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Molecular cloning of cyclin B transcript with an unusually long 3′ untranslation region and its expression analysis during oogenesis in the Chinese mitten crab, *Eriocheir sinensis*

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Abstract The meiotic maturation of oocyte in animals is regulated by maturation promotion factor (MPF), a complex of Cdc2 and cyclin B. Although the role of MPF during oocyte maturation has been well studied in a wide variety of eukaryotic organisms, little is known for crustacean species. In this study, a full-length cDNA of cyclin B was cloned from the Chinese mitten crab using degenerate RT-PCR and RACE methods. The crab cyclin B cDNA was 3,794 bp containing an unusually long 3′ untranslation region (UTR) of 2,403 bp and an open-reading frame encoding for a protein of 410 amino acids, with a calculated molecular mass of 45 kDa. The long 3′UTR harbors many cytoplasmic polyadenylation elements (CPE), and the GY-box, Brd-box, K-box that are perfectly complementary to the 5′-ends of various *Drosophila* microRNAs. The crab cyclin B transcript was predominantly expressed in ovary and testis. Semi-quantitative RT-PCR analysis revealed that the amount of cyclin B mRNA was high at previtellogenesis and late vitellogenesis stages, while low at early and middle vitellogenesis, suggesting that differential expression of cyclin B is closely related to oogonial proliferation (mitosis) and oocyte meiotic maturation.

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Introduction

The meiotic maturation of oocyte in animals is regulated by maturation promotion factor or M-phase promoting factor (MPF), a heterodimer composed of Cdc2 kinase and cyclin B [11, 13, 22]. As a regulator, cyclin B is required for activation of Cdc2 kinase during the transition from G2 to M-phase. In most of vertebrates, cyclin B protein is synthesized at the early G1 stage and combined with Cdc2 kinase to form inactive pre-MPF in the immature oocyte [2, 19, 20]. At the late G2 stage, MPF was activated through cyclin B by inducing phosphorylation of Cdc2 on Thr161, and dephosphorylation on Thr14 and Tyr15. The active MPF translocates into germinal vesicle (GV) and then germinal vesicle breakdown (GVBD) occurs at M phase I [2]. Conversely, cyclin B protein is absent in immature oocytes of fish and is synthesized immediately before GVBD [19].

The translational regulation of cyclin B has been well elucidated in amphibians and mammals. Cytoplasmic polyadenylation element (CPE) and translation control element (TCE) in the 3′ untranslated region (UTR) of cyclin B mRNA have been proved to play important roles in the translational regulation of gene expression in clam, *Xenopus* and mouse [7, 18]. CPE, a U-rich sequence with a loose consensus of A/UUUUUAU/A, also called adenylation control element (ACE), is required for deadenylation (storage) as well as the readenylation (translational activation) of the maternal cyclin B mRNAs during the oocyte maturation of the claw frog (*X. laevis*) and mouse [5, 12].

TCE, a bipartite consensus sequence of GUUGU-X23-AUUGUA, resembles a *nanos* response element (NRE) that controls translational repression of those mRNAs in the pole cells of the early *Drosophila* embryo [3, 4].

In crustacean, the role of cyclin B in the regulation of oocyte meiotic maturation is still unknown because of the lack of corresponding molecular data. We previously identified three forms of cyclin B transcripts in the ovary of kuruma prawn (*Marsupenaeus japonicus*) [14]. The three forms with the identical open reading frame differ only in the length of 3′UTRs. The number of TCE and CPE elements varies in the 3′UTR of the three forms. In this paper, a cyclin B transcript with an unusually long 3′UTR was identified and its differentiated expression was examined during oogenesis in the Chinese mitten crab (*Eriocheir sinensis*), one of the most important farmed species in Chinese aquaculture industry. The cloning and characterization of cyclin B transcript will provide us useful molecular information to further investigate molecular mechanism of oocyte maturation of the crab.

Materials and methods

Animals and tissues collection

Crabs were collected from a local fisheries market (Shanghai, China). Various tissues including testis, ovary, heart, muscle, hepatopancreas and gill were immediately frozen in liquid nitrogen and conserved at −80°C for total RNA extraction. The ovarian tissues at different developmental stages were fixed in Bouin's fixative (75% picric acid, 25% formalin, 5% acetic acid) for histological observation. Sections were routinely cut at 6–7 μm on a retracting microtome and stained with hematoxylin and eosin. The growing oocytes were staged according to histological characteristics and size of oocyte (Table 1, Fig. 1).

Total RNA isolation

Total RNA was prepared from the tissues using Trizol reagent according to manufacturer's instruction (Invitrogen). Genomic DNA contamination was prevented by treating the RNA sample with RNase-free DNase I (TaKaRa). Concentrations of isolated RNA were determined by measuring absorbance at 260 nm. The integrity of RNA was determined by agarose gel electrophoresis. Total RNA was stored at −80°C until use.

