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Characterization, Evolution and Expression of the Calmodulin1 Genes from the Amphioxus *Branchiostoma belcheri tsingtauense*

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Abstract Two full-length cDNAs, named *CaM1a* and *CaM1b*, encoding the highly conserved calmodulin1 (CaM1) proteins, were isolated from the cDNA library of amphioxus *Branchiostoma belcheri tsingtauense*. There are only two nucleotide differences between them, producing one amino acid difference between CaM1a and CaM1b. Comparison of the amino acid sequence of CaM1 reveals that the *B. belcheri tsingtauense* CaM1a is identical with CaM1 proteins of *B. floridae* and *B. lanceolatum*, *Drosophila melanogaster* CaM, ascidian *Halocynthia roretzi* CaMA and mollusk *Aplysia californica* CaM, and CaM1b differs only at one position (138, Asn to Asp). The phylogenetic analysis indicates that the *CaM1* in all three amphioxus species appears to encode the conventional *CaM* and *CaM2* might be derived from gene duplication of *CaM1*. Southern blot suggests that there are two copies of *CaM1* in the genome of *B. belcheri tsingtauense.* Northern blot and *in situ* hybridization analysis shows the presence of two *CaM1* mRNA transcripts with various expression levels in different adult tissues and embryonic stages in amphioxus *B. belcheri tsingtauense*. The evolution and diversity of metazoan *CaM* mRNA transcripts are also discussed.

Key words amphioxus; *Branchiostoma*; calmodulin1; evolution; expression

Calmodulin (CaM) is a calcium-binding EF-hand protein that mediates the calcium-dependent activity of a variety of different target enzymes and structural proteins. The primary structure of this protein has been determined in many organisms from different species and shows a remarkably high degree of conservation [1]. The protein contains four conserved canonical calcium-binding domains that might be derived from an ancestral one-domain precursor through events of gene duplication and translocation [2,3].

In vertebrates, CaM protein is encoded by multiple genes. For instance, six genes have been detected in zebrafish [4], three genes in humans [5–7] and rats [8, 9], at least two genes in frogs *Xenopus laevis* [10] and two genes in chickens [11,12]. Interestingly, all of these genes give rise to identical proteins, and this phenomenon has brought about the hypothesis of "multigene one-protein" for vertebrate *CaM* gene families [13,14]. Although the proteins are present in all cells of all eukaryotes and they play vital roles in cellular information transduction, the number of *CaM* genes in invertebrates is rather small. The exact number of CaM genes and proteins existing in metazoan is still unknown. It is possible that a single *CaM* gene (e.g., *Drosophila melanogaster* [15], mollusk *Aplysia californica* [16], ascidian *Ciona intestinalis* [1]) or two genes encode different CaM isoforms (e.g., echinoderm *Arbacia punctulata* [17], ascidian *Halocynthia roretzi* [18], *B. lanceolatum* and *B. floridae* [19]).

Although CaM is ubiquitous, the sizes and distributions of the transcripts vary in different tissues and embryonic stages in different species. For example, human *CaM1* gene is transcribed into two mRNAs of 1.7 kb and 4.2 kb. The 1.7 kb mRNA is uniformly present, whereas the 4.2 kb mRNA is particularly abundant in brain and skeletal muscle [7]. In chickens, four transcripts of 0.8 kb, 1.4 kb, 1.7 kb and 4.4 kb for *CaM1* gene are detected; two major transcripts of 1.4 kb and 1.7 kb are present in all chicken tissues, whereas the 4.4 kb *CaMI* transcript is

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plentiful in brain [20]. The frog *CaM* gene is transcribed into five mRNAs of 1.4 kb, 1.6 kb, 2.1 kb, 2.2 kb and 2.7 kb, and a major band of 1.4 kb has been observed in ovary, testis and brain [10]. In sea urchin, only a single size of 3.2 kb transcript for the *CaM* gene is detected in both embryonic and adult tissues. The mRNA is present in the unfertilized egg at the level of a typical rare-class mRNA and accumulates approximately 100-fold in pluteus-stage cells [21]. Fruit fly *CaM* gene is transcribed into two mRNAs of 1.65 kb and 1.9 kb, and the total amount of mRNA is highest in the larval stage compared to the embryo stage and the pupal stage [22].

Amphioxus, a cephalochordate, has long been known as an extant invertebrate that is most closely related to the proximate ancestor of vertebrates [23,24]. Karabinos and Bhattacharya have suggested the existence of two *CaM* genes both in *B. lanceolatum* and *B. floridae*, although it had been previously considered that only a single *CaM* gene existed in this taxon [25]. Even though they all belong to the same genus of *Branchiostoma* in taxonomic status, the exact number of *CaM* genes is sparse in *B. belcheri tsingtauense*, which is considered a different species to *B. lanceolatum* and *B. floridae*, both at the molecular level and histological level [26–29]. In addition, the expression pattern of *CaM* in amphioxus is still unclear. Our study is driven to explore the answers to these questions.

In this study, we isolated two full-length *CaM1* cDNAs (*CaM1a* and *CaM1b*) from the cDNA library of amphioxus *B. belcheri tsingtauense*, and determined the copy number of the gene and the expression pattern in different adult tissues and embryonic stages. We also explore the evolution and diversity of metazoan *CaM*s.

