> /1057 <sup>b</sup> |
| <i>Danio rerio</i> <sup>b</sup>       | 57                                 | 1981                                  | 110                                | 125                               | 66                               | 916                                | 199                                | 1495                               | 1017                                |

<sup>a</sup> Reported by Flink and Morikin, 1995.

<sup>b</sup> CNBP exon and intron numbers, and lengths from [www.ensembl.org](http://www.ensembl.org) site.

<sup>c</sup> Reported by Shimizu et al. (2003).

Morkin, 1995; Michelotti et al., 1995), amphibians (De Dominicis et al., 2000), chicken (van Heumen et al., 1997) and fish (this work), suggests that splicing events arose independently throughout evolution. A representation of the different intron 2 splicing events that take place among vertebrates is presented in Fig. 2-B.

### 3.4. Expression pattern of zCNBP-mRNA in adult tissues and embryos

zCNBP-mRNA distribution was examined in adult zebrafish tissues by RT-PCR using total RNA isolated from liver, brain, ovary, branchiae and gut. Relative to  $\beta$ -actin mRNA levels, CNBP-mRNA is detected in all the analysed tissues showing similar expression levels except in ovary where CNBP-mRNA is highly expressed (Fig. 3). Developmental CNBP-mRNA expression was analysed by semi-quantitative RT-PCR. The  $\beta$ -actin mRNA reactions were carried out for 25 cycles. The condition proved to be within the linear range of amplification (Fig. 4-A). CNBP-mRNA amplification was performed for 25 (Fig. 4-B), 30 and 35 cycles. Even the 35-cycles condition was in the linear range (not shown). At the 1-cell stage, CNBP-mRNA expression is higher than at later developmental stages such as the 256-cell or 512-cell stage. From early gastrulation (50% epiboly) through late neurula (70% epiboly) and late tailbud, CNBP-

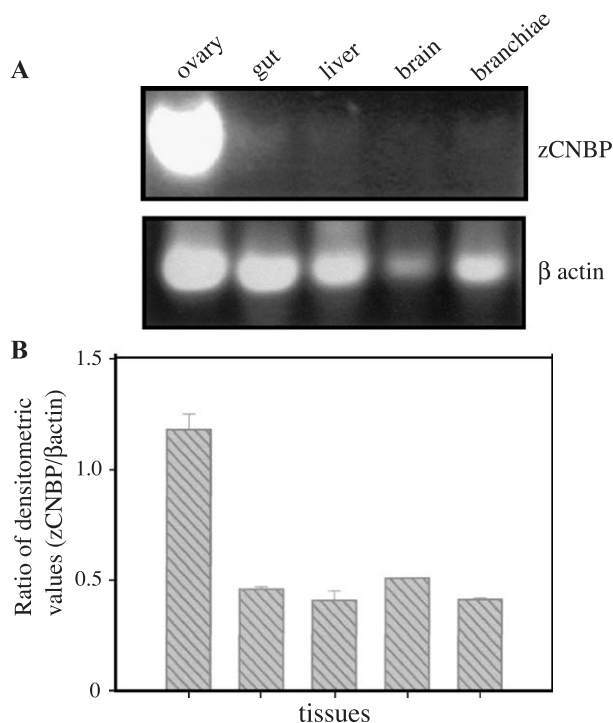


Fig. 3. RT-PCR on total RNA from adult zebrafish tissues. (A) The amplification products were resolved by 2% agarose gel electrophoresis and were visualised by Ethidium Bromide staining. RT-PCRs were performed for 40 cycles using CNBP or  $\beta$ -actin-specific primers. (B) Normalised zCNBP-mRNA levels corresponding to the same tissues described in panel A. The results are expressed as a mean of the ratio  $\pm$  S.E.M.;  $n=3$ .

mRNA expression increased, and the highest expression level was observed at the 26-somite stage (Fig. 4-C). Therefore, CNBP-mRNA level is high in 1-cell embryos, decreases while early development progresses until the midblastula transition (MBT) stage and increases again thereafter. CNBP-mRNA could be maternally inherited and used to synthesise the protein before the MBT. After that, the pool of CNBP-mRNA could be enriched by the zygotic transcription. A similar profile of CNBP expression was observed in *X. laevis* (De Dominicis et al., 2000).

