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Original Article

Expression of troponin C genes during development in the chicken

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ABSTRACT Expression of cardiac troponin C (C/STnC) and fast skeletal muscle troponin C (FTnC) genes during development was analyzed by *in situ* hybridization and Northern blot. At Hamburger-Hamilton stage 14, FTnC mRNA was detected in somites. At stage 20, FTnC mRNA was identified in truncus arteriosus. FTnC mRNA was undetectable in embryonic ventricle at the other developmental stages. This suggested that transcription of FTnC mRNA at stage 20 was stage and region-specific. At early stage 10, C/STnC mRNA was detected in truncus arteriosus, ventricle and vitelline veins at the proximal region where right and left veins bifurcate from ventricle. In the proximal region of vitelline vein, C/STnC mRNA was transcribed in both the rostral and caudal walls of the veins. The area of C/STnC gene expression in vitelline vein is wider than those of α-cardiac and smooth muscle actin genes (Ruzicka and Schwartz, *J. Cell Biol. 107:* 2575-2586, 1988). It is suggested that C/STnC plays an important role relating embryonic excitation-contraction-coupling in the wider area including vitelline vein. C/STnC mRNA was identified also in somites, embryonic breast muscle and adult breast muscle. Since C/STnC protein was undetectable in adult breast muscle by immunofluorescence microscopy (Toyota and Shimada, *J. Cell Biol. 91:* 497-504, 1981), the imbalance of C/STnC mRNA and protein levels suggested the operation of specific mechanisms that separately regulated the accumulation of C/STnC mRNA and protein.

KEY WORDS: *Ca binding protein, post-transcriptional control, embryo*

Introduction

Troponin C (TnC) is a Ca^{2+} binding component of the troponin complex that locates on the thin filament in striated muscles (Hanson *et al.*, 1973; Ohtsuki, 1975) and modulates their contraction in association with other troponin components (T and I) and tropomyosin (Ebashi *et al.*, 1971; Weber and Murray, 1973). Two different TnC isoforms have been identified in both birds and mammals by the studies of peptide maps and amino acid sequences (van Eard and Takahashi, 1976; Wilkinson, 1980). The analyses suggest that TnCs of cardiac and slow skeletal muscles (C/STnC) are derived from a single gene which is different from the gene of the fast skeletal muscle (FTnC). Structural studies have revealed that TnC is composed of four domains (I-IV), each of which consists of side chains of two helical regions and a Ca^{2+} binding loop (Collins *et al.*, 1973). FTnC possesses two high affinity Mg^{2+}/Ca^{2+} binding sites (domains III and IV) and two Ca^{2+}-specific binding regions (domains I and II) (Holroyde *et al.*, 1980). In contrast, C/STnC has a number of amino acid replacements in domain I and has lost one site with specific ability to bind Ca^{2+} (van Eard and Takahashi, 1976). Variation in the Ca^{2+} binding ability is thought to be important in the regulation of muscle contraction and tension control (Babu *et al.*, 1987).

TnC isoforms corresponding to C/STnC and FTnC have also been identified on the basis of immunological criteria (Dhoot and Perry, 1980; Toyota and Shimada, 1981). Studies with specific antibodies against C/STnC and FTnC (anti-C/STnC and -FTnC, respectively) have shown that in ventricular muscle, only C/STnC is expressed from early stages of cardiac muscle development. In contrast, in skeletal muscle, both C/STnC and FTnC isoforms are identified in somites. With C/STnC decreasing gradually during development, FTnC becomes predominant in the adult fast skeletal muscle (Dhoot and Perry, 1980; Toyota and Shimada, 1981).

cDNAs encoding the amino acid sequences for C/STnC and FTnC have been isolated from chicken (Putkey *et al.*, 1987; Reinach and Karlsson, 1988; Toyota *et al.*, 1989), rabbit (Zot *et al.*, 1987), human (Gahlman *et al.*, 1988) and murine muscles (Parmacek and Leiden, 1989; Parmacek *et al.*, 1990). DNA sequence analysis has shown that C/STnC cDNA has varying homologies with that of FTnC

Abbreviations used in this paper: TnC, troponin C; C/STnC, cardiac and slow skeletal muscle troponin C; FTnC, fast skeletal muscle troponin C; TnT, troponin T; TnI, troponin I; anti-C/STnC, specific antibody against cardiac troponin C; anti-FTnC, specific antibody against fast skeletal muscle troponin C; UTR, untranslated region; ALD, anterior latissimus dorsi; PLD, posterior latissimus dorsi; CaM, calmodulin.

