BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 200–205 (1996)
ARTICLE NO. 1333

Cloning of Chicken Slow Muscle Troponin T and Its Sequence Comparison with That of Human

Izuru Yonemura, Tsuyoshi Watanabe, Masashi Kirinoki,*
Jun-Ichi Miyazaki, and Tamio Hirabayashi[1]

*Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan; and *Department of Medical Zoology, School of Medicine, Dokkyo University, Shimotsuga, Tochigi 321-02, Japan*

Received July 24, 1996

A full-length cDNA coding for chicken slow muscle troponin T (TnT) was for the first time isolated from a cDNA library of 10-day-old embryos, using an RT-PCR product of chicken slow muscle TnT. It showed about 60% homology for chicken fast and slow muscle TnTs and 75.2% for human slow muscle TnT. The 16 amino acid sequence found in the carboxyl terminus of human slow muscle TnT was absent in the chicken slow muscle TnT. The 5E-5A-7E sequence found in the amino terminal region of chicken slow muscle TnT was partly similar to the counterpart of human slow muscle TnT, but not to those of chicken fast and cardiac muscle TnTs. With this report of chicken slow muscle TnT, cDNA information on chicken TnTs of all three striated muscles was completed following those of human TnTs.

Troponin is a regulatory protein of muscle contraction and composed of three components, troponin T (TnT), troponin I, and troponin C. Of the components, TnT is popularly known for its variation of isoform composition in skeletal (1-8) and cardiac (9-12) muscles. Especially, chicken fast skeletal muscle was well studied and found to express at least 4 kinds of mRNAs which cause many kinds of isoform although the functional significance could not be addressed yet. On the other hand, chicken slow skeletal muscle does not attract much concern, probably for the poor variation of TnT isoforms; the muscle expresses only two isoforms of different pIs (7). As for cDNA analysis of slow muscle TnT, Gahlmann *et al.* (13) reported two kinds of variants for human muscle.

In chicken muscle, cDNAs of fast (4) and cardiac (10) muscle TnTs were isolated and studied with precise description of exons, while no information about the cDNA of chicken slow muscle TnT was so far reported. In order to fill up the lack of information about chicken muscle TnTs, we carried the isolation of chicken slow muscle TnT cDNA to compare the sequences among three different muscles and find some clues to study differential expression of TnT isoforms.

MATERIALS AND METHODS

Materials. White leghorn chickens (*Gallus domesticus* (L)) were obtained from commercial sources.

Protein sequencing. Two-dimensional SDS-polyacrylamide gel electrophoresis (2D SDS-PAGE) was carried out according to the method of Hirabayashi (14). Slow muscle TnT spots of *anterior latissimus dorsi* (ALD) (7) were cut out from 2D SDS-PAGE gels stained with Coomassie Brilliant Blue R-250 (Sigma). The protein was digested with *Staphylococcus aureus* V8 protease (ICN ImmunoBiologicals) according to the Cleveland's method (15,16). The

The nucleotide sequence data reported in this article have been deposited with the DDBJ, EMBL, and GenBank Databases under Accession No. D85105.

[1] To whom correspondence should be addressed. Fax: +81–298–53–6649.

0006-291X/96 $18.00

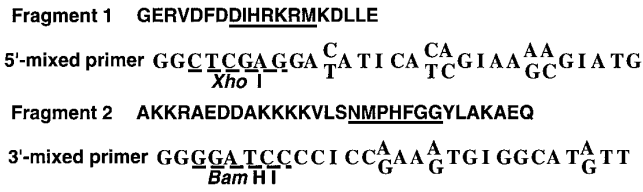

FIG. 1. Partial amino acid sequences of chicken slow muscle TnT and 29-mer and 28-mer oligonucleotide sequences. Fragments 1 and 2 were composed of 19 and 30 residues, respectively. 5′- and 3′-mixed primers which were designed based on the underlined amino acid sequences of the fragments are shown with recognition sites (dashed underlines) of *Xho*I and *Bam*HI.