### 3.5. zCNBP single-stranded nucleic acid binding capability

In order to evaluate whether the zCNBP protein is capable of binding single-stranded nucleic acids, band-shift assays were carried out using zCNBP fused to a GST tag. GST-zCNBP was incubated with labelled single-stranded DNA corresponding to the 5' UTR of the *Xenopus laevis* L4 ribosomal protein mRNA. This probe has a similar secondary structure compared with the RNA with the same sequence and has proved to be a specific target for *B. arenarum* CNBP binding, capable of displacing the RNA probe (unpublished results). A clear band-shift was observed (Fig. 5-A, line 3) which is a specific consequence of zCNBP binding capability because GST was unable to perform a similar band-shift under the same experimental conditions (Fig. 5-A, line 2). Band-shift assays using zebrafish embryo extracts showed a comparable behaviour (Fig. 5-A, line 4).

RNA band-shift assays were performed using riboprobes containing the same 5' UTR sequence and zCNBP fused to a histidine tag. His<sub>6</sub>-zCNBP was able to bind specifically this RNA probe (Fig. 5-B, lines 2 to 5). These results confirm the capability of zCNBP to bind to single-stranded nucleic acid targets.

### 3.6. Whole-mount in situ hybridisation of CNBP during embryogenesis

Whole-mount in situ hybridisation assays were carried out in order to analyse the embryonic zCNBP-mRNA expression pattern. We analysed embryos from the 2-cell stage to 26-somites stage, observing in all stages an even staining in all embryo cells (Fig. 6). At 26-somites stage, the staining was stronger than that from the first stages (Fig. 6-K). This finding suggests an increase in the zCNBP-mRNA levels from the late gastrula stage and is in agreement with the results obtained by RT-PCR (Fig. 4).

### 3.7. Immunolocalisation of CNBP during zebrafish oogenesis and embryogenesis

Whole-mount immunohistochemistry assays were performed to obtain a more detailed understanding of the precise zCNBP expression pattern. We examined CNBP protein expression in zebrafish embryos from the 8-cell stage to the 26-somites stage. At the beginning of the