Material and Methods

cDNA cloning and sequencing analysis

Gut cDNA library of adult amphioxus *B. belcheri tsingtauense* was constructed with the SMART cDNA Library Construction Kit (Clontech, Palo Alto, USA) using the method described previously [30]. In a large-scale sequencing of amphioxus gut cDNA library with an 377XL DNA sequencer (ABI Prism, Foster, USA), more than 5000 clones were analyzed for coding probability using the DNATools program (http://www.crc.dk/dnatools/downloads/accept.php?accept_url =setup/dt6_setup.exe) [31].

Initial comparison to the GenBank protein database was carried out using the BLAST network server at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) [32]. Multiple nucleotide and protein sequences were aligned using the CLUSTAL method from MegAlign in the DNAStar software package (version 5.0; Dr. Steve ShearDown, Madison, USA) [33]. A phylogenetic tree was constructed with 1000 bootstrap replicates using the neighbor-joining method (PHYLIP 3.6b software package, http://evolution.genetics.washington.edu/phylip.html) [34].

Southern blotting analysis

Genomic DNAs for Southern blotting analysis were isolated from adult amphioxus and digested with three restriction enzymes (37 ºC, 20 h): *Bgl*II, *Pst*I and *Hin*dIII (1 unit per μg DNA). The digested DNAs were separated on a 1% agarose gel using 1× TBE (89 mM Tris-borate and 2 mM EDTA) and transferred onto nylon membranes (Osmonics, Trevose, USA). The membranes were hybridized at high stringency with the digoxigenin (DIG)-labeled *B. belcheri tsingtauense CaM1a* cDNA probe produced with a DIG DNA labeling kit (Roche, Basel, Switzerland). Hybridized bands were visualized according to the instructions of the detection kit.

Northern blotting analysis

Total RNAs were prepared with Trizol (Gibco, Carlsbad, USA) from various tissues including muscle, notochord, testis, ovary, gut and gill of adult amphioxus and embryos at four developmental stages, including blastulae, gastrulae, neurula and 24 h larvae. A total of 3 μg RNAs each was detected using electrophoresis and was blotted onto nylon membranes (Osmonics). The blots were hybridized at high stringency with DIG-labeled *B. belcheri tsingtauense CaM1a* riboprobe. The hybridized bands were visualized by BM-Purple (Roche).

In situ hybridization histochemistry

Sexually mature amphioxus were dissected into three or four pieces and fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate-buffered saline, pH 7.4, at 4 ºC for 8 h. They were dehydrated, embedded in paraffin, and sectioned into 6 μm per slide. The sections were hybridized with the same DIG-labeled *B. belcheri tsingtauense CaM1a* riboprobe and the control sections were hybridized with the sense riboprobe. The hybridized signals were visualized by BM-Purple (Roche).

Results and Discussion

Identification and evolution of two amphioxus *CaM1* cDNAs

Obtained from the gut cDNA library of the amphioxus *B. belcheri tsingtauense* [30], two cDNA clones that encode CaM1s are named *CaM1a* (GenBank accession number: AY269783) and *CaM1b* (GenBank accession number: EF177448). *CaM1a* is 1412 bp long and contains three regions, a short 5′ untranslated region (UTR) of 64 bp, a longest open reading frame (ORF) of 450 bp and a 3′ UTR of 898 bp with a polyadenylation tail at the extreme 3′ end. The 3′ UTR shows the canonical polyadenylation signal (AATAAA) upstream of the poly(A) tail. *CaM1b* is 1459 bp long and contains a short 5′ UTR of 117 bp, a longest ORF of 450 bp and a 3′ UTR of 892 bp with a polyadenylation tail at the extreme 3′ end. The 3′ UTR also shows the canonical polyadenylation signal (AATAAA) upstream of the poly(A) tail (**Fig. 1**). There are only two nucleotide substitutions within their ORFs, producing one amino acid difference between CaM1a and CaM1b.

Comparison of the *B. belcheri tsingtauense* CaM1a and CaM1b with other known CaM1s reveals that the *B. belcheri tsingtauense* CaM1a is 100% identical to the CaM1 proteins of *B. floridae* (GenBank accession number: Y09863) and *B. lanceolatum* (GenBank accession number: Y09880), *D. melanogaster* CaM (GenBank accession number: AY118890), ascidian *H. roretzi* CaMA (GenBank accession number: AB018796) and mollusk *A. californica* CaM (GenBank accession number: AY036120), and the CaM1b differs at only one position, at 138, Asn to Asp (**Fig. 2**). The replacement of A with G at the first codon position generates an Asp instead of an Asn. The *CaM1b* sequence encoding this amino acid is further confirmed by genome amplification. Thus, two CaM1 proteins (CaM1a and CaM1b) are obtained from amphioxus *B. belcheri tsingtauense*. This finding indicates that the mutation of CaM1b might occur only in the lineage of amphioxus after the split of the amphioxus from a common ancestor approximately 550 million years ago. This evidence supports our previous hypothesis that amphioxus could represent a specialized form radiated from the chordate ancestor [30,35].