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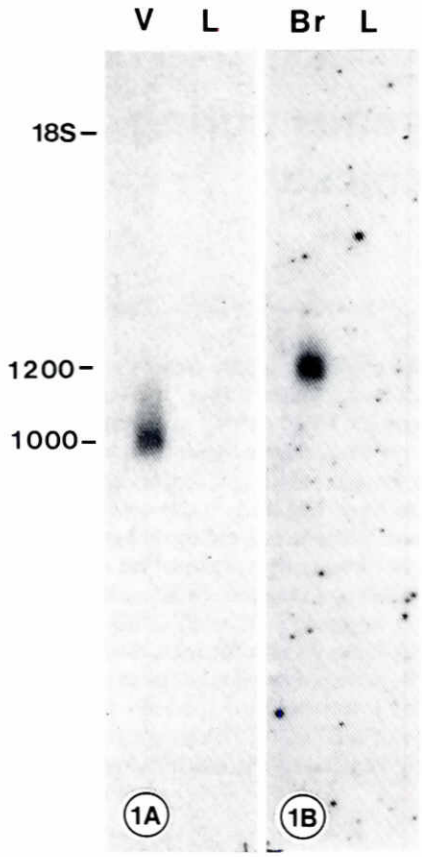


Fig. 1. Northern blot of RNAs from adult ventricular and breast muscles. *Total RNAs from adult ventricle (V), breast muscle (Br) and liver (L) were electrophoresed simultaneously in a large size agarose gel and hybridized with C/STnC-UTR* **(A)** *and FTnC-UTR* **(B)**.

(Toyota *et al.*, 1989). The coding region of the Ca^{2+} binding domains II-IV in C/STnC has a strong sequence conservation, whereas the 5', 3'-untranslated region (UTR) and domain I have a significant sequence divergence (Toyota *et al.*, 1989).

Expression of TnC isoforms was studied immunohistochemically and TnC cDNAs were cloned from various animals. However, little is known about expression of TnC gene in cardiac and skeletal muscles at early stages of development. In the present paper, the TnC gene transcripts were studied in chicken embryos during development by *in situ* hybridization and Northern blot. The results showed that C/STnC mRNA was transcribed in adult breast muscle and that FTnC mRNA was expressed in ventricular muscle at specific stages. These patterns of TnC gene expression at the RNA level were different from those at the protein level studied by immunohistochemical microscopy.

Results

In order to examine the specificity of C/STnC- and FTnC-UTR probes, Northern blot was performed with a large size agarose gel with the total RNAs from adult ventricle, breast muscle and liver. C/STnC-UTR showed a hybridizing band corresponding to an RNA size of approximately 10^3 nucleotides in ventricular muscle (Fig. 1A). When the RNA from breast muscle was probed with FTnC-UTR, the FTnC hybridization band was of a larger molecular size than that for C/STnC, corresponding to approximately $1.2x10^3$ nucleotides (Fig. 1B). This showed that the size of C/STnC mRNA is different from that of FTnC mRNAs and that C/STnC- and FTnC-UTRs recognize different mRNA species.

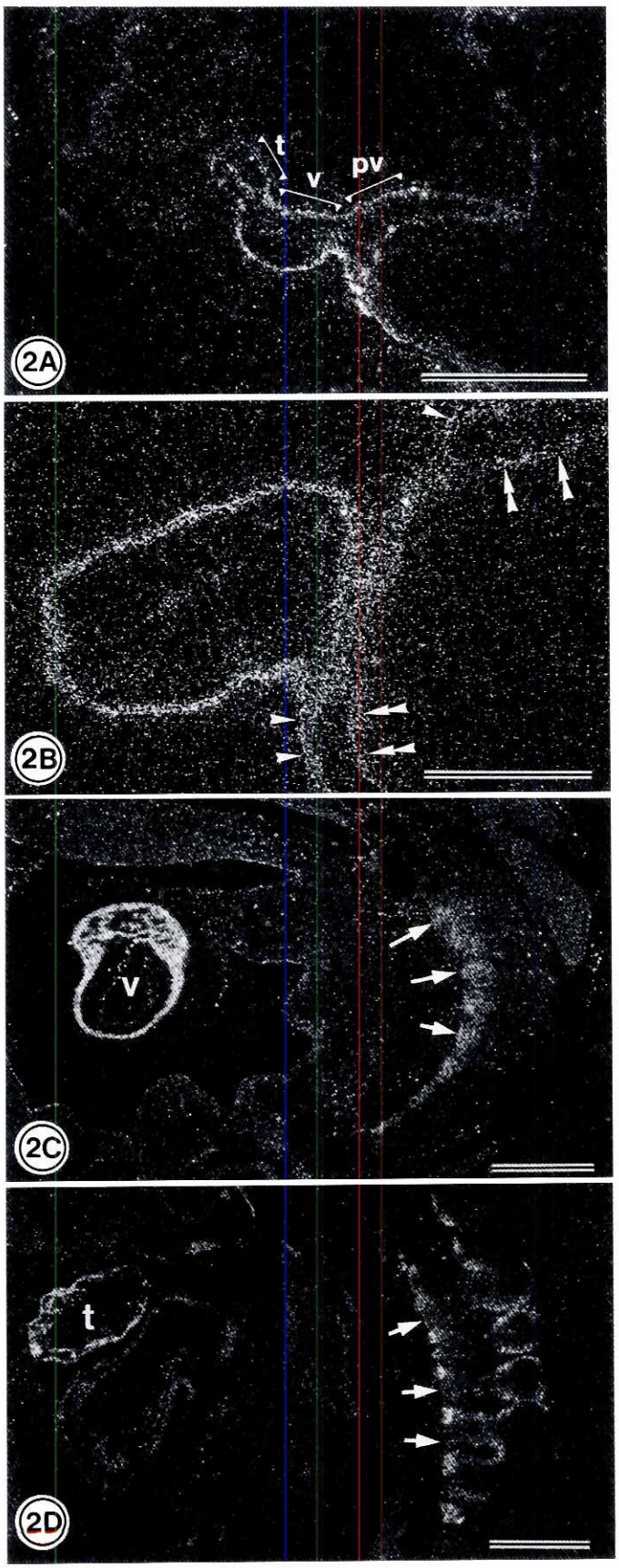
With these probes, expression of C/STnC and FTnC genes at the early stage of cardiac and skeletal muscle development was examined by *in situ* hybridization. At the earliest stage examined (stage 10; Hamburger and Hamilton, 1951), when ventricle began to beat, C/STnC mRNA was found in truncus arteriosus, ventricle and vitelline vein (Fig. 2A). At this stage, C/STnC-UTR hybridized in vitelline vein at the proximal region where the right and left veins branch from ventricle (Fig. 2A,B). C/STnC mRNA was identified in both rostral and caudal walls of vitelline veins (Fig. 2B). C/STnC mRNA was detected in somites as well as ventricle and truncus arteriosus from stage 14 (Fig. 2C). FTnC mRNA was detected in somites from stage 14 (not shown). FTnC mRNA was identified also in truncus arteriosus as well as somites at stage 20 (Fig. 2D).