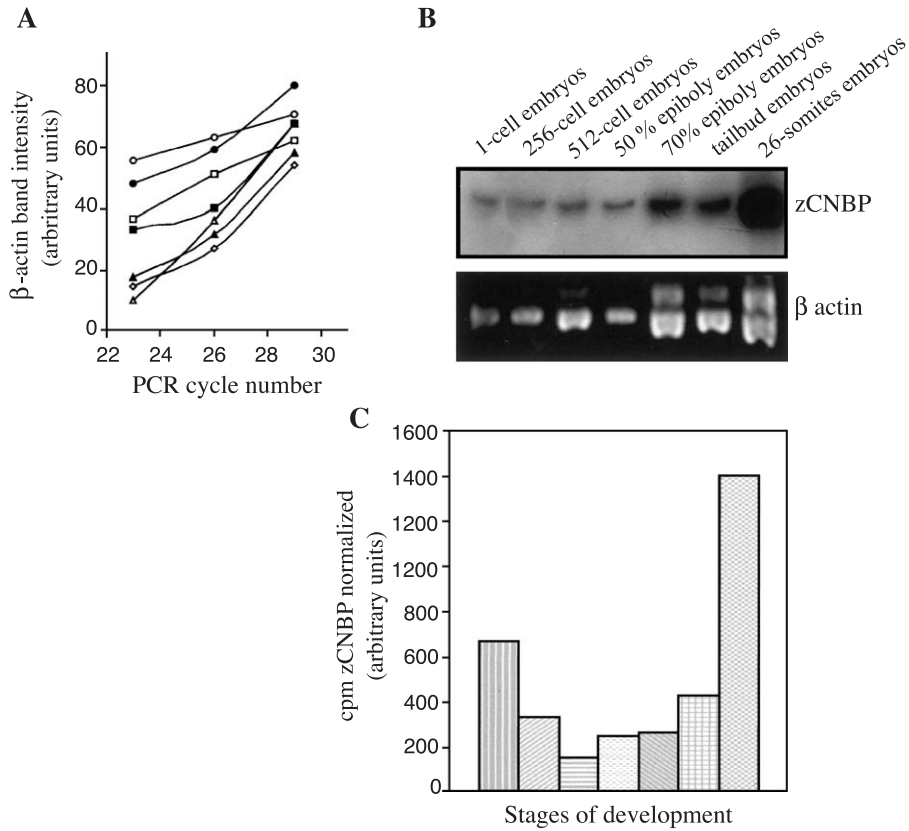


Fig. 4. Analysis of zCNBP-mRNA expression during zebrafish early development. (A) Establishment of linear amplification range condition for  $\beta$ -actin products. The  $\beta$ -actin amplification products were resolved by 8% polyacrylamide electrophoresis, visualized by Ethidium Bromide staining. Band intensities were analysed vs. PCR cycles number. ( $\Delta$ ) 1-cell embryos; ( $\diamond$ ) 256-cell embryos; ( $\blacksquare$ ) 512-cell embryos; ( $\blacktriangle$ ) 50% epiboly embryos; ( $\bullet$ ) 70% epiboly embryos; ( $\square$ ) tailbud embryos; ( $\circ$ ) 26-somites embryos. (B) Semi-quantitative RT-PCR on total RNA from different zebrafish developmental stages. The amplification products were resolved by 8% polyacrylamide electrophoresis.  $\beta$ -actin products were visualized by Ethidium Bromide staining whereas radiolabeled zCNBP products were exposed to X-ray film. Images correspond to 25 amplification cycles for zCNBP and  $\beta$ -actin. (C) For the same developmental stages as in panel B, the cpm corresponding to the zCNBP amplification product was normalised with respect to the band intensity of the  $\beta$ -actin amplification product.

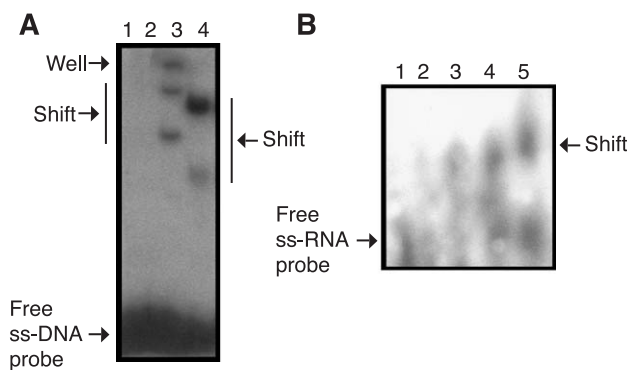


Fig. 5. Electrophoretic mobility shift assays (EMSA). (A) EMSA was performed using a single-stranded DNA probe corresponding to the 5' UTR sequence from the *Xenopus laevis* L4 ribosomal protein mRNA. Reactions were carried out by adding no protein (line 1), 10  $\mu$ M of purified GST (line 2), 10  $\mu$ M of purified GST-zCNBP (line 3), 200  $\mu$ g/ $\mu$ l of total protein extract from 1-cell embryo stage (line 4). (B) EMSA was performed using a single-stranded RNA probe corresponding to the 5' UTR sequence from the *X. laevis* L4 ribosomal protein mRNA. Reactions were carried out by adding no protein (line 1), 1  $\mu$ M (line 2), 3  $\mu$ M (line 3), 10  $\mu$ M (line 4) and 30  $\mu$ M (line 5) of purified His<sub>6</sub>-zCNBP.