The nucleotide sequence of the coding regions of *CaM*s was aligned in DNASTAR (**Fig. 3**). It has been found that the *B. belcheri tsingtauense CaM1a* or *CaM1b* have 11 and 21 base substitutions with *B. floridae CaM1* and *B. lanceolatum CaM1*, respectively. All substitutions appear at the third codon positions except one position of *CaM1b* (412, A to G) that attributes to the single mutation of the CaM1b amino acid sequence. *B. lanceolatum CaM1* and *B. floridae CaM1* have 13 base substitutions that all occur at the third codon positions. It suggested that the nucleotide sequence mutation between *B. belcheri tsingtauense CaM1*s and *B. lanceolatum CaM1* is maximal among the three amphioxus species. This finding reveals the evolutionary relationship of *CaM*s, which is still unclear. **Table 1** presents the number of substitutions of amino acids/nucleotides among CaMs in different species. In **Table 1**, the CaM1 from each of the three amphioxus species has similar amino acid/nucleotide substitutions to other CaMs. For example, *B. belcheri tsingtauense CaM1a* and *CaM1b*, *B. floridae CaM1* and *B. lanceolatum CaM1* show 74, 74, 73 and 70 nucleotide substitutions with *D. melanogaster CaM*, respectively. Furthermore, the nucleotide sequence of the respective 5′ and 3′ UTRs of *CaM1* and *CaM2* genes was compared, and they show 22%–30% identity (not higher than the identity among their coding regions). In addition, a phylogenetic tree was constructed using the nucleotide sequence of the coding regions of 28 known *CaM*s (**Fig. 4**), and the bacteria *Phytophthora infestans CaM* was added as the outgroup on the tree. The results show that *B. belcheri tsingtauense CaM1a* and *CaM1b* cluster together with *CaM1*s from the three amphioxus species, and two amphioxus *CaM2*s are on the separate branch. Our results also suggest that the amphioxus *CaM1*s are closer to *D. melanogaster CaM*, *Caenorhabditis elegans CaM*, *H. roretzi CaMA and CaMB* than the amphioxus *CaM2*s. Our findings further show that the *CaM1* sequence in all three amphioxus species appears to be the conventional CaM, and *CaM2* might be the gene duplication product of *CaM1*, which is consistent with the previous analyses of Karabinos and Bhattacharya [19]. We expect to find out the *CaM2* in *B. belcheri tsingtauense* in our future project. It would be more interesting to further compare their intron-exon structures of the *CaM1* and *CaM2* genes in *B. belcheri tsingtauense*, which is available in *B. lanceolatum* [19] and *B. floridae* (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Brafl1&id=132038, and http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Brafl1&id=120113). The results show that the gene organization of *CaM2* differs from that of *CaM1*: *CaM2* in *B. lanceolatum* and *B. floridae* has three introns, whereas *CaM1* has four introns.

Copy number of the amphioxus *CaM1* gene

To analyze the copy number of *B. belcheri tsingtauense CaM1* gene, we used the DIG-labeled cDNA probe of *B. belcheri tsingtauense CaM1a* to hybridize digests made from amphioxus genomic DNA by the restriction enzymes