fragments were separated by SDS-PAGE with 18% polyacrylamide gels and electroblotted onto a PVDF membrane (Pad). Major bands were cut out from the membrane and sequenced by ABI 477A Protein Sequencer.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA preparation was performed by using Isogen (Nippon Gene) following its protocol. Reverse transcription was performed using 10 μg of total RNA and 0.5 μg of oligo $(dT)_{12\text{-}18}$ at 37°C for 60 min with Superscript II (Gibco BRL). The reverse primer and forward primer are shown in Fig. 1. Thirty cycles of PCR were performed at 94°C for 1 min, at 37°C for 2 min, and at 72°C for 1 min with Taq DNA polymerase (Gibco BRL). Products were cloned in pBluescript II KS^+ plasmid and sequenced.

Cloning of chicken slow muscle TnT cDNAs and their sequencing. cDNA clones encoding chicken slow muscle TnT were isolated by screening a commercially available chicken cDNA library (whole tissues of 10-day old embryos, Stratagene) constructed in lambda gt11 with the RT-PCR product. Hybridization was carried out at 42°C for overnight in 6×SSC (1×SSC, 8.765 g of NaCl and 4.41 g of sodium citrate in 1 *l* of H_2O), 1% SDS, 5×Denhardt's reagent (1×Denhardt's reagent, 0.2 g of Ficoll, 0.2 g of polyvinylpyrrolidone, and 0.2 g of bovine serum albumin in 1 *l* of H_2O), 20 μg/ml ssDNA, and 50% formamide with [^{32}P] dATP-labeled probe. Filters were washed at 65°C in 2×SSC and 1% SDS. Positive clones were plaque-purified and cDNA inserts were subcloned into pBluescript II KS^+ plasmids. 5′-end amplification of cDNA (5′-RACE) method was performed according to the improved method of Frohman *et al.* (17). The gene-specific amplification primer was 3′-mixed primer (Fig. 1). Forty cycles of PCR were performed at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min with Taq DNA polymerase. PCR products were cloned in pBluescript II KS^+ plasmids. Nucleotide sequence analysis was carried out according to the method of ABI 373A DNA Sequencing System Protocol.

Northern blot analysis. Northern blotting was performed using 10 μg of total RNA electrophoresed on an agarose-formaldehyde gel and transferred to a nylon membrane. Hybridization was carried out at 42°C for overnight in 6×SSC, 1% SDS, 5×Denhardt's reagent, 20 μg/ml ssDNA, and 50% formamide with [^{32}P]dATP-labeled cDNA fragments (Fig. 2). Filters were washed at 65°C in 2×SSC and 1% SDS.

RESULTS AND DISCUSSION

In order to get amino acid sequence information, we cut out troponin T spots from gels of ALD extract separated by 2D SDS-PAGE. The spot identification was carried out according to Yao *et al.* (7) in which they used a specific anti-chicken slow muscle troponin