development, CNBP expression was initially symmetric and uniform in all embryonic cells until late gastrula state (Fig. 7A–K), agreeing with the CNBP-mRNA (Flink et al., 1998) and protein (Armas et al., 2001) distribution found in amphibians and with the zCNBP-mRNA distribution described here. At the 90% epiboly stage, the CNBP immunostaining signal became asymmetric, being stronger in the posterior region of the embryo (Fig. 7-L). The lower CNBP levels observed in the anterior region of *D. rerio* embryos could be a consequence of the antero-posterior progress of tissue differentiation, as was previously shown in mouse (Kingsley et al., 1996). At the stage of 26-somites, CNBP was evenly distributed in the whole embryo (Fig. 7-O). There was no signal localisation in the vitelline cell in any of the stages analysed. No differential expression of CNBP was observed in the dorsal shield region (Fig. 7-L). This finding contrasts with mouse CNBP expression pattern where both the CNBP-mRNA and the protein are localised to the anterior visceral endoderm (AVE) which is the mouse embryonic equivalent to zebrafish shield (Brewster and Dahmane, 1999). As CNBP has been involved in mitogenic

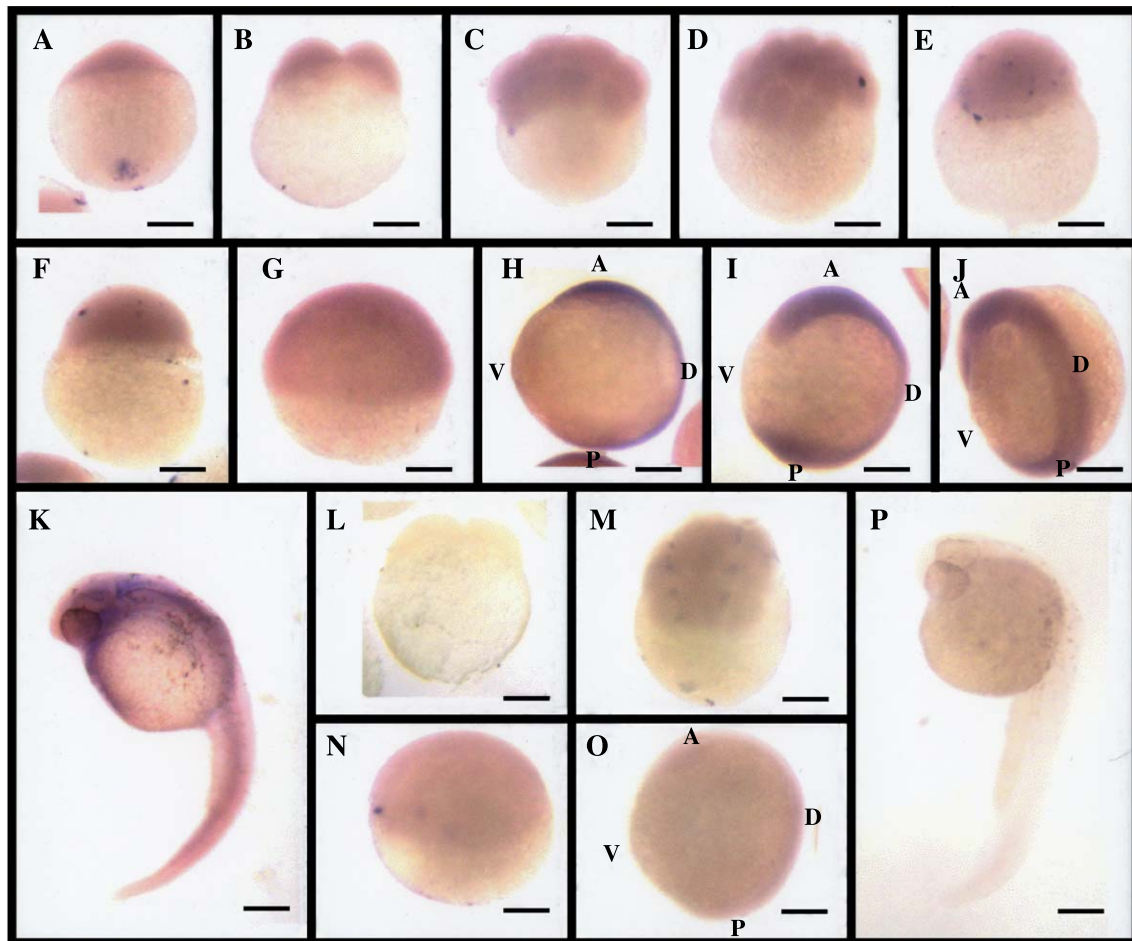


Fig. 6. zCNBP-mRNA distribution during embryogenesis. Whole-mount in situ hybridisation assays were carried out using antisense (A–K) or sense (controls, L–P) zCNBP-cDNA digoxigenin-labelled riboprobe. Embryos were staged as (A) 1-cell, (B, L) 2-cell, (C) 8-cell, (D, M) 16-cell, (E) 128-cell, (F) 512-cell, (G, N) 50% epiboly, (H) 100% epiboly, (I, J, O) late gastrula, (K, P) 26-somites. Panel J is a dorsal view of a late gastrula stage embryo. Bar represents 250  $\mu\text{m}$ . A, anterior; P, posterior; V, ventral; D, dorsal.

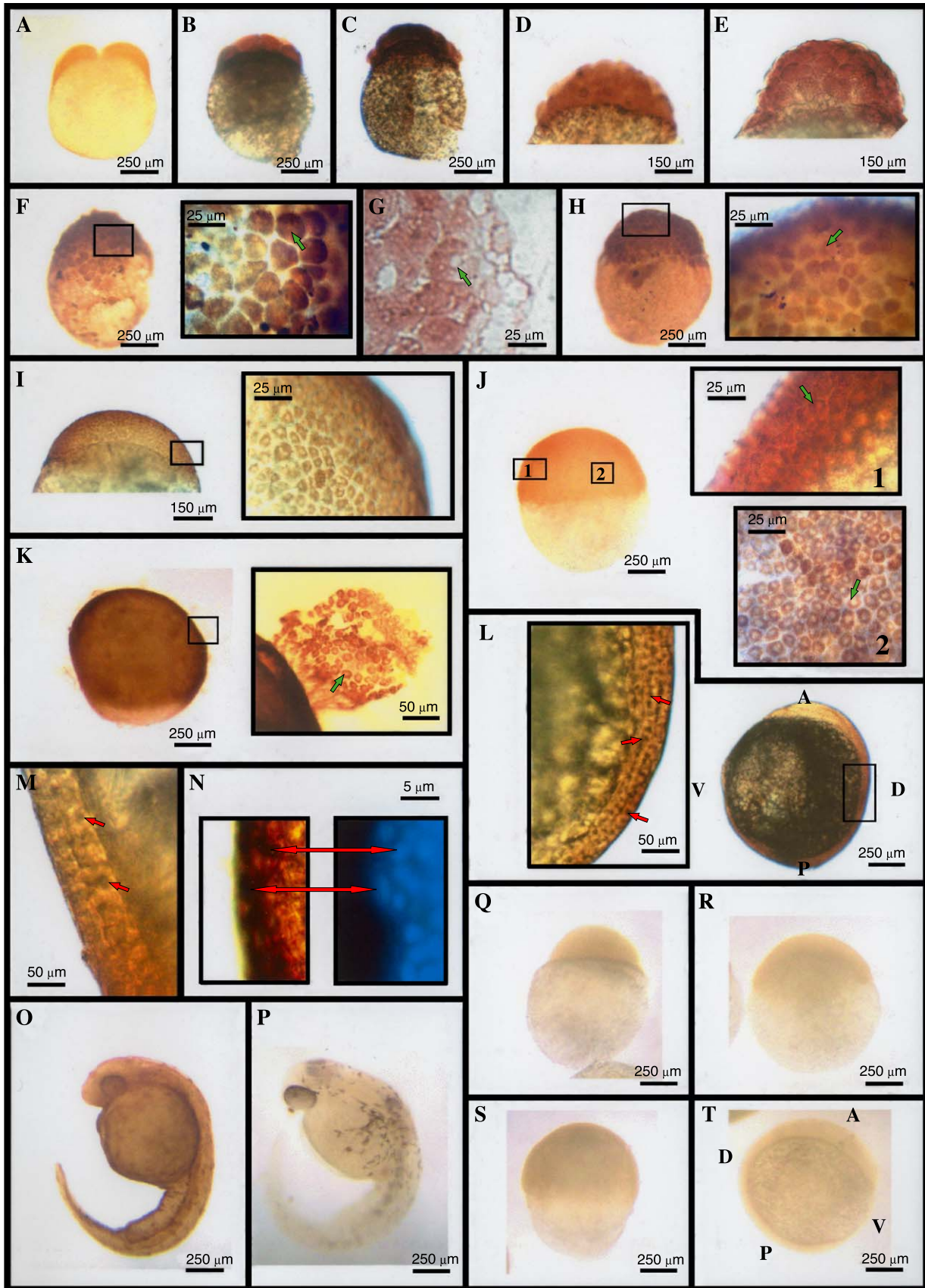
activity, CNBP behaviour could be slightly different in zebrafish and in mouse embryo cells as a cause or consequence of their different mitosis rates at the beginning of development. This hypothesis does not rule out the possibility that CNBP is actually acting in vertebrate cells in a common way.