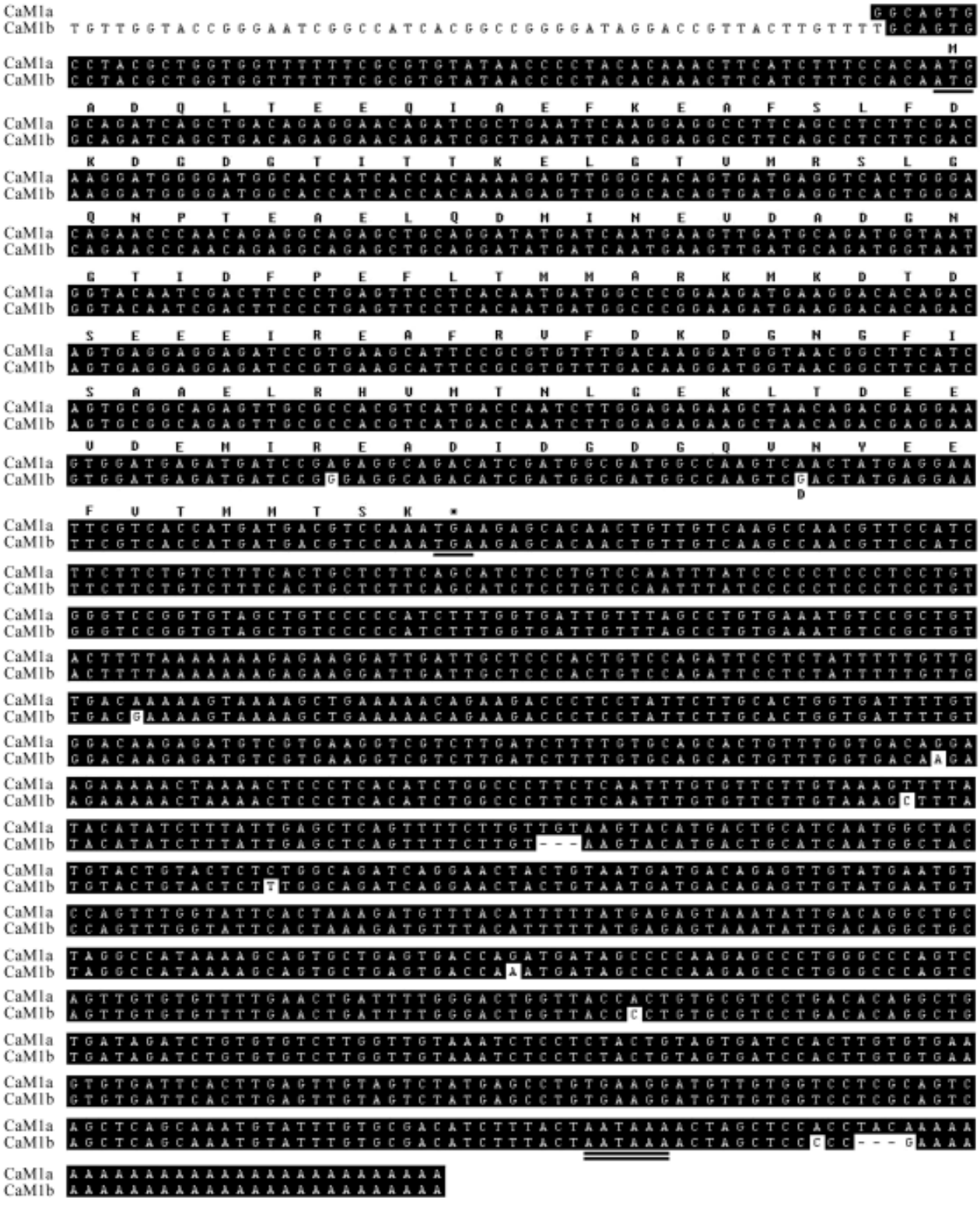

Fig. 1 Nucleotide and deduced amino acid sequences of *Branchiostoma belcheri tsingtauense calmodulin1* (*CaM1*)

The nucleotide sequences of two *B. belcheri tsingtauense CaM1* cDNAs were named *CaM1a* (GenBank accession number: AY269783) and *CaM1b* (GenBank accession number: EF177448). Conserved nucleotides in these two clones are shaded for identification. There was one difference in the amino acid sequences of *B. belcheri tsingtauense* CaM1a and CaM1b deduced from the open reading frames of these *CaM1* cDNAs. The translational start and terminal sites are underlined, and the asterisk represents the stop codon. The polyadenylation signal (AATAAA) upstream of the poly(A) tail is double underlined.