Expression of TnC genes of cardiac and breast muscles at subsequent developmental stages was examined with C/TnC- and FTnC-UTRs by Northern blots. C/STnC mRNA was readily identified in cardiac and breast muscles in embryos after 10 days and adults (Fig. 3A, 1-10). The level of the C/STnC mRNA accumulation increased in these embryonic muscles during development. In ventricular muscles, C/STnC mRNA levels in 10-, 13-, 16- and 19-day embryos were approximately 42, 48, 69 and 71% (SD<±5%) that of adult ventricular muscle, respectively. In breast muscle, the levels of C/STnC mRNA in embryos at 10, 13, 16 and 19 days and in adults were approximately 22, 36, 38, 39 and 91% (SD<±5%) that of adult ventricular muscle, respectively. A hybridizing band with FTnC-UTR was found in breast muscle from 10-day embryos to adults (Fig. 3B, 6-10), while the band was undetectable in embryonic and adult cardiac muscles (Fig. 3B, 1-5).

Expression of TnC mRNAs in adult various tissues were examined by Northern blot. A hybridization signal of C/STnC mRNA was identified in RNAs of adult ventricular, breast, ALD (anterior latissimus dorsi) and PLD (posterior latissimus dorsi) muscles (Fig. 4A, 1-4). In contrast, a signal of FTnC mRNA was found only in breast and PLD muscles (Fig. 4B, 2,4). C/STnC and FTnC mRNAs were not detectable in gizzard, brain, liver and lung (Fig. 4A and B, 5-8). Bands of actin mRNA were detected in all the tissues examined (Fig. 4C). These results suggested that C/STnC gene was actively expressed in skeletal muscles during developmental stages and in the adult.

To determine whether adult breast muscle transcribed authentic C/STnC mRNA or other RNA(s) having a similar sequence to C/STnC mRNA, *S1* nuclease protection assay was carried out with RNAs and a probe containing the whole pCTnC1 (Fig. 5A, c). Ventricle, ALD, PLD, smooth muscle and nonmuscle tissues were also examined in addition to adult breast muscle. An autoradiograph obtained after electrophoresis revealed that there was an *S1* nuclease-protected DNA band approximately 660 nucleotides long, corresponding in size to pCTnC1 in adult ventricular, breast, ALD and PLD muscles (Fig. 5B, 3-6). These results showed that the mRNA that was identical to C/STnC mRNA is transcribed in these striated muscles. C/STnC mRNA was undetectable in smooth muscle and non-muscle tissues (Fig. 5A, 7-10).

