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Full Length Research Paper

Molecular Cloning and Characterization of the Tpn I Gene in Bombyx mori

Zhao Yuan[1, 2], Yao Qin[1], Tang Xudong[1], Wang qiang[1], Yin huijuan[1], Hu Zhigang[1], Lu Jian[1] and Chen Keping[1]*

[1]Institute of Life Sciences, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, P. R. China.
[2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, 212018, China.

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The troponin complex is composed of three subunits, Troponin C (the calcium sensor component) and Troponin T and I (structural proteins). Tpn C is encoded by multiple genes in insects, while the Tpn T and Tpn I proteins are encoded by single genes. Tpn I binds to actin and Tpn T binds to tropomyosin. We cloned and sequenced the Tpn I (AY873787) gene from *Bombyx mori* that encodes 225 amino acids and contains a conserved motif seen in *Drosophila virilis* and *Anopheles gambiae.* Bioinformatic analysis suggests that its deduced amino sequence shares 81.3 and 78.7% homology with the Tpn I genes of *A. gambiae* and *D. virili*, respectively.

Key words: Tpn I, *Bombyx mori*, troponin, homology.

INTRODUCTION

The troponin complex is responsible for the regulation of the thin filaments response to calcium when muscular contraction is required in the different types of muscular fibres (Henrikson and Chandra-Strobos, 2004). The complex is constituted of three subunits, Troponin C (TpnC), a four EF-Hand protein that bind to calcium when the intracellular level of this cation is increased, Troponin I (TpnI), a subunit that inhibits the transduction role of the third component, and Troponin T (TpnT) (Cullen et al., 2004). The last two subunits have mainly a structural role, while TpnC is the sensor component. Extensive studies have been performed on structure-function of mammals of this complex (Filatov et al., 1999; Gordon et al., 2000, 2001). When a nervous stimulus reaches the muscle fibre, the subsequent calcium increase triggers the interaction of the myosin filaments with the thin fila-ments of actin to promote the contraction event. In basal calcium conditions, tropomyosin locks the actin site of myosin binding. When the Calcium level increases in the sarcoplasm, Troponin C is able to capture it and change its dumbbell extended structure into a more compact form affecting the rest of the troponin complex elements (Reinach et al., 1997; Vassylyev et al., 1998). It has been proposed that the TpnI amino terminal domain interacts with both the carboxy terminal TpnC and TpnT domains. The TpnT amino terminal domain interacts with tropomyosin. After calcium sensing by TpnC, TpnI interacts also with the carboxy-terminal domain of TpnC, allowing TpnT to change its interaction with tropomyosin, unlock-king the myosin binding site in actin and thus promoting the sliding mechanism.

Silkworm, *Bombyx mori*, is an important economic insect and the model insect of Lepidoptera. In our fluorescence differential display of transcription research on resistance to densonucleosis in silkworm *B. mori* we found a cDNA fragment of 523 nts with high homology to that of *B. mori* EST (Access Number in GenBank: CK494310). In order to identify what it encodes, special primer was designed for 5'-RACE. An 814 bp cDNA clone containing a 675 bp open reading frame (ORF) was identified. It contained whole troponin motif. This is the first report on Tpn I in *B. mori* . In insects, Tpn I of *Anopheles gambiae* and *Drosophila virilis* have been reported. Alignment of the amino acid sequence of Tpn I from 16 organisms, *A. gambiae* (EAA44286), *Drosophila melanogaster* (A38594), *D. virilis* (AAR24602), *Lethocerus indicus* (CAF18234), *Branchiostoma belcheri* (BAA96549), *Chlamys nipponensis akazara* (Q7M3Y3), *Chlamys nipponensis* (BAE43658), *Ciona intestinalis* (AAD09271), *Clupea harengus* (AAB05825), *Coturnix coturnix* (A41030), *Halo-*

*Corresponding author. E-mail: kpchen@ujs.edu.cn. Tel: 86-511-8791923. Fax: 86-511-8791923.