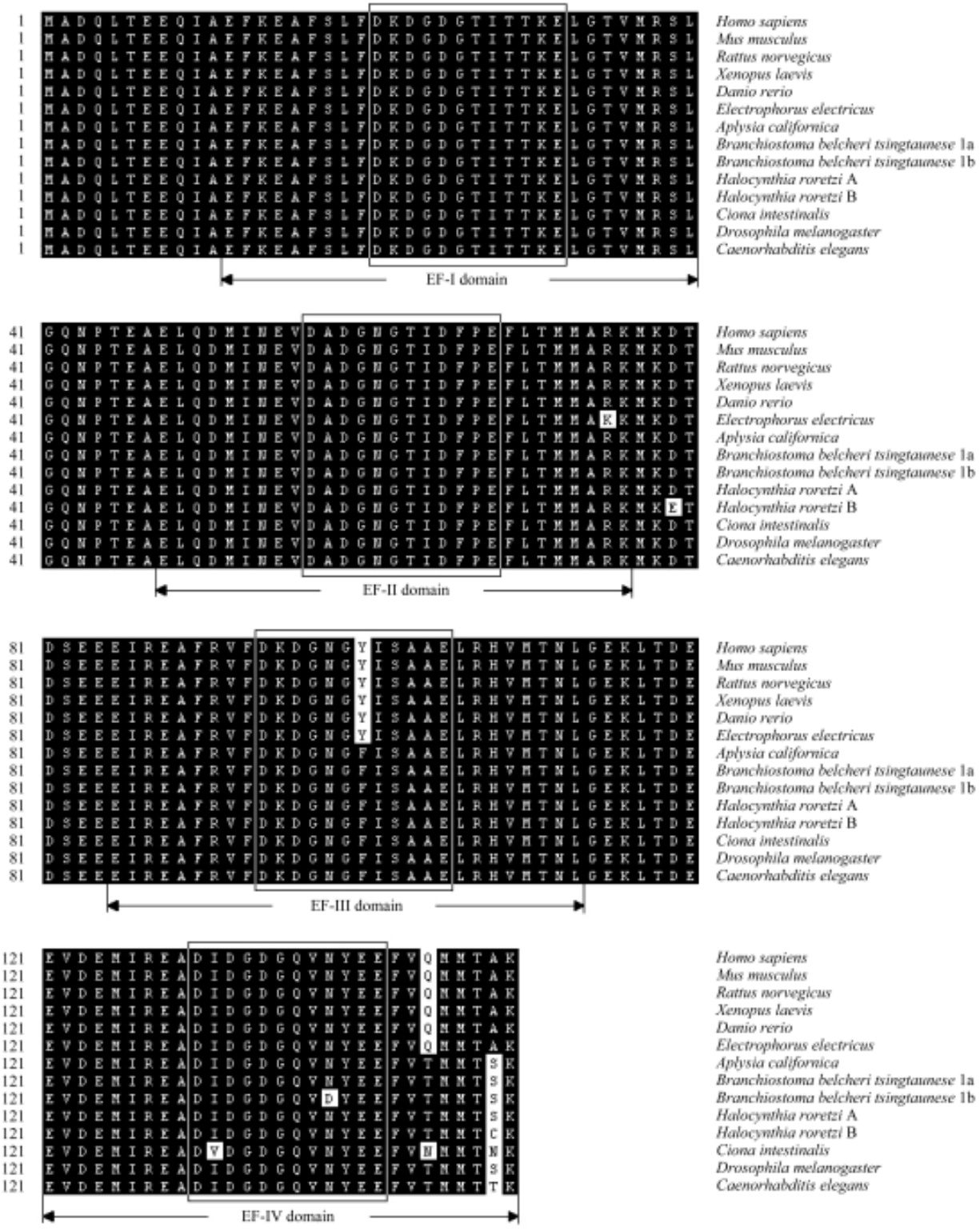

Fig. 2 Alignment of calmodulin (CaM) amino acid sequences including *Branchiostoma belcheri tsingtauense* CaM1a and CaM1b using the CLUSTAL method from MegAlign in the DNASTAR software package

Conserved amino acid residues in these protein sequences are shaded for identification. The four EF-hands (EF-I, EF-II, EF-III and EF-IV) are marked by arrows, and the Ca^{2+}-binding loop in the helix-loop-helix structure of individual EF-hands was boxed. The amino acid sequences of CaM in representative species were downloaded from GenBank.

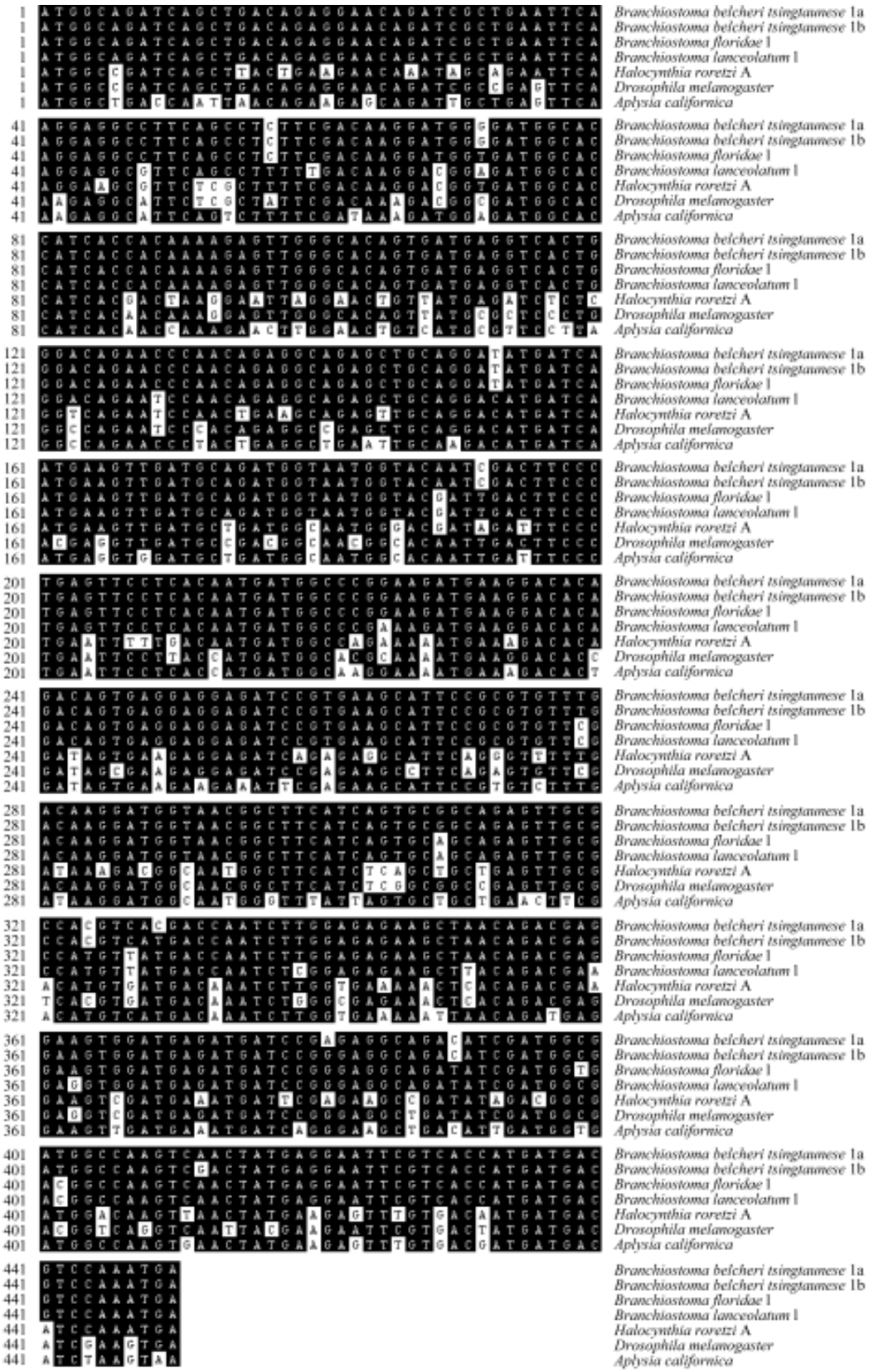

Fig. 3 Alignment of *CaM* nucleotide sequences of coding regions using the CLUSTAL method from MegAlign in the DNASTAR software package

Conserved nucleotides are shaded for identification.