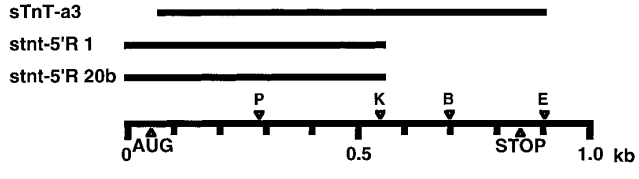

FIG. 2. cDNAs and restriction endonuclease cleavage map of the slow muscle TnT mRNA. The bottom line represents slow muscle TnT mRNA with translation initiation and termination sites. The heavy lines above this are three cDNAs, sTnT-a3, stnt-5′R1, and stnt-5′R20b, which include 5′- and 3′-untranslated regions and open reading frame. The sTnT-a3 was used as a probe for RNA hybridization experiment. Restriction endonuclease cleavage sites : P, *Pst*I; K, *Kpn*I; B, *Bam*HI; E, *Eco*RI.

```
        10         20         30         40         50         60         70
GAGCCAACAGGACCGACCCCACCGGACCCCCCCGACCCGCAGCCATGTCCGAAGCTGAGGAGGAATACGA
                                                  M  S  E  A  E  E  E  Y  E

        80         90        100        110        120        130        140
GGAGGAGCAGCCCGAAGAGGAGGAGGAGGCGGCGGCGGCGGCGGAGGAGGAGGAGGAAGAGGAAGCAGAG
 E  E  Q  P  E  E  E  E  E  A  A  A  A  A  E  E  E  E  E  E  E  E  A  E

       150        160        170        180        190        200        210
GCTTCCAAACCTCATGAGGAGCCAGAAGAGGAGCGGCCTCGGCCTCGGCCTGTGGTGCCCCAGCTGGCCC
A  S  K  P  H  E  E  P  E  E  E  R  P  R  P  R  P  V  V  P  Q  L  A  P

       220        230        240        250        260        270        280
CCCCCAAGATCCCCGAGGGGGAGCGCGTGGACTTCGATGACATCCACCGGAAGCGCATGGAGAAGGACCT
  P  K  I  P  E  G  E  R  V  D  F  D  D  I  H  R  K  R  M  E  K  D  L

       290        300        310        320        330        340        350
GCTGGAGCTGCAGACCCTCATTGACGCCCACTTCGAGCAGCGGCGCCGCGAGGAGAACGAGCTGGTGGCA
 L  E  L  Q  T  L  I  D  A  H  F  E  Q  R  R  R  E  E  N  E  L  V  A

       360        370        380        390        400        410        420
CTGATGGAGCGCATTGAACGGCGCCGGGCGGAGCGCAACGAGCAGCTGCGGAGCCGCACGGAGAAGGAGC
L  M  E  R  I  E  R  R  R  A  E  R  N  E  Q  L  R  S  R  T  E  K  E  R

       430        440        450        460        470        480        490
GCGAGCGGCAAGCAAGGCTGGCAGAGGAGAAGCTCCGCAAGGAGGAGGAGGAGGCCAAGAAGCGAGCCGA
  E  R  Q  A  R  L  A  E  E  K  L  R  K  E  E  E  E  A  K  K  R  A  E

       500        510        520        530        540        550        560
GGATGACGCCAAGAAGAAGAAAGTCCTGTCCAACATGCCCCACTTCGGGGGGTACCTGGCCAAGGCGGAG
 D  D  A  K  K  K  K  V  L  S  N  M  P  H  F  G  G  Y  L  A  K  A  E

       570        580        590        600        610        620        630
CAGCGGCGTGGGAAGCGGCAGACGGGGCGGGAGATGAAGCTGCGCATCCTGGCTGAGCGCAAGAAGCCCC
Q  R  R  G  K  R  Q  T  G  R  E  M  K  L  R  I  L  A  E  R  K  K  P  L

       640        650        660        670        680        690        700
TCCACATTGAGCACATGCGGGAGGATGAGCTGCGGGCCAAGGCCAAGGAGCTGCACGACTGGATCCAGCA
  H  I  E  H  M  R  E  D  E  L  R  A  K  A  K  E  L  H  D  W  I  Q  Q

       710        720        730        740        750        760        770
GCTGGAGTCGGAGAAGTTCGACCTGATGGAGAAGCTGCGGCGCCAGAAATACGAGATCAACGTTCTGTAC
 L  E  S  E  K  F  D  L  M  E  K  L  R  R  Q  K  Y  E  I  N  V  L  Y

       780        790        800        810        820        830        840
AACCGCATCAGCCATGCCCAGAAATTCAAGAAGGTGGTGGGCAAAGGCCGCGTGGGGGGGCGCTGGAAGT
N  R  I  S  H  A  Q  K  F  K  K  V  V  G  K  G  R  V  G  G  R  W  K  *

       850        860        870        880        890        900
GACCCCTCCCCCCGCCCGGTGCTGCTGCGGCCCAATAAAGCTCTGCTCGTCCGCTCCG
**
```

FIG. 3. Complete nucleotide sequence of chicken slow muscle TnT cDNA and the deduced amino acid sequence. A stop codon, TGA, is marked with asterisks. The polyadenylation signal, AATAAA, is underlined.