Figure 1. Nucleotide sequence and deduced amino acid sequence of the Tpn I gene of *B. bombyx*. The start codon of ATG and the deduced polyadenylation signals are underlined. The termination codon is shown by asterisk. This cDNA seq-uence has been deposited in GenBank under accession number AY873787.

cynthia roretzi (BAA19425), *Homo sapiens* (AAH12601), *Mizuhopecten yessoensis* (BAA22853), *Polyandrocarpa misakiensis* (BAB83806) and *Sus scrofa* (NP_001027530) was performed and the polylogenic relationship among them was compared.

MATERIALS AND METHODS

Materials

Rneasy Mini Kit was purchased from QIAGEN, and BD SMART RACE cDNA Amplification Kit was from BD Bioscience Clontech. PCR reagents and pMD18-T vector were obtained from Takara Company (Dalian China). Other reagents were purchased from Shanghai Sangon Bio-technology Corporation. The silkworm *B. mori* was inbred in our laboratory. About 50 silkworms were collected for one RNA pool.

RNA extraction

The midgut was dissected from the larvae at the 3rd day of the 5th instar, frozen with liquid nitrogen and ground into powder. Total RNA was extracted used the Rneasy Mini Kit according to the user manual. Finally, the consistency of the total RNA was inspected with Gene spec (Naka Instruments Co., Ltd.) and stored at -70 ºC for further use.

RT-PCR and 5'-RACE

1µg total RNA was used as a template in the first-strand cDNA synthesis. And a specific primer, 5'-cttcgaccagtcaggctttttctctttg-3', was designed for 5'-RACE based on the known sequence of our former research. PCR reaction was carried out for 35 amplification cycles (94ºC for 30 s, 58ºC for 30 s, 72ºC for 3 min) in a Gene Amp 2400 System thermocycler (Applied Biosystem, USA). PCR products were examined by electrophoresis in 1% agarose gel with ethidium bromide staining.

Cloning and sequencing

The specific fragment from 5'-RACE was ligated into pMD18-T vector and then transformed into *E. coli* (DH5 strain). Plasmid was purified with MiniBEST Plasmid Purification Kit (Takara). The sequencing was performed using an automatic sequencer: CEQ8000 (Beckman USA).

Software and database used for bioinformatic analysis

The primer used to perform RACE was designed by GeneFisher in the website of Bielefeld University Bioinformatics Server (http://www.genefisher.de/). The DNA and amino acid sequence analysis were performed using the software Lasergene 6. The homologous analysis of deduced amino acid sequence was perfor-med using the BLAST Tool in GenBank (Blastp). Phylogenetic relationships and pairwise identities and similarties of the deduced amino acid sequence was performed using Clustal W on http://www.ebi.ac.uk/clustalw. MotifScan, which scans a sequence against protein profile databases (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was also performed.

RESULTS AND DISCUSSION

We sequenced a cDNA clone with an 814 bp fragment inserted, which contains a 675 bp ORF, having a poten-tial to encode a peptide of 225 amino acid residues. The initiating codon ATG and the stopping codon TGA are at the positions of 57 and 732, respectively (Figure 1). Two polyadenylation signals (ATTAA and AATAA) are 19 and 42 bp downstream from the termination condon.

The result of the Blast search using the amino acid sequence in the NCBI database showed that the peptide encoded by the 675 bp ORF was a deduced Tpn I gene. The length of Tpn I in *B. mori* is very similar to those of other reported in insects, which ranges from 201 to 225 amino acid residues. Lasergene module for multiple and pairwise sequence alignments and phylogenetic tree construction Aligning its sequences with the amino acid sequences of other 7 organisms using ExPASy Proteomics Server *(http*://us.expasy.org) ClUSTALW program, revealed that the sequence of Tpn I of *B. mori* shares 86.8, 24.2, 84.4, 21.9, 23.5, 23.5 and 25.3% similar with the Tpn I of *A. gambiae* (Access number: EAA44286), *Danio rerio* (Access number: NM_205742), *D. virilis* (Access number: AAR24602) (Barbas et al., 1993), *Gal-lus gallus* (Access number: NM_205417) (Murakami et al., 2005), *R. norvegicus* (Access number: NM_017184) (Kedar et al., 2004), *H. sapiens* (Access number: BC012601) (Strausberg et al., 2002) and *X. laevis* (Acc-ess number: BC044282) (Klein et al., 2002), respectively (Table 1). Among the 225 amino acid residues of *Bm*Tpn I, only 28 amino acid residues are different from *D. virilis* and *A. gambiae* (Figure 2). However the similarity with the Tpn I of others species was very low.