Degenerate RT-PCR

About 500 ng of total RNA isolated from ovary at vitellogenic stages was applied for synthesizing the first strand cDNA using BcaBEST^{TM} RNA PCR Kit (TaKaRa). A pair of degenerate primers of cyclin B, sense (5′-ATT/A GCA/T AGT/C AAA TAT/C GAA GAA/G ATG TA-3′) and antisense (5′-TC CAT IAG/A G/ATA T/CTT T/GGC A/TAG/A IGT ATG-3′), were designed based on the highly conserved cyclin B nucleotide sequences from cattle (*Bos taurus*) (GenBank accession number BC118382), human (*Homo sapiens*) (NM_031966.2), kuruma prawn (*M. japonicus*) (AY769095), zebrafish (*Danio rerio*) (BC045492), sea urchin (*Arbacia punctulata*) (Y00608.1), and fruit fly (*Drosophila melanogaster*) (NM_143046.2). A total volume of 25 μl PCR mixture contained 2.5 μl first strand cDNA, 1.5 μl 25 mM $MgSO_4$, 4 μl 5× Bca 2nd buffer, 0.125 μl Bca-Optimized Taq, 0.75 μl cyclin B sense primer (10 μM), 0.75 μl cyclin B antisense primer (10 μM), 0.125 μl dNTP (10 mM). The PCR cycling parameters included 40 cycles: denatured at 94°C for 30 s, annealed at 45°C for 30 s and prolonged at 72°C for 1 min. The amplified products were purified and ligated with plasmid T vector. The recombinant plasmid was transformed into competent cells and positive clones were picked up for sequence.

Rapid amplification of cDNA end (RACE)

Full-length cyclin B cDNA was amplified by 3′ and 5′ RACE method using BD SMART^{TM} RACE cDNA Amplification Kit according to manufacturer's instruction (BD Biosciences Clontech). The gene-specific primers were sense (5′GATCACCGACAAGGCCTAC3′) and antisense (5′CTTGCTGTTCCGCCTAAG3′) designed according to the cyclin B cDNA fragment obtained by degenerate

Table 1 Developmental stages of oocytes in the Chinese mitten crab

Stage	Abbreviation	Histological characteristics	Oocyte diameter (μm)
Previtellogenesis	pVt	Cytoplasm with strong staining of hematoxylin	<70
Vitellogenesis			
Early vitellogenesis	eVt	Appearance of oil globules	70–100
Middle vitellogenesis	mVt	Appearance of yolk granules	250–300
Late vitellogenesis	lVt	GV shrinking and migrating toward cytoplasmic membrane	>350
Final maturation	GVBD	Occurrence of GVBD	>350

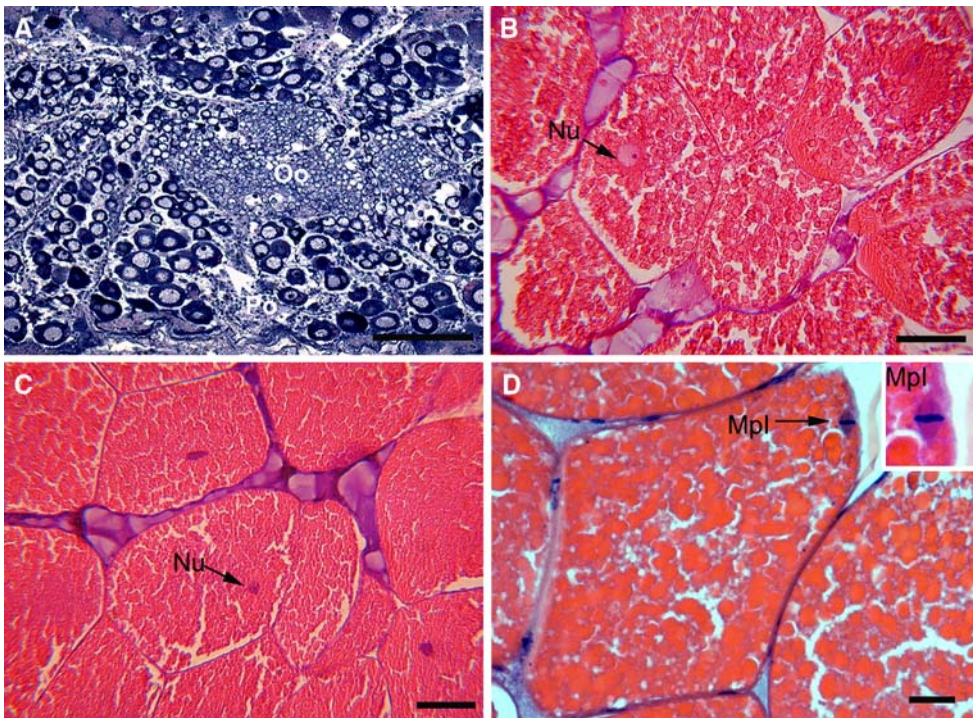

Fig. 1 Histological photographs of the crab ovarian development. Tissue sections (thickness 6–7 μm) were stained with hematoxylin and eosin. (**A**) Previtellogenesis stage; (**B**) vitellogenesis stage; (**C**) late vitellogenesis stage; and (**D**) GVBD stage. Oo, Oogonium; Po, perinucleolar oocyte; Nu, nuclear; MpI, meiotic M-phase I. The scale bar indicates 50 μm

RT-PCR. For 3′ RACE, touchdown PCR strategy was employed as follow: denatured at 94°C for 30 s, annealed at 60°C (1°C down per 4 cycles) for 30 s and last 10 cycles at 58°C for 30 s, and prolonged at 72°C for 1 min. For 5′ RACE, Thermal cycle parameters were 94°C for 30 s, 68°C for 30 s, 72°C for 1 min. RACE products were purified, cloned and sequenced.