T serum for the identification. After Cleveland's digestion and separation of TnT spots, three major bands were found and their partial amino acid sequences were analyzed. Two of the three showed the same sequence. Their partial amino acid sequences, 19 residues (fragment 1) and 30 residues (fragment 2), were determined as shown in Fig. 1. Based on these amino acid sequences, two mixed primers were designed and used to generate cDNAs by RT-PCR. Amplification of total RNA isolated from *gastrocnemius* of 1-day old chicks generated cDNA products of approximately 300 nucleotides (nt) (data not shown). Using one of the products as a probe, cDNAs of slow muscle TnT were isolated from a chicken cDNA library and one of the cDNA was designated sTnT-a3 (Fig. 2) which had the polyadenylation signal, AATAAA, but lacked the translation initiation site, ATG, unfortunately. To compensate the lack of information we performed 5′-RACE method and isolated 2 clones, stnt-5′R1 and stnt-5′R20b (Fig. 2), which were revealed to contain the translation initiation site and 5′-untranslated region. Nucleotide sequences from the two clones were the same except three bases (bases 135 ~ 137 in Fig. 3), that is, these bases were absent

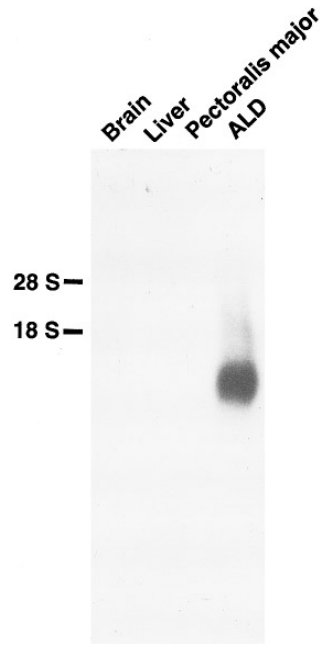

FIG. 4. Specific expression of slow muscle TnT mRNA in ALD, demonstrated by Northern blot hybridization with sTnT-a3. 10μg of total RNAs from adult chicken brain, liver, pectoralis major, and ALD were separated on an agarose-formaldehyde gel and transferred onto a nylon membrane. Chicken slow muscle TnT mRNA is specifically present in the RNA from ALD.

in stnt-5′R1. The absence of three bases may not cause one of the two spots of different pIs in the 2D SDS-PAGE pattern of ALD (7), since the absence of alanine can not cause any pI shift. With sTnT-a3 and stnt-5′R20b, the nucleotide sequence of slow muscle TnT was completed and the deduced amino acid sequence is shown in Fig. 3. The full length of the slow muscle TnT cDNA is 898 nt, and codes for 265 amino acids.

Northern blotting of the total RNAs from adult brain, liver, pectoralis major, and ALD showed specific expression of slow muscle TnT gene only in ALD with no signal for the others (Fig. 4).