Table 1 Numbers of substitutions of the aligned calmodulin (CaM) amino acids (aa) and nucleotides (nt) in Figures 2 and 3

aa/nt	Bb 1b	Bf 1	Bl 1	Dm	Hr A	Ac	Bf 2	Bl 2	Hr B
Bb 1a	1/2	0/11	0/21	0/74	0/89	0/96	6/86	17/117	2/91
Bb 1b		1/11	1/21	1/74	1/91	1/96	7/88	18/119	3/93
Bf 1			0/13	0/73	0/89	0/97	6/89	17/121	2/90
Bl 1				0/70	0/85	0/103	6/90	17/125	2/87
Dm					0/86	0/95	6/87	17/127	2/88
Hr A						0/90	6/104	17/140	2/92
Ac							6/104	17/145	2/3
Bf 2								22/84	8/106
Bl 2									18/146

Ac, *Aplysia californica* CaM; Bb 1a, *Branchiostoma belcheri tsingtauense* CaM1a; Bb 1b, *B. belcheri tsingtauense* CaM1b; Bf 1, *B. floridae* CaM1; Bf 2, *B. floridae* CaM2; Bl 1, *B. lanceolatum* CaM1; Bl 2, *B. lanceolatum* CaM2; Dm, *Drosophila melanogaster* CaM; Hr A, *Halocynthia roretzi* CaMA; Hr B, *H. roretzi* CaMB.

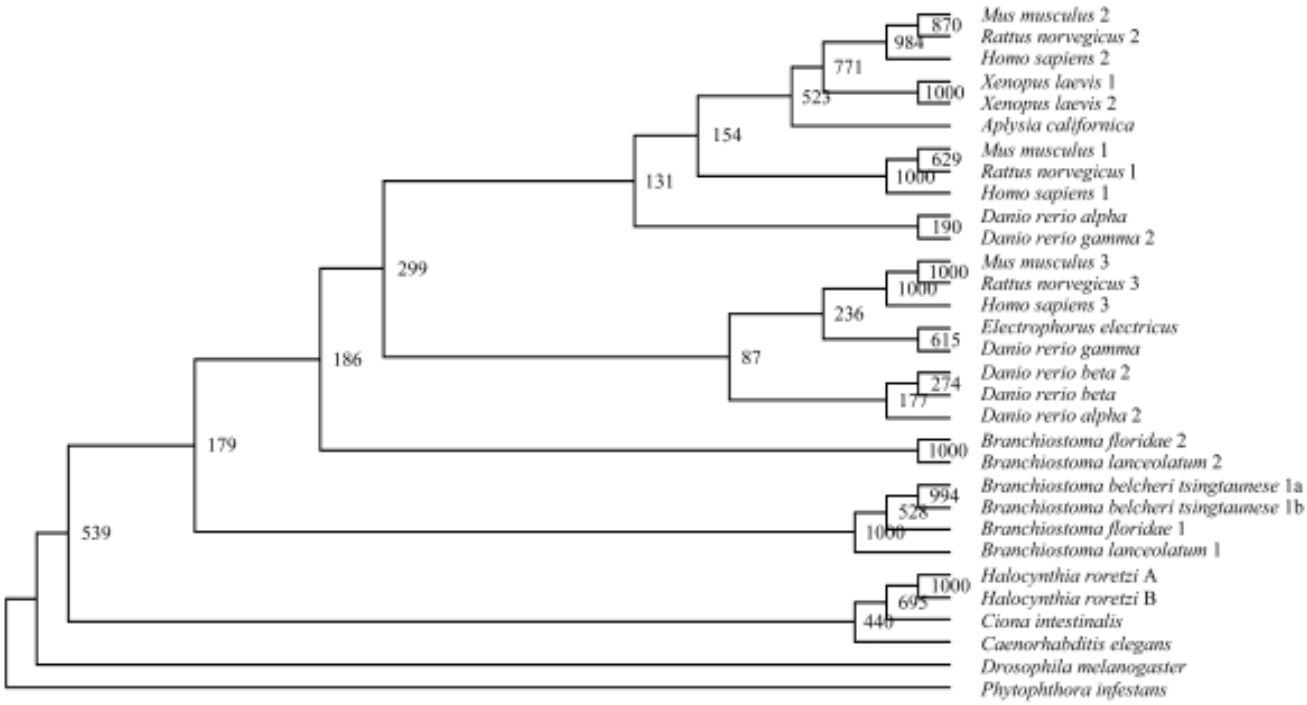

Fig. 4 Phylogenetic tree of *CaM* nucleotide sequences of coding regions constructed using the neighbor-joining method (PHYLIP 3.6b software package)

The bacteria *Phytophthora infestans CaM* was added as the outgroup on the tree. Bootstrap majority consensus values on 1000 replicates are indicated at each branch point.