To examine whether C/STnC mRNAs present in adult breast muscle were active, poly(A)$^+$RNA prepared from adult breast muscle



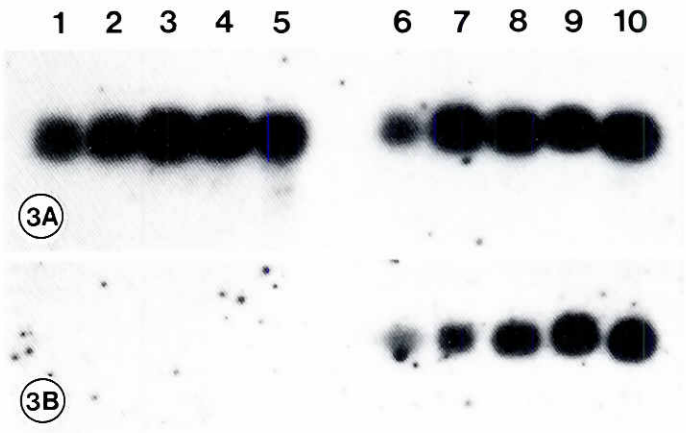


Fig. 3. Expression of TnC mRNAs in ventricular and breast muscles during development. *Northern blot was carried out with total RNAs from embryonic ventricular (1-4) and breast muscles (6-9) as well as adult ventricular (5) and breast muscles (10). RNAs were prepared from embryos at 10 (1,6), 13 (2,7), 16 (3,8) and 19 (4,9) days and adults (5,10). DNA probes are C/STnC-UTR* **(A)** *and FTnC-UTR* **(B)**.

was incubated with rabbit reticulocyte lysates in the presence of ^{32}S methionine. Proteins were translated with the poly(A)$^+$RNA from adult breast muscle (Fig. 6C). Anti-C/STnC was added to the translated product. Immunoreactive proteins were precipitated and examined by SDS polyacrylamide gel electrophoresis. A radioactive protein with a molecular weight of 18 k corresponding in size to C/STnC was precipitated and detected on the autoradiogram (Fig. 6A and D).

Discussion

The present study shows that C/STnC- and FTnC-UTRs are specific for the respective transcripts of C/STnC and FTnC genes. These probes enabled us to study precisely the expression of TnCs in ventricular and skeletal muscles during development and in the adult.

In situ hybridization shows that, at early stage 10, C/STnC mRNA is transcribed in the proximal region of vitelline vein as well as ventricle and truncus arteriosus. Vitelline vein is a primordium of sinus-venosus and does not beat at this stage (Patten, 1956). The area of C/STnC gene expression in vitelline vein is different from those of α-cardiac and smooth actin genes in the developing chicken heart (Ruzicka and Schwartz, 1988). These actin mRNAs are transcribed in the wall of only the rostral side in vitelline vein.

Fig. 2. TnC transcripts in the early stages of ventricular and skeletal muscles. *At stage 10, C/STnC mRNA was detected in truncus arteriosus (t) ventricle (v), and the proximal region of vitelline vein (pv)* **(A)**. *Higher magnification of ventricle and vitelline vein at early stage 10* **(B)**. *C/STnC mRNA was transcribed in both rostral (arrowheads) and caudal (double arrowheads) walls of vitelline veins. C/STnC mRNA was expressed in somites (arrows) as well as cardiac muscle (v) at stage 14* **(C)**. *FTnC mRNA was transcribed in somites (arrows) and truncus arteriosus (t) at stage 20* **(D)**. *Bars: (A) 1 mm; (B) 500 μm; (C) and (D) 1 mm.*

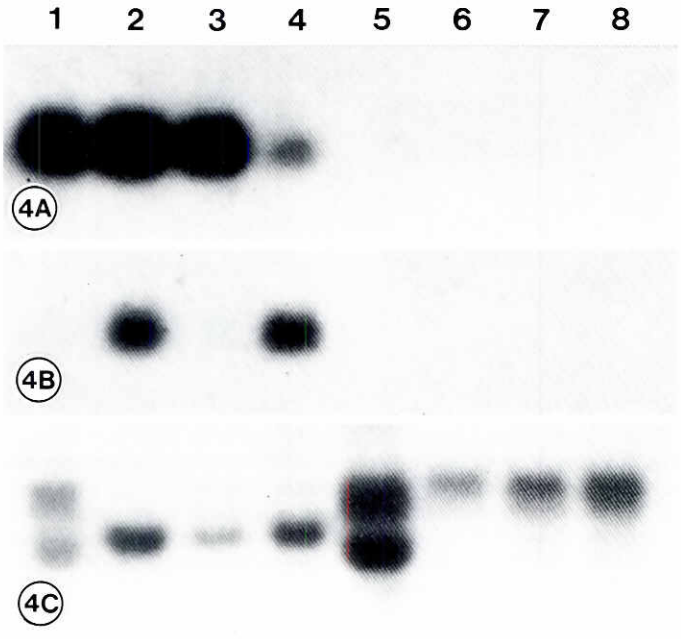


Fig. 4. Northern blot showing expression of TnC genes in various tissues. *Total RNAs prepared from adult ventricular (1), breast (2), ALD (3), PLD muscles (4), gizzard (5), brain (6), liver (7) and lung (8) were hybridized with C/STnC-UTR* **(A)**, *FTnC-UTR* **(B)** *and a genomic DNA fragment of human ß-actin* **(C)**.