Tpn I is the actomyosin ATPase inhibitory subunit present in the thin filament regulatory complex. The actin binding domain of *Bm*- tpn I displays 95% amino sequence homology with both insect and mammal Tpn I (Figure 2, arrow pointed). The consensus sequence of this doma-

Table 1. Pair distances of untitled clustal W among 16 species (percent similarity in upper triangle)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		81.3	23.6	32.0	28.0	24.4	19.6	23.1	78.7	78.7	24.9	22.2	73.8	32.0	26.2	22.7	1
2	11.6		25.9	34.1	30.2	26.8	22.0	25.4	85.9	85.9	27.8	25.4	80.5	34.1	29.8	23.9	2
3	154.9	153.8		31.1	31.1	46.1	43.3	38.9	30.6	30.0	48.9	44.4	27.8	31.1	46.1	47.8	3
4	121.4	120.9	127.4		56.2	17.1	16.8	18.5	24.0	24.0	18.2	17.5	23.6	96.6	16.4	14.7	4
5	116.7	119.2	127.4	0.0		30.5	29.9	31.1	37.8	37.8	32.3	31.1	36.6	95.7	29.3	26.2	5
6	152.3	150.3	87.0	150.1	150.1		51.6	43.4	31.9	31.9	75.8	55.5	29.1	28.6	72.0	50.5	6
7	187.8	180.3	93.2	144.4	144.4	69.0		46.6	27.8	27.8	51.1	55.1	25.0	28.4	47.7	53.4	7
8	186.0	181.6	114.1	161.0	145.4	96.2	86.3		25.0	25.0	39.9	48.6	23.6	24.0	39.9	42.3	8
9	16.1	15.7	149.2	120.9	119.2	142.9	165.4	184.9		98.1	29.8	24.5	78.8	33.7	31.7	25.0	9
10	15.1	14.7	149.4	120.9	119.2	142.9	164.2	181.6	0.5		30.2	24.9	80.0	34.1	31.7	25.4	10
11	148.1	142.9	78.3	139.8	139.8	28.6	73.9	88.5	130.6	130.6		58.2	31.3	29.7	79.7	50.5	11
12	171.7	161.8	96.0	146.5	146.5	65.6	66.4	67.0	168.9	165.5	58.9		26.2	27.8	55.6	56.7	12
13	22.0	22.7	168.3	123.2	124.4	158.2	186.4	195.0	23.9	22.3	143.9	178.2		14.3	12.0	9.5	13
14	121.4	120.9	127.4	3.5	4.4	143.1	140.9	176.3	120.9	120.9	136.6	143.1	123.2		17.1	15.0	14
15	154.5	146.5	89.3	176.6	158.8	33.8	83.0	103.7	135.6	135.5	22.0	64.0	174.4	168.3		42.6	15
16	164.4	170.0	82.2	178.1	178.1	76.9	70.5	81.4	160.7	160.7	76.2	59.5	184.4	173.4	75.5		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 *Bombyx mori* AAW66633
2 *Anopheles gambiae str. PEST* EAA44286
3 *Branchiostoma belcheri* BAA96549
4 *Chlamys nipponensis akazara* Q7M3Y3
5 *Chlamys nipponensis* BAE43658
6 *Ciona intestinalis* AAD09271
7 *Clupea harengus* AAB05825
8 *Coturnix coturnix* A41030
9 *Drosophila melanogaster* A38594
10 *Drosophila virilis* AAR24602
11 *Halocynthia roretzi* BAA19425
12 *Homo sapiens* AAH12601
13 *lethocerus indicus* CAF18234
14 *Mizuhopecten yessoensis* BAA22853
15 *Polyandrocarpa misakiensis* BAB83806
16 *Sus scrofa* NP_001027530