*Bgl*II, *Pst*I and *Hin*dIII. Two hybridization bands were observed on a Southern blot of the *Bgl*II and *Pst*I restriction digest, and three bands were seen in the *Hin*dIII digest alone (**Fig. 5**). The presence of the three bands generated with the enzyme *Hin*dIII is possibly due to *Hin*dIII's restriction site on the *B. belcheri tsingtauense CaM1* intron, as the same restriction site of *Hin*dIII has also been seen in the *B. lanceolatum CaM1* intron [25]. These findings suggest the presence of two copies of the *CaM1* gene in the genome of amphioxus *B. belcheri tsingtauense*. A high level of nucleotide sequence homology (98.3% identity), which has been noticed between *CaM1a* and *CaM1b* cDNAs, raises the possibility that the two genes might be the products of a gene duplication event that occurred only in the lineage of amphioxus.

Expression analysis of the amphioxus *CaM1a* gene

To detect the distribution of *CaM1a* mRNA in tissue,

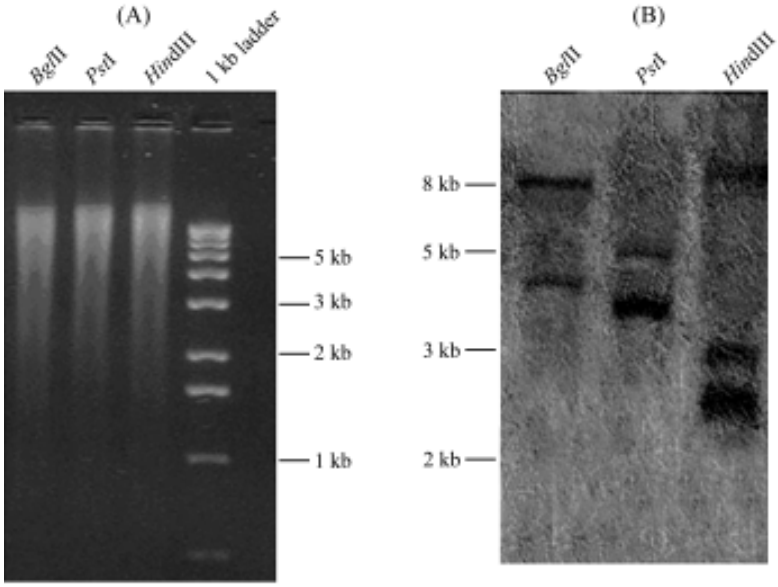

Fig. 5 Southern blotting analysis of genomic DNAs extracted from adult amphioxus *Branchiostoma belcheri tsingtauense*
(A) The digested DNAs were separated on a 1% agarose gel. (B) The blot was hybridized with the digoxigenin-labeled *B. belcheri tsingtauense CaM1a* cDNA probe.

we applied *in situ* hybridization on tissues of adult amphioxus *B. belcheri tsingtauense*, using a specific probe, the *B. belcheri tsingtauense CaM1a* cDNA (**Fig. 6**). The results show a strong expression of *CaM1a* in ovary, hepatic caecum, hind-gut, testis, gill, endostyle and theca of notochord. A weak expression is found in neural tube, muscle and notochord. It would also be interesting to test for coexistent expression between *CaM1a* and *CaM1b* in amphioxus *B. belcheri tsingtauense* in further studies.

In addition, the expression patterns of *CaM1a* in different adult tissues and embryonic stages in amphioxus *B. belcheri tsingtauense* were analyzed using Northern blot. As shown in **Fig. 7**, two different sizes of mRNA are seen at 1.4 kb and at 3.2 kb. The 1.4 kb mRNA shows strong