However, C/STnC mRNA is expressed in both walls of the rostral and caudal sides of the vein. The same pattern of C/STnC protein appearance is confirmed by immunofluorescence microscopy (not shown). Since the pace maker of heart beating exists in sinus-venosus at later developmental stages (Patten, 1956) and TnC is the regulatory protein of muscle contraction coupling with excitation (Ebashi and Endo, 1968), the wider active area of C/STnC gene than α-actin genes suggests the significant role of C/STnC in the conduction system of embryonic heart.

FTnC mRNA is transcribed in somites from stage 14 when the formation of myotome is just beginning (Freeman and Bracegirdle, 1978). At stage 20, FTnC mRNA is accumulated in truncus arteriosus corresponding to the relative rostral segment of the cardiac tube. However, the FTnC gene is not active in this area at the other developmental stages. This reveals that the FTnC mRNA gene is expressed stage-specific. In the rostral segment of the cardiac tube, other mRNA species and proteins also display distinct patterns of expression during development. α-smooth muscle actin mRNA and protein are present along the entire length of the myocardium at stage 9-10 in the chicken. As development proceeds, α-smooth muscle actin mRNA persists in truncus arteriosus but this actin gene is down-regulated in other cardiac regions at later stages (Ruzicka and Schwartz, 1988; Woodcook-Mitchell *et al.*,

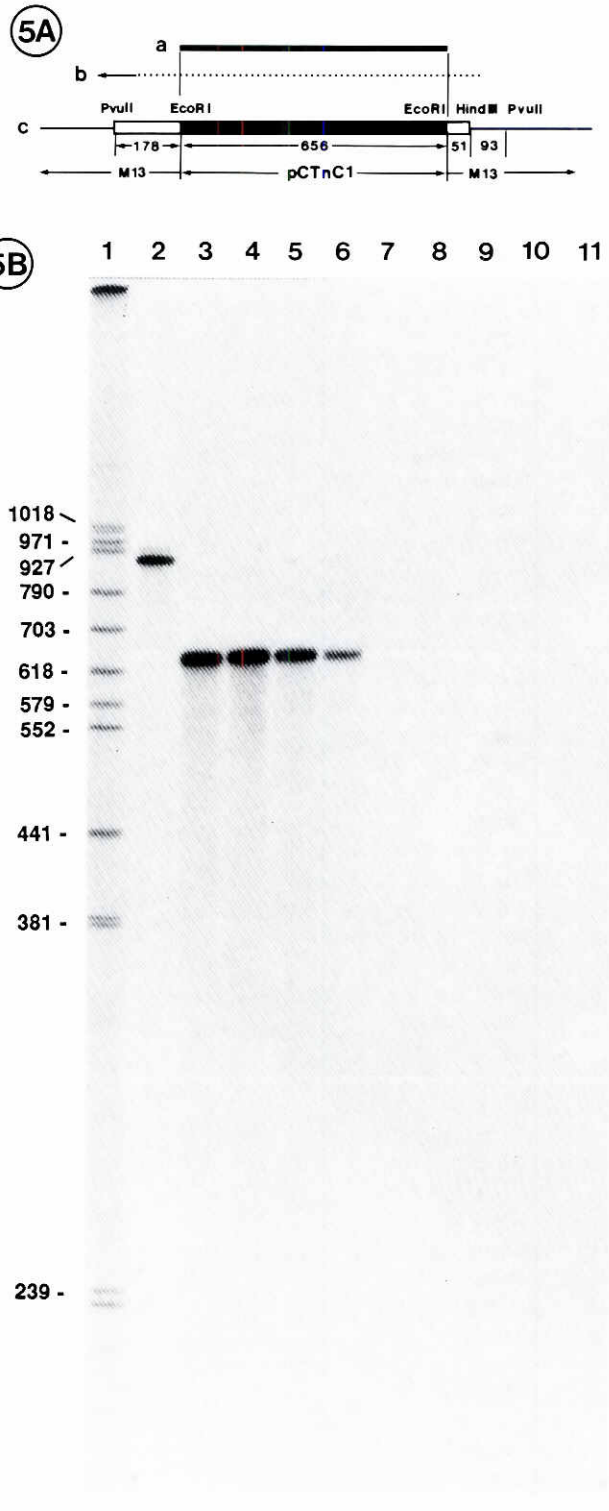


Fig. 5. *S1* nuclease protection assay with RNAs from various tissues and schematic representation of a DNA probe used for the assay. (A) *DNA probe for S1 nuclease protection assay. Protected DNA fragment (a), C/STnC mRNA (dotted line) (b) and the probe (c). The probe (885 bp) consists of pCTnC1 (656 bp, filled box) and two M13 DNA fragments (178 and 51 bp, open boxes) that are both sides of pCTnC1.* **(B)** *S1 nuclease protection assay. Size markers generated from M13mp18 DNA digested with Taq I (1). The positions of the size markers are indicated on the left. A labeled probe used for protection assay (2). RNAs from ventricle (3), breast (4), ALD (5), PLD (6), gizzard (7), brain (8), liver (9), lung (10) and tRNA (11) were examined.*