On comparison of amino acid sequences among chicken slow muscle, human slow muscle (13), chicken fast muscle (4), and chicken cardiac muscle (10) TnTs, the highest homology, 75.2%, was found between chicken and human slow muscle TnTs. When the sequence found in only human slow muscle (in brackets of Fig. 5) is excluded in the calculation, the homology rises up to 80.2%, while it is around 60% between chicken slow muscle TnT and fast muscle or cardiac muscle TnT. This shows, as in other examples (18,19), that homology is higher between homologous organs from differents species than between different organs of the same species. The sequence in brackets of Fig. 5 could be found neither in human fast muscle (8) and cardiac muscle (12) TnTs nor in chicken slow muscle TnT, for which we have tried RT-PCR five times to detect variant(s) with the sequence using total RNA isolated from adult ALD, young and adult gastrocnemius muscles. Therefore, the sequence seems to be specific to one variant of human slow muscle TnT and not to be shared with other slow muscle TnTs, although the number of slow muscle TnT investigated so far is limited.

In chicken slow muscle TnT, a seemingly novel 5E-5A-7E sequence (underlined in Fig. 5) is in the amino terminal region. This sequence is absent in other chicken TnTs, and seems to

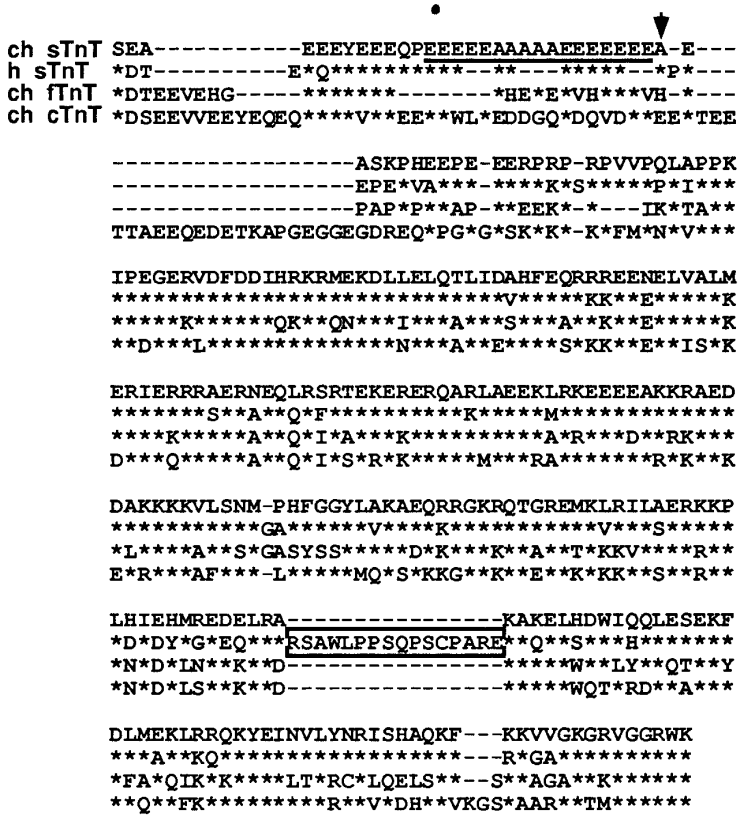

FIG. 5. Comparison of the amino acid sequence of chicken slow muscle TnT (ch sTnT) with those of human slow muscle (h sTnT : H22h) (13), chicken fast muscle (ch fTnT : TNT-3) (4), and chicken cardiac muscle (ch cTnT : form I) (10) TnTs. The amino-termini of human slow, chicken fast, and chicken cardiac muscle TnTs start with S-D-, while only chicken slow muscle TnT starts with S-E-. The counterpart of the exon(s) encoding 16 amino acids (in brackets) inserted in the carboxyl-terminus of human slow muscle TnT is not detected in any of chicken TnTs (see text). The amino acid marked with an arrow was absent in stnt-5′R1. The 17 amino acid sequence underlined in the amino-terminal of chicken slow muscle TnT seems to be an unusual sequence, although the counterpart in human TnT is similar to some extent. Identical amino acids to those in chicken slow muscle TnT are shown as asterisks (*). Deletions introduced to maximize homologies are shown as dashed lines (−).

be restricted to chicken slow muscle TnT. But, close inspection of Fig. 5 reveals that this sequence has some similarity to the counterpart of human slow muscle sequence and dissimilarity to those of chicken fast muscle and cardiac muscle sequences. The significance of the repeated sequence is to be studied, and might reflect some function specific to slow muscle, since it follows a 7 residue sequence (residues 4∼10 from the amino acid terminus of chicken slow muscle TnT) of high homology among the 4 kinds of TnTs, although it is in the region out of the functional domain (20,21).