Regular RT-PCR

In order to examine the expression of cyclin B mRNA in different tissues, equal amount of total RNA from various tissues was applied for RT-PCR. The first strand cDNA was synthesized as described in degenerate RT-PCR. The gene-specific primers were the same as used in RACE-PCR reaction. Thirty cycles of amplification were performed in a temperature cycler with denaturation (94°C) for 30 s, annealing (54°C) for 30 s and extension (72°C) for 45 s, followed by a 10-minute incubation (72°C). Meanwhile, beta-actin (200 bp) was amplified as a positive control.

Semi-quantitative RT-PCR

Equal amounts of RNA samples from the ovaries at five developmental stages, pVt, eVt, mVt, lVt and GVBD, were submitted to semi-quantitative RT-PCR. Total RNA (500 ng) was reverse transcribed into first strand cDNA in a 20 μl final reaction mixture in the presence of M-MLV transcriptase (TaKaRa). To ensure RNAs were free of genomic DNA, negative control cDNAs were prepared by reverse transcription reactions without adding the reverse transcriptase. Semi-Quantitative PCR was performed for each cDNA sample in 25-ml reaction volumes containing cDNA derived from 50 ng of total RNA and two primer sets, one for cyclin B gene and the other for beta-actin gene. Primers for the gene cyclin B and beta-actin are the same as used in regular RT-PCR. Preliminary experiments were carried out to establish the optimal ratio between beta-actin and cyclin B. The PCR included 33 cycles: denatured at 94°C for 30 s, annealed at 54°C for 30 s and extended at 72°C for 1 min. The amplified products were analyzed by electrophoresis on a 2% agarose gel together with a negative control. The gel was stained with ethidium bromide and photographed under ultraviolet. PCR products were quantified using Quantity One software (BandScan).

Statistical analysis

Semi-quantitative PCR was repeated three times using each ovarian sample of five individuals. The band intensities for the target gene of interest obtained from each aliquot of PCR products were normalized against those of the beta-actin mRNA. The ratio of the amount of the target gene to that of the reference gene within the same sample was calculated and expressed as relative amounts of mRNA with arbitrary units. Results were expressed as means ± SEM. The significance of the difference between stages was analyzed by *t*-tests with a confidence interval of 95% (SPSS software, version 13.0). *P*-values below 0.05 were considered significant.

Results

cDNA cloning and sequence of the Chinese mitten crab cyclin B

A partial cDNA fragment (about 220 bp) of the Chinese mitten crab cyclin B cDNA was obtained by degenerate RT-PCR based on a pair of degenerate primers (Fig. 2). To retrieve the entire cDNA, 5′- and 3′-RACE PCR were performed using an adaptor and gene-specific primers designed based on the obtained fragment sequence. The sequences of the 3′- and 5′-RACE products were merged into a complete cDNA. The merged sequence was confirmed to be from the mRNA of a single gene by an additional RT-PCR using gene-specific primers at the 5′ and 3′ terminal of cDNA (data no shown). The full length of cyclin B cDNA was 3,789 bp containing a 5′UTR of 132 bp, a putative coding region of 1,233 bp, and an unusually long 3′UTR of 2,403 bp with a poly(A) tail (Fig. 2, Table 2). There are two potential polyadenylation signal sequences (AATAAA) in the 3′UTR. Searching the UTResource database (http://www.ba.itb.cnr.it/UTR) with the crab cyclin B 3′UTR sequences revealed eight potential CPEs in the 3′UTR but no TCEs, which was reported to exist in 3′UTR of cyclin B mRNA in other species [5, 14]. Interestingly, GY-box (GTCTTCC), Brd-box (AGCTTTA), and K-box (TGTGAT) motifs, which are microRNA-binding sites and perfectly complementary to the 5′-ends of various *Drosophila* miRNAs [10], were also found in the 3′UTR of the crab cyclin B cDNA (Fig. 2).

The coding region of the crab cyclin B cDNA encoded a protein of 410 amino residues, with a calculated molecular mass of 45 kDa. A BLAST searching GenBank database revealed that the deduced amino acid sequence of the crab cyclin B shared 62% identity with kuruma prawn (AAV37462.1). A consensus putative amino-terminal cyclin B destruction box (RXALGXIXN) (residues 22–31) [16], which serves as a signal for the degradation of the B-type cyclin at the transition from metaphase to anaphase, is only one amino acid residue substitution of isoleucine (I) for valine (V) in the crab cyclin B (Fig. 3). The B-type cyclin characteristic FLRRXSK motif [21], known as cyclic AMP-dependent phosphorylation sequence, was also present in the deduced protein (residues 285–291).

Tissue distribution of cyclin B mRNA in Chinese mitten crab

The tissue distribution of mRNA expression of the mitten crab cyclin B was examined with RT-PCR method. The expression of the crab cyclin B mRNA was detected mainly in the ovary and testis, little in the heart, but no in muscle, hepatopancreas and gill (Fig. 4). A predominant expression in the ovary and testis suggested that cyclin B has important roles in oogenesis and spermatogenesis.