1988). Transcription of slow TnI mRNA is earlier than that of cardiac TnI mRNA in rat truncus arteriosus (Gorza *et al.*, 1993). The pattern of FTnC gene expression is different from those of the α-smooth muscle actin gene and TnI isogenes in that the FTnC mRNA is expressed stage-specific. This reveals that FTnC gene expression is distinct in myofibrillar proteins in the embryonic cardiovascular system. Since FTnC protein is not detectable in embryonic cardiac muscle cells in the previous immunohistochemical studies (Toyota and Shimada, 1981), expression of FTnC gene at the early stages may be regulated at the level of post-transcription. However, the possibility that FTnC isoform distinct for truncus arteriosus is expressed at the specific stages can not be ruled out.

FTnC mRNA shows fast muscle-specific expression in embryos after 10 days and adults. In contrast, expression of C/STnC gene is up-regulated in both cardiac and breast muscles during development and persists in various adult striated muscles examined. C/STnC mRNA is transcribed in adult breast and ALD muscles. There are several reports suggesting that C/STnC mRNA is expressed in fast skeletal muscle. Analysis by *S*1 mapping and nuclear run-on assay with quail C/STnC genomic DNA shows that C/STnC mRNA is expressed in both adult breast and cultured breast muscles (Bucher *et al.*, 1988). C/STnC cDNA is cloned from a library of neonatal chicken breast muscle (Putkey *et al.*, 1987). A weak hybridizing signal of C/STnC is detected in adult breast muscle on a longer-exposed autoradiograph by Northern blot (Toyota *et al.*, 1989). Human C/STnC cDNA hybridizes with mRNA from human and rabbit skeletal muscles (Gahlman *et al.*, 1988). In murine, however, C/STnC mRNA is not detectable in fast skeletal muscle but transcribed in well differentiated C2C12 muscle cells (Parmacek and Leiden, 1989).

Adult skeletal muscles are usually a mixture of fast and slow muscle fibers, but chicken breast muscle is predominantly a homologous muscle with approximately 98-99% of its mass consisting of the fast muscle fiber (Gauthier *et al.*, 1982; Toyota and Shimada, 1984). A small red strip area on the deep surface of chicken breast muscle is known to display immunological reactivity with antibodies to both fast and slow myosins (Matsuda *et al.*, 1983). However, the red strip area is eliminated from adult breast muscle in the present study. Therefore, C/STnC mRNA is transcribed not in small numbers of slow muscle fibers but in fast muscle fibers of adult breast muscle. Further, the *S*1 nuclease protection assay demonstrates that C/STnC mRNA in adult breast muscle is the authentic C/STnC mRNA and that its signal is not due to cross-hybridization among C/STnC-UTR, FTnC mRNA and/or RNA which has a similar sequence.

The persistence of C/STnC mRNA in adult breast muscle differs from C/STnC protein expression studied by immunofluorescent microscopy (Toyota and Shimada, 1981). C/STnC protein is not detectable in adult breast muscle. Taken together, these results suggest that post-transcriptional control plays an important role in adult breast muscle. *In vitro* translation assay with mRNA from adult breast muscle reveals that C/STnC mRNA is active and translatable in rabbit reticulocyte lysates. Although it is unknown whether the translational mechanism in the rabbit reticulocyte lysate is the same as that in chicken breast muscle, the result suggests that the translational and post-translational mechanisms are significant steps rather than the pre-translational step in C/STnC gene expression.

The physiological significance of the C/STnC mRNA persisted in adult breast muscle is unclear. However, the existence of C/STnC mRNA in adult breast muscle may relate to the molecular evolution of Ca^{2+} binding proteins of which expression is tissue-nonspecific. There is a possibility that TnCs are evolved from an ancestral protein common to calmodulin (CaM), myosin light chain and parvalbumin by gene duplication (Collins *et al.*, 1973; Goodman *et al.*, 1979). The analysis of genomic DNAs among CaM, C/STnC and FTnC shows that there are purine-rich DNA sequences in promoter regions of these genes and that there are similarities in the positions of exon and intron among these genes (Gahlman and Kedes, 1990). Parmacek and Leiden (1989) also described the intragenic homologies between Ca^{2+} binding domains of the mouse genomic C/STnC gene. It is becoming clearer that TnC genes have many traces of the gene of the primitive Ca^{2+} binding protein. It may be a disadvantage for C/STnC gene to regulate tissue-specific expression with regulatory motif(s) and trans-acting factor(s) which evolve from the ubiquitous Ca^{2+} binding proteins. Extensive studies concerning cis- and trans-acting factors of chicken C/STnC gene will be necessary to explore transcription of C/STnC mRNA in adult breast muscle.