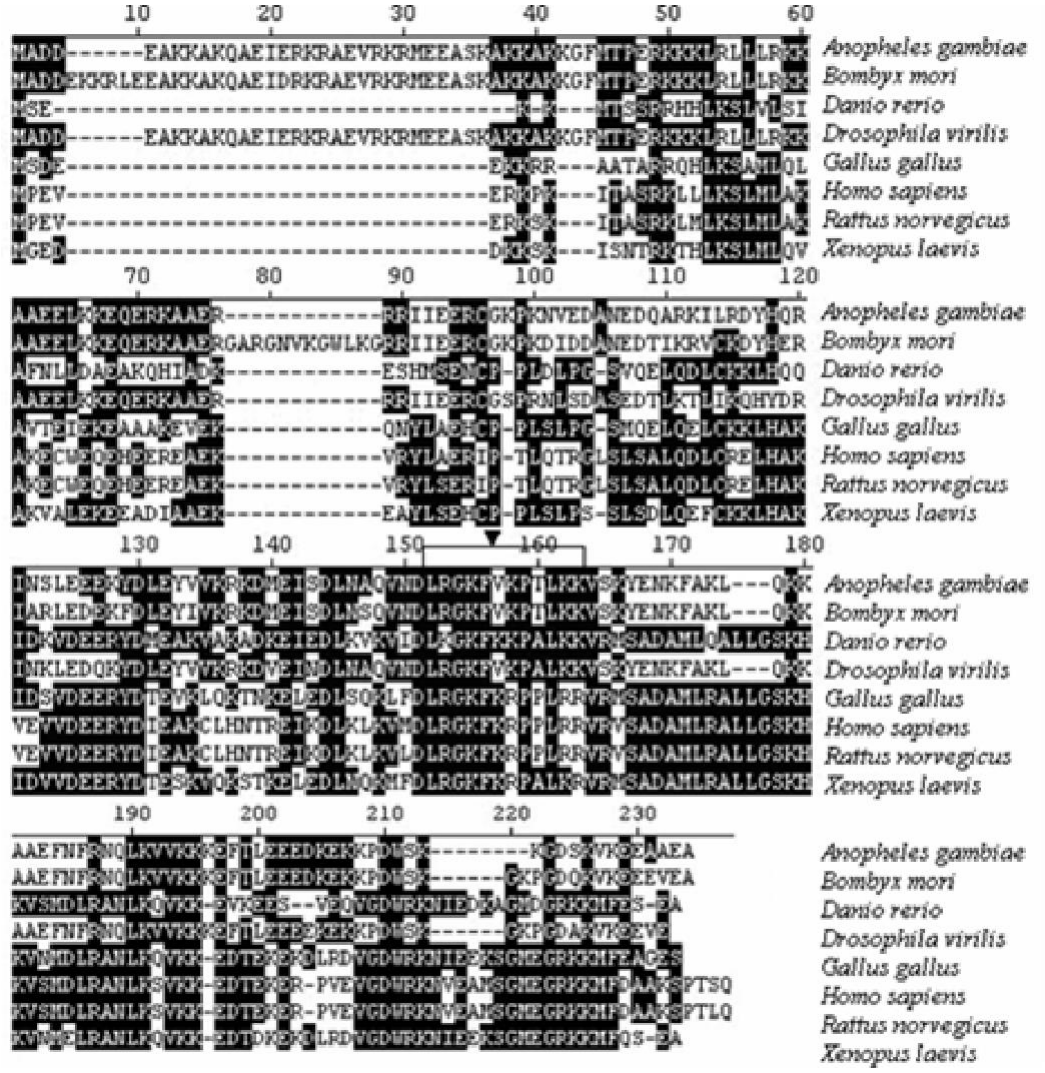

Figure 2. Comparison of deduced amino acid sequences of Tpn I in 8 species. The actin binding area is indicated with single arrowhead. Residues same to *B. mori* were in black shading.