Fig. 2 The nucleotide and deduced amino acid sequence of the Chinese mitten crab cyclin B cDNA (GenBank accession number EU622123). The sequences of primers used for degenerated PCR and RACE-PCR are double-underlined and underlined, respectively. The potential cytoplasmic polyadenylation elements (CPE) (U/AU-UUUAU/A) are marked by shadow. The GY-box (GTCTTCC), Brd-box (AGCTTTA), and K-box (TGTGAT) are separately marked by frame, broken frame, and broken line. The potential cytoplasmic polyadenylation signals AATAAA are in bold and italic ▶

Expression profiles of cyclin B transcripts during oogenesis

The development of the mitten crab oocyte experiences three main stages, previtellogenesis (pVt), vitellogenesis (early, eVt; middle, mVt; late, lVt), and final maturation (Table 1, Fig. 1). At the lVt stage, oocytes undergo germinal vesicle (nuclei) shrinking, nucleolus disappear, and germinal vesicle migrating toward peripheral cytoplasmic membrane. Finally, germinal vesicle breaks down (GVBD) at final maturation stage (Fig. 1D). To compare the relative amounts of mRNA for cyclin B between stages during oogenesis, ovarian samples from different developmental stages were subjected to semi-quantitative RT-PCR analysis using beta-actin as an internal reference. As shown in Fig. 5, beta-actin mRNA level is constant between all stages, confirming equal loading of RNA in the different samples. The level of cyclin B transcript is the highest at pVt stage ($P < 0.05$), but drops remarkably at eVt and reaches the lowest point at mVt stage, after which the amount of cyclin B mRNA began to increase from lVt and climb back to a high point at GVBD stage ($P < 0.05$) (Fig. 5).

Discussion

In this study, a full length of cyclin B cDNA with an uniquely long 3′UTR was cloned and characterized in the ovary of the Chinese mitten crab. The 2.4 kb long 3′UTR is the longest one of B-type cyclin cDNA registered so far in the GenBank database. As shown in Table 2, the 3′UTR of invertebrates cyclin B is much longer than that of vertebrates. Considering that the subtypes cyclin B1 and B2, that have only been reported in vertebrate species, diverge from invertebrate cyclin B, the mitten crab cyclin B with unique long 3′UTR might represent a primitive type in the evolution of B-type cyclin.