Immunohistochemistry assays on zebrafish ovary sections were performed. Results showed a uniform cytosolic distribution of CNBP among immature and mature oocytes (Fig. 8). An association of CNBP with ovary membranes was not detected. Gathering these results, it is possible that CNBP is being stored as a cytosolic protein during oogenesis in order to be used by the embryos during the first steps of development.

zCNBP subcellular location during embryogenesis was also studied. Whole-mount immunostaining signal was detected in the cytoplasm of the early blastomeres (Fig. 7-A–E). From the 256-cell stage until 70% epiboly (Fig. 7-F–K), it was possible to detect CNBP located mainly in the cytoplasm of periderm cells. This was confirmed by immunostaining embryo sections (Fig. 7-G). However, at the end of gastrulation, CNBP was observed in the nucleus of those cells fated to give rise to embryonic tissues (Fig. 7-L–M). Embryos were subsequently treated with DAPI and the fluorescence overlapped the immunostaining demonstrating CNBP nuclear localisation (Fig. 7-N). The CNBP nuclear localisation signal, combined with its basic  $pI$ , agree with CNBP's possible function as a transcription

Fig. 7. Developmental behaviour of zCNBP. Immunohistochemistry was performed on whole embryos using affinity-purified anti-CNBP antibodies. Embryos were staged as (A) 2-cell, (B) 8-cell, (C) 32-cell, (D) 64-cell, (E) 128-cell, (F, G) 256-cell, (H, Q) 512-cell, (I, R) dome, (J, S) 50% epiboly, (K) 80% epiboly, (L–N, T) late gastrula, (O, P) 26-somites. Green arrows indicate exclusion of CNBP from the nuclei, red arrows indicate nuclear CNBP. Nuclei were visualised by DAPI staining (N). Controls were carried out omitting the anti-CNBP antibody (P–T). A, anterior; P, posterior; V, ventral; D, dorsal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)





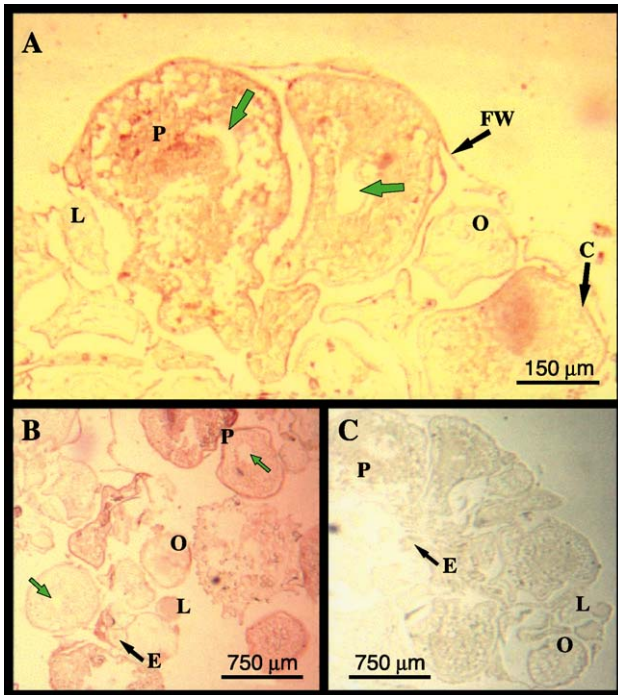


Fig. 8. zCNBP expression in adult ovary. Immunohistochemistry was performed on ovary sections using affinity-purified anti-CNBP antibodies. (A) Immunostained ovary section (X 20). (B) Immunostained ovary section (X 4). (C) Control carried out omitting the anti-CNBP antibody (X 4). Green arrows indicate exclusion of CNBP from the germinal vesicle. E, early perinucleolus stage oocyte; L, late yolk vesicle stage oocyte; O, oil drop stage oocyte; P, primary yolk globule stage oocyte, C, chorion; FW, follicle wall. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