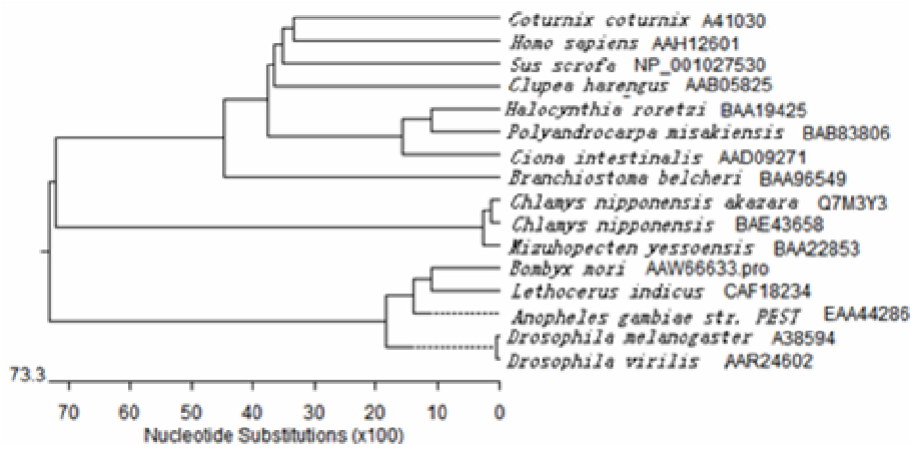

Figure 3. Phylogenetic tree of Tpn I among 16 different species. The resulting tree was divided into two main branches.

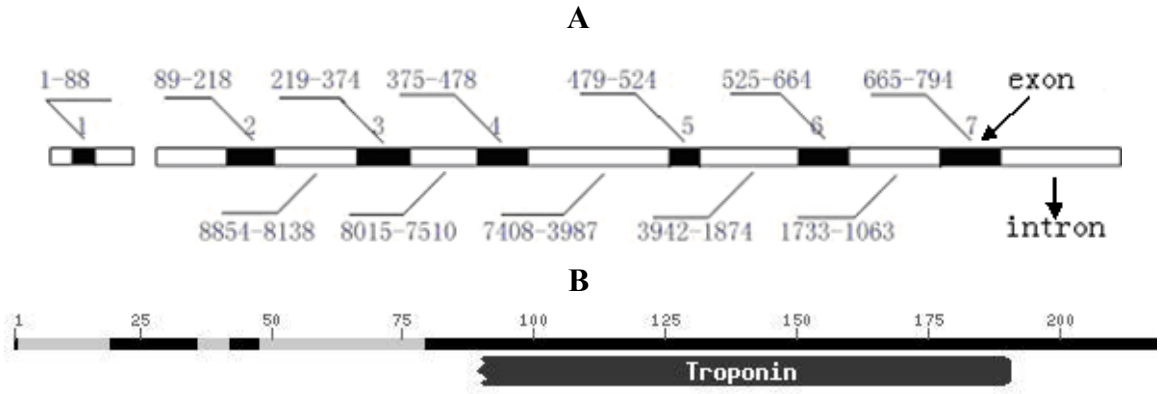

Figure 4. A. *Bombyx mori* Tpn I gene coding (exon) and noncoding (intron) regions. **B.** Troponin motif in Tpn 1.

in in all 8 organisms we studied is as follows: D -L-R-G-K-F-R^+-R*-P-X-L- R*-R*-V, where R^+ stands for V/K, R* stands for R/K, and X for any amino acid sequence (Margaret et al., 1989). Tpn I possesses two Ca^{2+} dependent interactive sites for troponin C; one partly overlaps the actin binding domain and highly conserved, and the other corresponding to the 30-residue-long segment following the N-terminal extension is poorly conserved in different organisms. Tpn I also interacts with Troponin T. The consensus sequence for the interacting site is as follows: h-D-X-R^+-Y-D-h-E-h, where h stands for a hydrophobic residue, D for Asp/Glu, R^+ for Arg/Lys, and X for any residue. We have found this domain in the Bm- Tpn I, and its evolutionary conservation suggests that this domain is involved in protein-protein interaction.

The analysis of the Tpn I amino acid sequence in *B. mori* with the SignalP program at the website (www.expasy.org) did not show any deduced signal peptide clea--vage site in the N terminal, which means that the protein was not a secretary protein. A single *Drosophila* Tpn I gene described and studied by Ferrus group (Barbas et al., 1991; Barbas et al., 1993) contains a total number of 13 exons and is able to yield 10 different isoforms by alternative splicing processes. According to our study, we found Bm-Tpn I contains at least 7 exons. But whether there is any post-transcriptional modification of Bm-Tpn I need further study.