Although 3′UTR of eukaryotic mRNAs were once thought to be unimportant trailers following protein coding regions, growing evidence indicates that 3′UTRs in fact

```
AAGCTTCCCAGGCCTGGCGCGGGACGGACCCCTCAGTGTTACCCTCATTGTACAGTGTGCACGCCT 66
CCATTAGCCAACGACTACCACCCCACCTTCTTCGCCGTGGCTCTGAGGGCAAGACAGCTTAACAAG 132
ATGACGGACCAGAGCATTGGGCCGCGCAAGGTGGAGGCCAAAGTGTACCAGGGCCCCACCTTGCAC 198
M  T  D  Q  S  I  G  P  R  K  V  E  A  K  V  Y  Q  G  P  T  L  H    22
CGCACGGCCCTTGGGGAGATGAGCAACAGCAACCTGCCACGCATCAGCCTCAGGGGGTCTAAGGCC 264
R  T  A  L  G  E  M  S  N  S  N  L  P  R  I  S  L  R  G  S  K  A    44
ATGGAACTGCTGAAGAAACAGACACAGCAGCCACAACCACCCCCAGCACCAGCACCAGACTGTGGG 330
M  E  L  L  K  K  Q  T  Q  Q  P  Q  P  P  P  A  P  A  P  D  C  G    66
GACCCTTCACAGCTCAAGGGCCTGTCCAGGGCGTCCTCACTCTCCTTCAAGAGATACAATGGTAAG 396
D  P  S  Q  L  K  G  L  S  R  A  S  S  L  S  F  K  R  Y  N  G  K    88
GAAAACATTCAGCCCAAACCAGTGCTGGAGAAGGTGAAGGAGGTGAACAAGAAGGAGGAGGATGTG 462
E  N  I  Q  P  K  P  V  L  E  K  V  K  E  V  N  K  K  E  E  D  V   110
GTAGAGGAGATGGAGGTGGAGGAGCTGGCCGTCGCATTCTCCACCCAGAGGTTCAACGTGGAGGAC 528
V  E  E  M  E  V  E  E  L  A  V  A  F  S  T  Q  R  F  N  V  E  D   132
ATCGACTCCCAGGATGCCGACAACCCCCAGCTGGTGTCGGAGTATGTGTGTGACATCTACAAATAC 594
I  D  S  Q  D  A  D  N  P  Q  L  V  S  E  Y  V  C  D  I  Y  K  Y   154
CTCAGGACTTTAGAGGACAATTCACCAGTCCAGCAACAGTACCTCGAGGGCCAGATCATTACCCAC 660
L  R  T  L  E  D  N  S  P  V  Q  Q  Q  Y  L  E  G  Q  I  I  T  H   176
AAGATGCGTGCCATCCTTGTCGACTGGCTGGTGCAGGTCCATCACCGCTTCACCCTCATGCAGGAG 726
K  M  R  A  I  L  V  D  W  L  V  Q  V  H  H  R  F  T  L  M  Q  E   198
ACGCTCTACCTCACAGTGGGCACCCTCGACAGATACCTCCAGGTCGTGAGGAACACCCCCCGCAAC 792
T  L  Y  L  T  V  G  T  L  D  R  Y  L  Q  V  V  R  N  T  P  R  N   220
ATGCTGCAGCTGGTGGGGGTGACGGCCATGTTCATCGCCTGCAAGTTTGAGGAGATGTACTGCACT 858
M  L  Q  L  V  G  V  T  A  M  F  I  A  C  K  F  E  E  M  Y  C  T   242
GACGTGGGCGACCTGTCCTTGATCACCGACAAGGCCTACACCAAGCGGGAGATCCTCGCCATGGAG 924
D  V  G  D  L  S  L  I  T  D  K  A  Y  T  K  R  E  I  L  A  M  E   264
GTCAAGATGCTGAAGGCGCTCAAGTTCAACATCTCCTTCCCTCTGCCGCTGCACTTCCTTAGGCGG 990
V  K  M  L  K  A  L  K  F  N  I  S  F  P  L  P  L  H  F  L  R  R   286
AACAGCAAGGCTGGCTTGGTGGACTCCAGGCACCACACACTAGCCAAGTATCTGATGGAGCTGTGT 1056
N  S  K  A  G  L  V  D  S  R  H  H  T  L  A  K  Y  L  M  E  L  C   308
CTGCCGGAATACTCCATGTGTCACTTCAAGGCGTCCATCCTTGCTGCTGCTGCTCTCTGCCTCACA 1122
L  P  E  Y  S  M  C  H  F  K  A  S  I  L  A  A  A  A  L  C  L  T   330
CTCAAGCTGCTGGATGGAGGAGAGTGGAATGACACGCTGATTTACCACTCAAGCTACACGGAAGAG 1188
L  K  L  L  D  G  G  E  W  N  D  T  L  I  Y  H  S  S  Y  T  E  E   352
CAGCTGATGCCCGTCATGTGCAAGATTGCCACCATCGTTGTCAAGAGCCATCACTCAAAACAACAG 1254
 Q  L  M  P  V  M  C  K  I  A  T  I  V  V  K  S  H  H  S  K  Q  Q   374
GCGGTGAGGCAGAAGTACGACTCAGCCAAGTTAATGAAGATCAGCAAGATTCCGCAGCTGAAGTCT 1320
A  V  R  Q  K  Y  D  S  A  K  L  M  K  I  S  K  I  P  Q  L  K  S   396
GACCTGATATCCAAGCTGGCCGAGAGAAGCGCCTCTTTCTCGTGAGTGTCCTGCGCGCCCTCGGAT 1386
D  L  I  S  K  L  A  E  R  S  A  S  F  S  *                        410
GGACCCCCTGGCTCGCTTTGTAAATACTAGTCTGTAGAGAAACCTGATTTATTTTGTACCTTCCTT 1452
GTGATCTGTATTATTTAATGAAGATAATGATATTCTTTTTGCGCTGTATGCCATATATATATTCTG 1518
TACTGAGGGTGGGTTTGCCGGTTCTTGCACACTCCTCCAGTTTTCTAGTTCTTGTTTCAGGGAAAT 1584
TTACGATGGAAAATATGACGGAGGTATAAAAGTTTTCAAAGTAAAGATTAATTTTTCTTTTATTGA 1650
TTTTTTTTAATAACTTACTTACCTCTACTCAGTTTTCCATAGGAGTAGTTTCCCTGATGTGAGCACA 1716