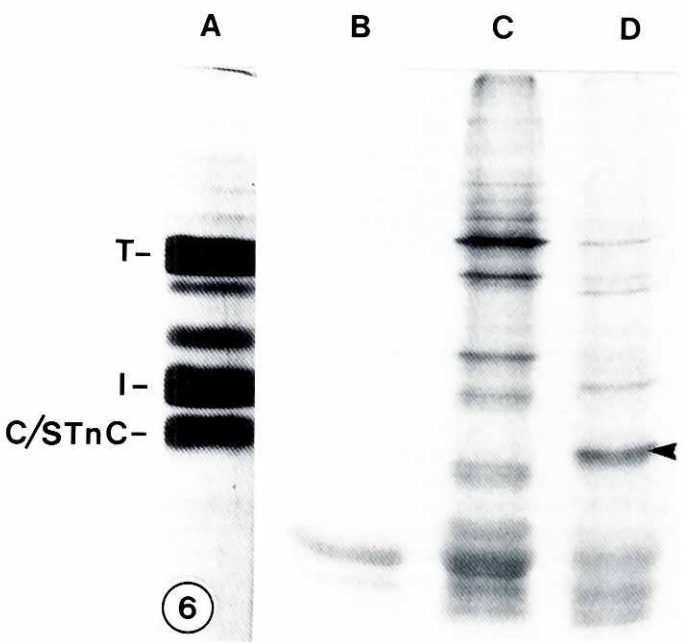


Fig. 6. Autoradiogram of the translation products synthesized in a cell-free system. *A crude extract of cardiac Tn components separated with an apparatus of disc type electrophoresis (12% polyacrylamide gel)* **(A)**. *T and I indicate TnT and TnI, respectively. In vitro translation products incubated with distilled water* **(B)** *and poly(A)⁺RNA prepared from adult breast muscle* **(C)**. *Anti-C/STnC were added to a reticulocyte lysate after the in vitro translation of poly(A)⁺RNA from adult breast muscle* **(D)**. *An arrowhead indicates immunoreactive protein with a molecular weight of 18 k corresponding in size to C/STnC.*

Materials and Methods

DNA probes

pCTnC1 was isolated from a chicken ventricular library as described previously (Toyota *et al.*, 1989).

To distinguish between transcripts of C/STnC and FTnC genes, the distinct sequence area of pCTnC1 including 5' UTR and domain I (Reinach

and Karlsson, 1988; Toyota *et al.*, 1989) was digested with *Taq*I to yield a C/STnC-UTR fragment. The DNA sequence of C/STnC-UTR is 5'GCCTCCCTGGCCCGGCCATGGATGACATCTATAAGGCGGCGGTTGAGCAGTTGACAGAA-GAACAAAAAAATGAGTTTAAGGCTGCCT3'. For a probe of FTnC, an oligonucleotide of 5' UTR in FTnC mRNA 5'GGCAAGGAAGAGGTGCT-CCCTGCGGGAGGAGAGCAG3' was synthesized and used as template for a label. The sequence data of FTnC is available from EMBLE/GenBank under accession number M19027 (Reinach and Karlsson, 1988). Restriction fragment (446 bp) of human ß-actin gene (Nakajima-Iijima *et al.*, 1985), including a third exon which was prepared by digestion with *Hinf*I, was used as control.

In situ hybridization

Chicken embryos were staged according to Hamburger and Hamilton (1951).

C/STnC-UTR and FTnC-UTR were subcloned into the vector pGEM-7Zf(-) (Promega Corporation, Madison, WI, USA), and antisense cRNAs were synthesized according to the manufacturer's instructions and labeled with [α-^{32}P]UTP (>800 Cim/mol). *In situ* hybridization experiments of C/STnC and FTnC were performed by the method of Sassoon *et al.* (1988). Aminoalkylsilane-treated glass slides were used to mount paraffin sections (Rentrop *et al.*, 1986). Hybridization of C/STnC-UTR cRNA was performed in a solution containing 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM Na-P04 (pH 8.0), 10% dextran sulfate, 1xDenhardt's and 50 µg/ml tRNA. Sections were incubated with 10^7 cpm of ^{32}P-labeled C/STnC-UTR cRNA at 42°C overnight.

All procedures for hybridization of FTnC-UTR were the same as those for C/STnC. However, concentrations of NaCl and Tris-HCl (pH 8.0) in the hybridization buffer were substituted to 1 M NaCl and 10 mM Tris-HCl (p H6.8), respectively. The sections were incubated with $4x10^7$ cpm of ^{32}P-labeled FTnC-UTR cRNA at 47°C overnight and washed at 50°C. The slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 1-4 weeks in tight light boxes with desiccant at 4°C. Photographic development was carried out with Kodak D-19. Slides were observed under a Nikon Optiphot microscope equipped with dark field optics.

Preparation of RNAs

Total RNAs were prepared by the guanidine isothiocyanate (GI) procedure as described by Davis *et al.* (1986) from the following tissues: ventricular, breast, ALD and PLD muscles, gizzard, brain, liver and lung of adult chickens, and embryonic ventricular and breast muscles. In adult breast muscle, a red stripe area which contains slow red muscle was removed and a posterior region of pectoral muscle was used. To increase the yield of RNAs, the concentration of the GI solution was increased from 4 to 6 M.