The polygenetic analysis of Tpn I was performed following the method of Clustal W, based on the amino acid sequence. The resulting tree (Figure 3) was divided into two main branches. One corresponds to the insect group, including *A. gambiae, B. mori,* and *D. virilis.* And the other one corresponds to the other species, including *D. rerio*, *G. gallus*, *R. norvegicus* , *H. sapiensand* and *X. laevis.* According to the tree, the phylogenic relationship between *A. gambiae* and *B. mori* is closer than it between *B. mori* and *D. virilis.*

The full-length cDNA of Bm-Tpn I was blast with the silkworm genome sequence at the website (http://silkworm.genomics.org.cn/jsp/tools.jsp).The results shows that the Bm-Tpn I gene was consisted of seven exons (Figure 4). Further research work such as expression, catalyzing activity and mutagenesis of this gene are under our consideration.

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REFERENCES

Barbas JA, J Galceran (1993). Abnormal muscle development in the heldup3 mutant of Drosophila melanogaster is caused by asplicing defect affecting selected troponin I isoforms. Mol. Cell Biol. 13:1433-1439.

Barbas JA, J Galceran, I Krah-Jentgens, JL de la Pompa, I Canal, O Pongs, A Ferrus (1991): Tpn I is encoded in the haplolethal region of the Shaker gene complex of *Drosophila*. Genes Dev. 5: 132-140.

Barbas JA, J Galceran, L Torroja, A Prado, A Ferrus (1993) .Abnormal muscle development in the heldup3 mutant of *Drosophila melanogaster* is caused by a splicing defect affecting selected Tpn I isoforms. Mol Cell Biol. 13: 1433-1439.

Cullen ME, KA Dellow, PJ Barton (2004). Structure and regulation of human troponin genes. Mol. Cell Biochem. 263: 81-90.

Filatov VL, AG Katrukha, TV Bulargina, NB Gusev (1999). Troponin: structure, properties, and mechanism of functioning. Biochem. Mosc. (64): 969-985.

Gordon AM, E Homsher, M Regnier (2000). Regulation of contraction in striated muscle. Physiol. Rev. 80: 853-924.

Gordon AM, M. Regnier, E Homsher (2001). Skeletal and cardiac muscle contractile activation: tropomyosin "rocks and rolls". News Physiol. Sci. 16: 49-55.

Henrikson CA, N Chandra-Strobos (2004). Troponin and outcomes. J. Am. Coll. Cardiol. 44: 1933-1934.

Kedar V, H McDonough (2004). Muscle-specific RING finger 1 is a bona fide ubiquitin ligase that degrades cardiac troponin I. Proc. Natl. Acad. Sci. USA. 101: 18135-18140.

Klein, SL, RL. Strausberg et al. (2002). Genetic and genomic tools for Xenopus research: The NIH Xenopus initiative. Dev. Dyn. 225: 384-91.

Margaret VW, RB Andrea (1989). Amino Acid Sequence of Crayfish Tpn I. J. Biol. Chem. 264: 1551-1557.

Murakami K, Yumoto F et al. (2005). Structural basis for Ca2+-regulated muscle relaxation at interaction sites of troponin with actin and tropomyosin. J. Mol. Biol. 352: 178-201.

Reinach FC, CS Farah, PB Monteiro, B Malnic (1997). Structural interactions responsible for the assembly of the troponin complex on the muscle thin filament. Cell Struct Funct. 22: 219-223.

Strausberg RL et al. (2002). Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc. Natl. Acad. Sci. USA 99: 16899-16903

Vassylyev DG, S Takeda, S Wakatsuki, K Maeda, Y Maeda (1998). The crystal structure of troponin C in complex with N-terminal fragment of Tpn I. The mechanism of how the inhibitory action of Tpn I is released by Ca (2+)-binding to troponin C. Adv. Exp. Med. Biol. 453: 157-167.