GATTGAAAGGGTGTGTGCAATACTGTAAAAATACCCACCGTTCTCGGGACTTGTTCTATAGGTATG 1782
TATAATAACTTAAGTGAGGCACTGTGATCTTTTTTTCACCGATCAAAGCATTGCTGCATCTTCATAT 1848
TTTACCATCAGACAGGAAAGATTGGTAGCAGCAGTTCTCGGAGTAGCCATACAAGAAGGTAGTGTT 1914
CTGTATGGACTGGTTATCTGTTTTTTATACTTTTTAATCCATAACTTCTTGTATCCCGTTGGGGAGT 1980
TCTTTTTACAAGCTTTAGCAACAGTGTGTGACAGAGGGCAAGTGTGGGGGACTGGAGGCATTAATT 2046
TCCCCTGTGTTATAAATCAGAGTTAGAGAATAGAAGAGTGTGACCAACTTCATCACAGCTGCCATG 2112
TTGTTATCAGAAGAACCCTTAGTGATGGACATACAGTAGTGTTTTTTGTGCTTTTGGTACTTCTTC 2178
GAGATTCCAACTATACATAGAGCACAAGAAAGGTATTATTGGATTAGCATCAGTCTGAATTTCATG 2244
TAGCTCTTTTTTGTAACAATGTGTTGCCTGTGATGAAAGTGGCCAAAGTCTCCTGTTCTATGGCTCT 2310
GGTATGAAGTTCCCTGCTTGTCCTCACACTCTAAACATACTTTTTAACAATGGATCTGTGAGTAGT 2376
CTTGTAGCATCAACAGTACGCTCATGGGATTAGGGAAGGTTTGCCAGAATAGTTTATCTCTTTAGG 2442
GGGTAACTTAACTCTCCTGTTCTTGTCTCATCACTTACTTTTACTCTGTAGTGGTTAACTCTCCTC 2508
TAAGTCTACACAGACTGTCCATCTCTCCTGGGTCTCTACTGTCCTCCTCCTCTAGATTGCCATAAC 2574
TTGTATATCCATGGTCATTATCTGTTAGTTCATTGACTTTCATTTATGTACTTCAGATAATTAAGG 2640
TCTTCATGCATCTTTTACTCTTACACAGTCTATCCACTTCCTCCTGGGTCTTCCTATTGGCCTCCT 2706
TCCTCTAGGTTGCCATGATATGTATGTCCATCTGTTAGTTTATTTGGACGTTCATTTATATACTTC 2772
AGATAAATCCTCTAGGGTAGTTATTGCCTTAACATGAATGCTGTTTGGAAGTGCATGAGGAAATAA 2838
GTGGACTTGCTCTGTGTTGTAATGTCTGGCTAATGTTCTTCACACGCAAATGTTAGAAGAATTCGG 2904
ACCAGAAGTTATATTATTTTTTATATTTTTTTACGTCAAAGGAAATAGCTCAAGGGC***AATAAA***CAGA 2970
ATCAGAGCCCGTTAATCGCTGCTCCTGCAAAACCAAACAAAGGAGTGGCCAGAAGAGAGGTCAGTG 3036
GTTGGTTGGTATGGTTGGTTAGACAAGTATCCATGCATGAGTTCGATTTTTAGTTCGCTTTTTTTAA 3102
AGACGATATTGTACATTTTACATCCCTCAGTACCTCAGGGTGTGTTGGACCATTTGTACTCATCAG 3168
TTAGAGTAGTATAAAAAAAAAGTTCTTGAGAAAAGTGATGTATTAAAGTGAGTCAAAGTGAGGTTG 3234
ATTATACTTAATTTTGACAGTATCCTTTTTGTATATTAGCTTATTTCGCAGAGCTTTTTTTCCTTT 3300
GAGTACTAATTGGATTGGTACATTACTAATTGGTGAACACATCTTAAGTTGTTACCACTGCTGTTA 3366
TTAGTCCTTTTTTTATTGTTTTTCTCTCCGTGAGCCGTGGTCTGAACATGGCTGGTGAGGATACTTG 3432
TGTGTGGAGTGTGGATTCAGGAAAGCTCATTATTACTATTATGTTTTAGTCAGCCAGAGAGTGTGT 3498
GTGTGTGTGTGTGTGTGTAATTGCCATTCCCATTTCTTGAACAAAGTGAGGATAAATTTTAAGTTA 3564
CTGGTGGAAGAAGACTATGAATGTGTGTGCGTGTGTGTGTAATTGCTATTCTCATTTCTTAAACAA 3630
AGTGAGAAGTTTTAAGTTAGTGGTGGAAGTTGACTATGAATGTGTGTGTGTCACCCATGTTGCA 3696
TGCTACCAAGCTGTCAGTCATATCACTTATTGTCATTAGATGTAAGAGATTATTCAGTGTATG***AAT*** 3762
***AAA***AAAATAGAACCCAAAAAAAAAAAA                                  3789
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Table 2 A comparison of the 3′UTR length of B-type cyclins transcripts in various species

Species	Types	Length of 3′UTR (bp)	Accession number
Vertebrates			
Human (*Homo sapiens*)	Cyclin B1	615	NM031966
	Cyclin B2	168	AF002822
	Cyclin B3	183	AJ416458
Cattle (*Bos taurus*)	Cyclin B1	117	L26548
	Cyclin B2	165	BC118382
Frog (*Xenopus laevis*)	Cyclin B1	165	CR761287
	Cyclin B2	189	NM001087799
	Cyclin B3	223	BC106306
Zebrafish (*Danio rerio*)	Cyclin B1	204	BC153626
	Cyclin B2	246	BC066507.1
	Cyclin B3	383	LOC564956
Invertebrates			
Fruit fly (*Drosophila melanogaster*)	Cyclin B	781	AY102682
Zebra mussel (*Dreissena polymorpha*)	Cyclin B	1,260	AF086634
Kuruma prawn (*Marsupenaeus japonicus*)	Cyclin B	1,156	AY769095
Spiny starfish (*Marthasterias glacialis*)	Cyclin B	1,440	AJ512968
Chinese mitten crab (*Eriocheir sinensis*)	Cyclin B	2,412	EU622123

may influence polyadenylation efficiency, translational efficiency, transcript localization and stability [17]. Especially in recent years, the discovery of microRNAs (miRNAs), noncoding RNAs of ~22 nucleotides that regulate gene expression at the post-transcriptional level by binding to 3′UTR of target gene [1].