factor (Rajavashisth et al., 1989; Flink and Morkin, 1995; Michelotti et al., 1995; Konicek et al., 1998; Shimizu et al., 2003) and with its participation in nuclear RNA metabolism (Yasuda et al., 1995). However, the exclusive nuclear localisation is not supported by the experimental observations previously reported (Warden et al., 1994; Calcaterra et al., 1999; Armas et al., 2001) and with the results presented here which show CNBP in the cytoplasm of zebrafish early embryonic cells. This apparent discrepancy may be resolved when analysing CNBP's developmental behaviour. CNBP is in the cytoplasm of zebrafish blastomeres and then, as development proceeds, its localisation becomes nuclear. This same behaviour was shown during amphibian development (Armas et al., 2001). It seems that CNBP could have a dual localisation being a nuclear as well as a cytoplasmic protein, depending on the developmental stage. The cytoplasmic localisation of CNBP together with its single-stranded nucleic acid binding capability suggests that CNBP might be interacting with maternal mRNAs before the MBT. CNBP could be localised to the nucleus once zygotic transcription starts in order to modulate transcription and/or to associate with newly synthesised transcripts. In mammals, zygotic transcription begins at the two-cell stage, earlier than in other

animal organisms. This fact could explain the detection of CNBP in the nuclei of mouse embryonic cells as was recently reported (Shimizu et al., 2003).

#### 4. Conclusions

- (1) A cDNA coding for zCNBP has been found. It encodes a protein of 163 amino acids. The coding region, as well as the 3' UTR, show striking homology with CNBPs from other species. Yet, the 5' UTR is moderately conserved among mammals but not conserved among vertebrates. zCNBP has a shorter linker peptide between the first and second Zn knuckle conforming a novel type of short CNBP characteristic of fish.
- (2) The zCNBP gene organisation is conserved among vertebrates. The zebrafish CNBP gene shows an absence of a putative TATA box and CAAT box in the region near the transcription start site. zCNBP is organised in five exons and four introns. No alternatively spliced zCNBP isoforms were found. Two isoforms are present in mammals and amphibians but only one in fish and chicken. The larger isoforms are very similar whereas the shorter ones are different. Consequently, it is possible that the mammalian and amphibian CNBP alternative splicing in intron 2 arose independently during evolution.
- (3) Zebrafish CNBP-mRNA is ubiquitously expressed in all adult tissues including uniquely fish organ such as the branchiae. The highest level of transcripts was observed in ovary. CNBP-mRNA detected during early developmental stages could be maternally inherited. It decreases until MBT stage and increases again thereafter, presumably due to zygotic transcription. The CNBP-mRNA distribution is homogeneous in all the embryonic cells until the 26-somites stage.
- (4) zCNBP protein is evenly distributed in the cytosol of immature and mature oocytes and in the cytosol of embryo cells until advanced epiboly. At the end of gastrulation, the protein locates to the nucleus of embryonic cells, showing a slight preferential staining in immunolocalisation assays at posterior cells.
- (5) The fact that CNBP has seven retroviral CCHC motifs and an auxiliary RGG box, together with its cytoplasmic localisation, are in agreement with its possible function in translational regulation of ribosomal protein mRNAs. Moreover, zCNBP binds in vitro single-stranded DNA and RNA probes containing the 5' UTR of the L4 ribosomal protein mRNA. Nonetheless, the CNBP dual subcellular localisation observed during early development suggests that it might be involved in maternal and/or newly synthesised RNA binding as well as in zygotic transcription modulation. Further functional analysis needs to be performed to address the biological role of CNBP.

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