Isolation of chicken slow muscle TnT is considered to be significant in studying the mechanism of tissue specific expression of TnT genes. In fact, chickens are the second material in which cDNAs of all three striated muscle TnTs were isolated. But, unlike human material, chickens are much popular in developmental study of muscle differentiation, and with the addition of this information on chicken slow muscle cDNA, it became possible to carry a comparative study on the troponin T expression mechanism in three striated muscles along

developmental processes. We have now isolated the 5′ promoter region of the chicken slow muscle TnT, and are investigating its regulatory mechanism.

ACKNOWLEDGMENT

We thank Miss Mariko Katoh for helpful discussion along the course of this work.

REFERENCES

1. Medford, R. M., Nguyen, H. T., Destree, A. T., Summers, E., and Nadal-Ginard, B. (1984) *Cell* **38,** 409–421.
2. Abe, H., Komiya, T., and Obinata, T. (1986) *Dev. Biol.* **118,** 42–51.
3. Breibart, R. E., and Nadal-Ginard, B. (1987) *Cell* **49,** 793–803.
4. Smillie, L. B., Golosinska, K., and Reinach, F. C. (1988) *J. Biol. Chem.* **263,** 18816–18820.
5. Nakamura, M., Imai, H., and Hirabayashi, T. (1989) *Dev. Biol.* **132,** 389–397.
6. Bucher, E. A., Charles de la Brousse, F., and Emerson, C. P., Jr. (1989) *J. Biol. Chem.* **264,** 12482–12491.
7. Yao, Y., Nakamura, M., Miyazaki, J.-I., Kirinoki, M., and Hirabayashi, T. (1992) *Dev. Biol.* **151,** 531–540.
8. Wu, Q.-L., Jha, P. K., Raychowdhury, M. K., Du, Y., Leavis, P. C., and Sarkar, S. (1994) *DNA Cell Biol.* **13,** 217–233.
9. Cooper, T. A., and Ordahl, C. P. (1984) *Science* **226,** 979–982.
10. Cooper, T. A., and Ordahl, C. P. (1985) *J. Biol. Chem.* **260,** 11140–11148.
11. Cooper, T. A. (1992) *J. Biol. Chem.* **267,** 5330–5338.
12. Mesnard, L., Samson, F., Espinasse, I., Durand, J., Neveux, J.-Y., and Mercadier, J.-J. (1993) *FEBS Lett.* **328,** 139–144.
13. Gahlmann, R., Troutt, A. B., Wade, R. P., Gunning, P., and Kedes, L. (1987) *J. Biol. Chem.* **262,** 16122–16126.
14. Hirabayashi, T. (1981) *Anal. Biochem.* **117,** 443–451.
15. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
16. Fujiwara, Y., Hatano, K., Hirabayashi, T., and Miyazaki, J.-I. (1994) *Differentiation* **56,** 13–20.
17. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
18. Ausoni, S., de Nardi, C., Moretti, P., Gorza, L., and Schiaffino, S. (1991) *Development* **112,** 1041–1051.
19. Hastings, K. E. M., Koppe, R. I., Marmor, E., Bader, D., Shimada, Y., and Toyota, N. (1991) *J. Biol. Chem.* **266,** 19659–19665.
20. Hitchcock, S. E., and Zimmerman, C. J. (1981) *J. Mol. Biol.* **147,** 125–151.
21. Mak, A. S., and Smillie, L. B. (1981) *J. Mol. Biol.* **149,** 541–550.