It has been demonstrated that the CPE and TCE in 3′UTRs, have important roles in the translation regulation of cyclin B in clam, mouse, *Xenopus* and *Drosophila* [23]. The CPE can activate translation of cyclin B during oocyte maturation by helping consort with the polyadenylation signal [6]. Microinjection of the CPEs into oocytes was sufficient to induce cyclin B1 protein synthesis [5]. On the contrary, TCE in 3′UTR is involved in translational repression of the transcripts in the pole cells of the early *Drosophila* embryo [4]. In kuruma prawn, three forms of cyclin B transcripts were found to have identical coding region and differ only in the length of 3′UTR. The three forms have differential localization during oogenesis. The long form of cyclin B transcript bears multiple potential CPEs and TCEs in the 3′UTR and was thought to have a role in regulation expression at translational or post-transcriptional level [14]. In this study, eight potential CPEs but no TCE are found in the 3′UTR of the crab cyclin B, suggesting that the translation or post-transcription might be regulated mainly through CPEs.

Interestingly, the miRNA-binding sites, GY-box, Brd-box, and K-box motifs, are simultaneously present in the 3′UTR of the crab cyclin B (Fig. 2). These conserved motifs, were initially identified in the 3′UTR of the Enhancer of split Complex [E(spl)-C] and the Bearded-Complex [Brd-C] family genes in *Drosophila*. Most members of the E(spl)-C and Brd-C are negatively regulated by GY-box-, Brd-box-, and/or K-box class miRNAs. GY-boxes, Brd-boxes, and K-boxes are necessary and sufficient for regulation by corresponding miRNAs [10]. The deletion of K box resulted in a strong increase of transcript and protein levels of the heterologous reporter gene lacZ that linked with a 3′UTR of the gene E(spl) *m8* [9]. The presence of GY-box, Brd-box, and K-box motifs in the crab 3′UTR of cyclin B transcript suggested that the translation or post-transcription of the crab cyclin B might be regulated cooperatively via CPE and the miRNA-binding sites.

In the kuruma prawn ovary, the three forms of cyclin B transcripts were generated by alternative usage of potential polyadenylation signal sequences (AATAAA) at various sites in 3′UTR. In the mitten crab, only a single form of cyclin B transcript was identified in ovary, even though there are two potential polyadenylation signals in the extremely long 3′UTR. This result is similar with those of spiny starfish (*Marthasterias glacialis*) [8], in which one form of cyclin B transcript harbors more than one AATAAA signals in 3′UTR. The polyadenylation signal, AATAAA, located near the polyadenylation, is required for cleavage in almost all the eukaryotic mRNAs, but the polyadenylation signal was not enough for cleavage and polyadenylation [15]. Therefore, the presence of the signal does not absolutely mean cleavage and polyadenylation [24]. Whether the first potential polyadenylation signal (nucleotide 2961–2966) in the 3′UTR can be used to produce a short form of the crab cyclin B transcript need to further demonstrated by Northern blot analysis.

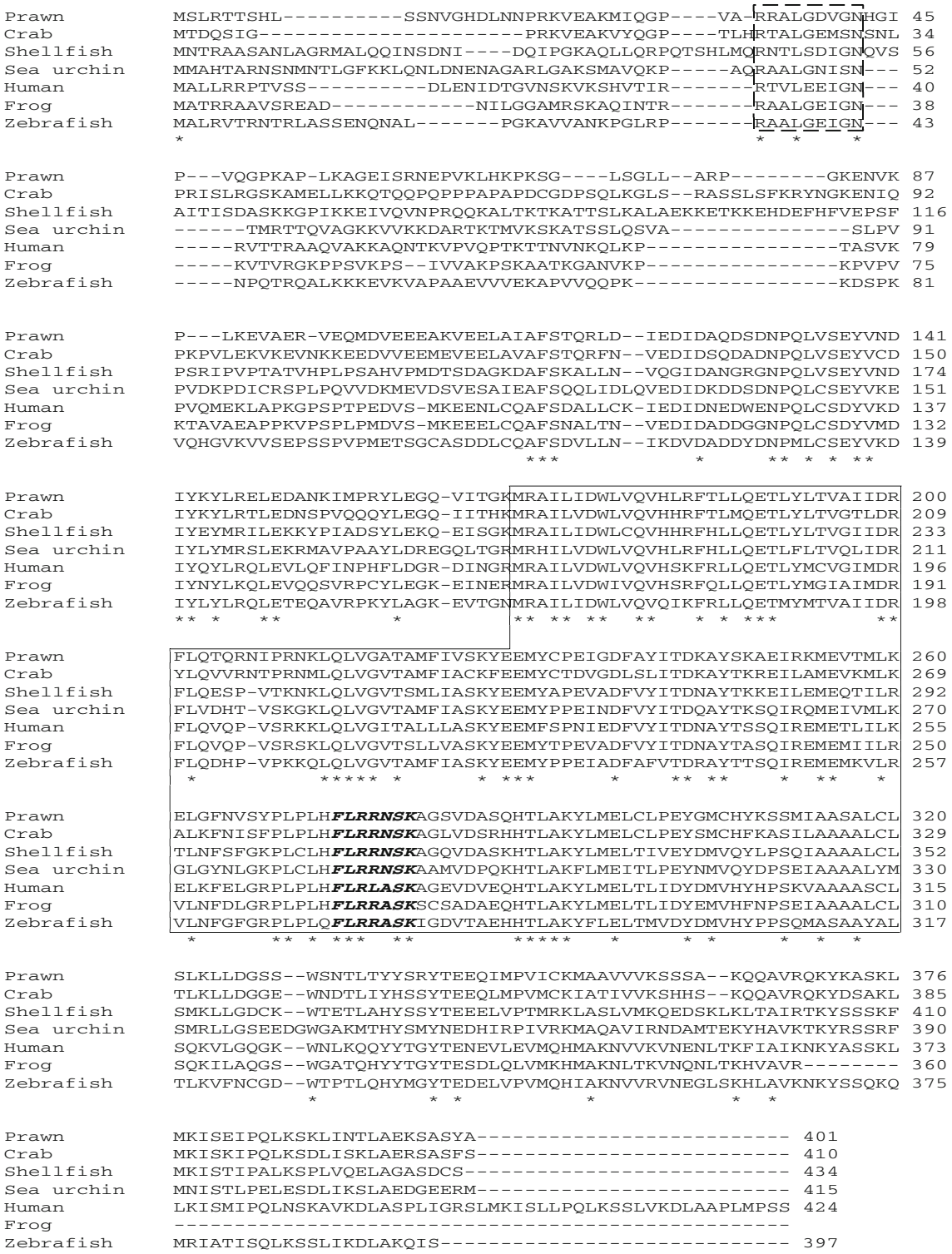