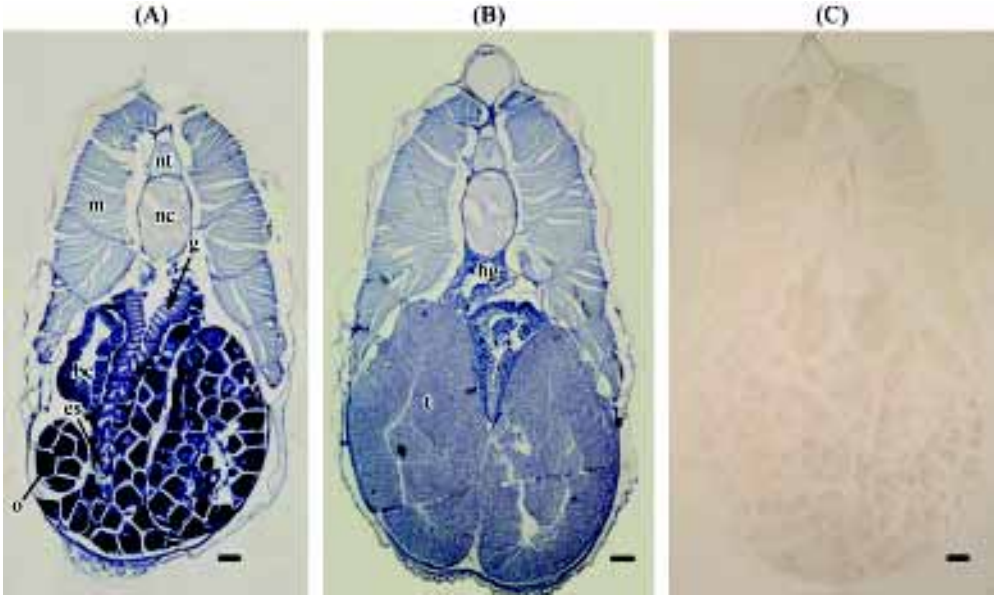

Fig. 6 Localization of *Branchiostoma belcheri tsingtauense CaM1a* transcripts in different tissues of adult amphioxus
(A, B) *CaM1a* transcripts were observed in the digestive system including endostyle, hepatic caecum, hind-gut and in the gill, ovary and testis. es, endostyle; g, gill; hc, hepatic caecum; hg, hind-gut; m, muscle; nc, notochord; nt, neural tube; o, ovary; t, testis. (C) Control sections using the sense probe. Bar=100 μm.

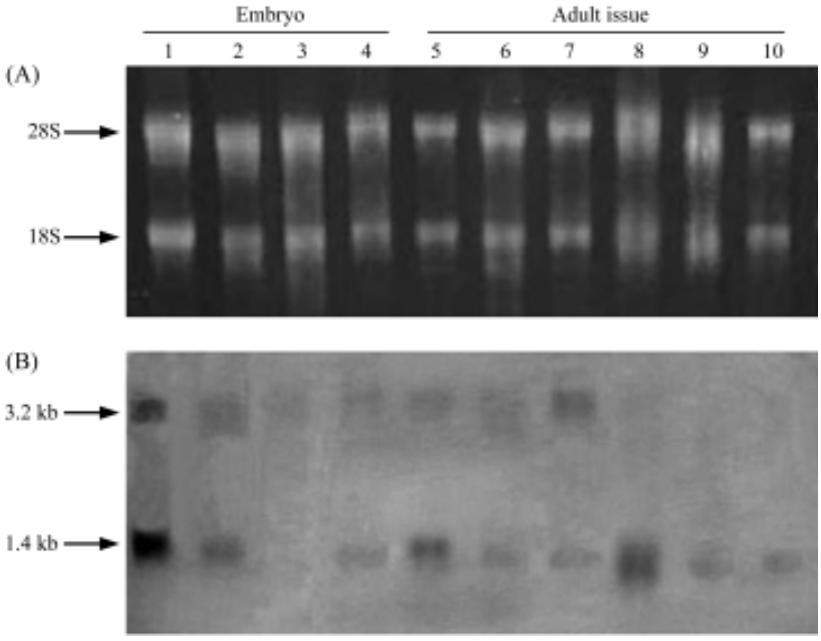

Fig. 7 Northern analysis for *Branchiostoma belcheri tsingtauense CaM1a* transcripts in different tissues of the adult and embryos at four developmental stages

(A) A total of 3 μg RNA for each sample was analyzed in 1.3% agarose formaldehyde denaturing gel. (B) The blot was hybridized with digoxigenin-labeled *B. belcheri tsingtauense CaM1a* riboprobe. Embryo: blastula (3.5 h after fertilization, lane 4), gastrula (6 h after fertilization, lane 3), neurula (16 h after fertilization, lane 2) and 24 h larva (24 h after fertilization, lane 1). Adult tissues: gill (lane 5), gut (lane 6), ovary (lane 7), testis (lane 8), notochord (lane 9) and muscle (lane 10).

signals in the tissues of testis and gill, although weak signals are seen in other tissues including gut, muscle, notochord and ovary. During embryonic development, the 1.4 kb mRNA starts from extremely low density in blastulae and gastrulae, gradually increases in neurula, and reaches its maximum at 24 h larvae.

In contrast, the 3.2 kb mRNA is transcribed significantly in ovary, immaterially low in gut and gill, and rarely in testis, muscle and notochord. Its expression pattern is notably different to that of the 1.4 kb mRNA in adult amphioxus tissues. However, the expression pattern of the *CaM1a* mRNA (the 3.2 kb and the 1.4 kb mRNA together) agrees consistently with the result detected by *in situ* hybridization. The expression pattern of the 3.2 kb mRNA is similar to that of the 1.4 kb mRNA in each stage of the embryo, showing the weak appearance from blastulae to gastrulae, then a significant increase in neurula, and finally reaching the maximum elevation in 24 h larvae. The progression of the positive signal of both mRNA, starting from the neurula stage, probably reflects the relationship between the differentiation of the neural system and the onset of gene transcription. These results concur with the expression pattern of *CaM* transcript in ascidians that are developmentally regulated and specifically restricted to the larval neural system [1].

We propose that the two different sizes of mRNA for *CaM1a* in amphioxus *B. belcheri tsingtauense* might have arisen from a common nuclear precursor of the gene through differential polyadenylation. It has been reported that three different *CaM* mRNAs in eel electroplax tissue are derived from a single nuclear transcript of approximately 5500 nucleotides, which represents a primary transcript of the gene [36]. However, further experiments are needed to determine whether the two different sizes of mRNA for *CaM1a* in amphioxus *B. belcheri tsingtauense* originated from one gene.

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