Fig. 3 Alignments of the deduced amino acid sequence of the Chinese mitten crab cyclin B with those of prawn cyclin B (Genbank accession number AY769095), sea urchin cyclin B (number AAO73601), frog cyclin B1 (number AAH41302), zebrafish cyclin B1 (number BAA92876), human cyclin B1 (number P14635) and shellfish cyclin B (number AF086634). Conserved residues are indicated with asterisks. The putative destruction signal is marked by broken frame, the cyclin box is marked by box, and the amino acid residues of pkA site are in bold and italic

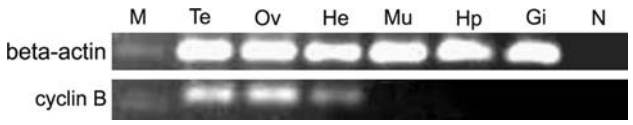

Fig. 4 Tissue distribution of the crab cyclin B mRNAs as examined by RT-PCR using beta-actin as an internal reference. Te, testis; Ov, ovary; He, heart; Mu, muscle; Hp, hepatopancreas; Gi, gill; M, molecular weight standards; N, a negative control containing no cDNA template

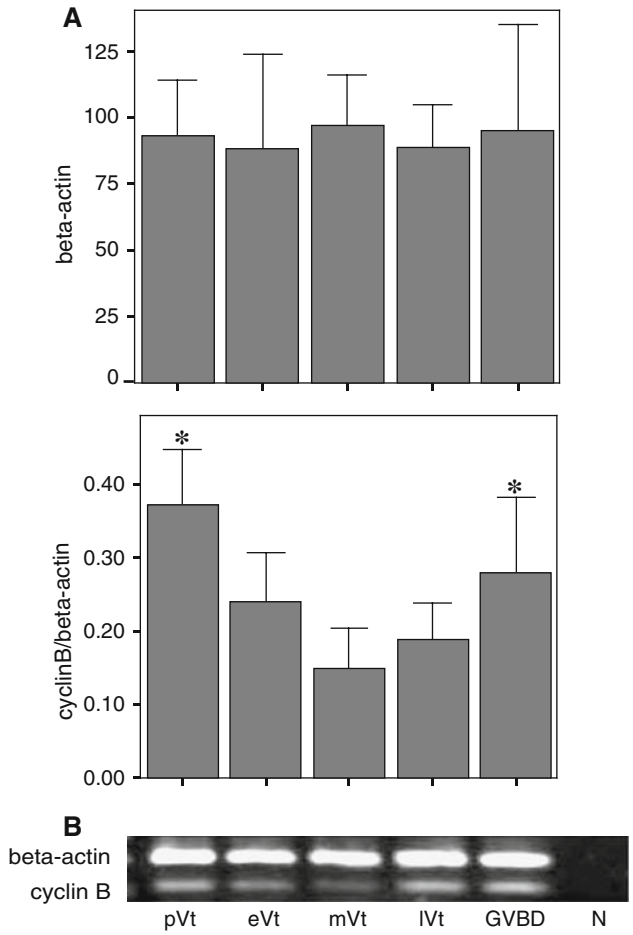

Fig. 5 Semi-quantification by RT-PCR of cyclin B transcripts in the crab ovaries at various stages of development. pVt, previtellogenic stage; eVt, early vitellogenic stage; mVt, middle vitellogenic stage; lVt, late vitellogenic stage; GVBD, germinal vesicle breakdown; N, a negative control of non-reverse-transcribed RNA submitted to PCR amplification. (**A**) The quantities of cyclin B transcripts were normalized to the beta-actin mRNA level. The values (means ± SEM) were expressed as arbitrary units of relative abundance of the target gene. Significant difference was indicated by asterisks. (**B**) Representative photograph of typical 2% agarose gels stained with ethidium bromide, showing the presence of the expected products yielded after RT-PCR using primers for target cyclin B (120 bp) and beta-actin (200 bp)

The crab cyclin B mRNA was expressed predominantly in ovary and testis, little in heart, and no signal was detected in liver, gill and muscle (Fig. 4). Considering the exceedingly low content of proliferating cells in most somatic tissues whereas high in developing gonad, the tissue distributions of the crab cyclin B mRNA are consistent with the role of the cyclin B in cell cycle regulation. Semi-quantitative RT-PCR analysis further revealed that the amount of cyclin B mRNA in ovary fluctuated according to oogenesis. At pVt stage, the crab oogonia undergo active mitosis proliferation for increasing their number. The cyclin B mRNA level was significantly high. At eVt and mVt stages, the growing oocytes accumulate yolk with low activity in proliferation and arrest at the first meiotic prophase. The cyclin B mRNA level drops to the lowest point. After completion of yolk accumulation, the fully-grown oocytes resume meiosis at lVt stage and geminal vesicle (nuclei) breakdown (GVBD) occurs at final maturation stage (Fig. 1). The cyclin B mRNA level increase again. Although the mitten crab can spawn three times during a breeding season, and therefore, there are three stages of oocytes in a matured ovary, the most predominant oocytes were visually at lVt or GVBD stage (Fig. 1C, D). The second and third batch oocytes at pVt were found in only a very small portion and most of them are resting primary oocytes without mitotic activity. Therefore, the increase of cyclin B mRNA level at pVt, lVt and GVBD stages strongly suggests that cyclin B is closely related to oogonial proliferation (mitosis) and oocyte meiotic maturation in the crab ovary.

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