Northern blots

10 µg of total cellular RNA was electrophoresed on a 1.5% agarose gel (150 mm in length) in the presence of 6% formaldehyde and transferred to nitrocellulose filters. C/STnC-UTR and ß-actin probes were labeled with [α-^{32}P]dCTP (>800 Cim/mol, Amersham International, Amersham) by a random primer method (Feinberg and Vogelstein, 1983). For the label of FTnC-UTR, a single primer (5'CTGCTCTCCTCCCG3') was used. Hybridization with C/STnC-UTR and ß-actin DNA was performed at 42°C for 16 h in the presence of a 40% formamide solution containing 10% dextran sulfate, 4xSSC (1xSSC, 0.15 M NaCl, 0.015 M sodium citrate), 20 mM Tris (pH7.5), 1xDenhardt's solution and salmon sperm DNA (ssDNA) (20 µg/ml). The filters were then washed with a solution of 2xSSC and 0.1% SDS three times for 20 min at room temperature, and with a solution of 0.1xSSC and 0.1% SDS for 20 min at 68°C. Hybridization with FTnC-UTR was carried out at 47°C for 16 h in the presence of 50% formamide solution containing 10% dextran sulfate, 6xSSC, 20 mM Tris (pH7.5), 1xDenhardt's solution and ssDNA (20 µg/ml). Filters were washed (2xSSC, 0.1% SDS for 30 min at room temperature, and 0.1xSSC, 0.1% SDS, for 5 min at 42°C) and exposed to Fuji New RX films for 1-2 days at -70°C.

In some experiments, a large size gel (250 mm in length) was used to separate TnC mRNAs. 30 µg of RNAs was electrophoresed for 6 h at 200V and hybridized with TnC-UTR probes.

S1 analysis

*S*1 nuclease protection assay was performed as described by Davis *et al.* (1986). pCTnC1 was subcloned into an *Eco*RI site of M13mp18. An M13 clone whose primer-extended product was complementary to C/STnC mRNA, was selected by a sequencing procedure. A uniformly labeled, single-stranded DNA was synthesized with the M13 template by primer extension reactions using [α-^{32}P]dCTP (>400 Cim/mol^{-1}), dATP, dGTP, dTTP, universal primer and Klenow fragment. Labeled pCTnC1 was digested with *Pvu*II and *Hind*III. ^{32}P labeled DNA was separated by electrophoresis with 1% of TBE (50 mM Tris-HCl, pH 8.3, 1 mM EDTA and 0.24 M boric acid) agarose gel. Hybridization of sample RNAs (20 µg) or tRNA (10 µg) with the labeled probe (60,000 cpm) was performed in 40 µl of a hybridization buffer (75% formamide, 0.46 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA, 0.1% SDS) at 75°C for 15 min followed by 60°C for 6 h. *S*1 nuclease digestion was carried out in 400 µl of 0.1 M NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$ and 8 µg ssDNA with 100 units of S1 nuclease for 1 h at 37°C. The samples were precipitated with ethanol and analyzed on a 5% acrylamide-urea gel. The size markers were the restriction fragments of M13mp18 digested with *Taq*I. The 3' recessed termini of the *Taq*I sites were labeled by filling with [α-^{32}P]dCTP. Gels were exposed to X-ray film for 6 h at -70°C.

Determination of intensities of mRNA level

The levels of C/STnC mRNA were quantitated by scanning densitometry (Sakura PDS15, Tokyo) of the bands analyzed by Northern blot. Each RNA sample was examined in at least three different experiments.

Antibody

Anti-C/STnC was the same as that used previously (Toyota and Shimada, 1981).

In vitro translation

In vitro translation by rabbit reticulocyte lysates (Amersham International, Amersham) was performed by the method of Jackson and Hunt (1983). Poly(A)$^+$RNA was isolated from total RNA by oligo(dT)-cellulose column chromatography according to the method described in Davis *et al.* (1986). 3 µg of poly(A)$^+$RNA and 110 units of RNasin were added to 25 µl of the lysate solution. The concentration of ^{35}S methionine was adjusted to provide ~20 µCi/25 µl. Translations were incubated at 37°C for 90 min. Approximately 100 µg of anti-C/STnC was added to the lysate solution and reacted at 4°C overnight. The mixture was centrifuged at 15,000 rpm for 30 min at 4°C. The precipitate was treated with SDS sample buffer and subjected to a 12% polyacrylamide gel in the presence of 0.1% SDS according to the procedure of Weber and Osborn (1969). After fixation with 50% methanol containing 10% acetic acid, the gel was immersed in EN^{3}HANCE (Dupont/NEN Research Products, Boston) for 60 min and rinsed in water for 60 min. The gel was dried under vacuum and used to expose Fuji New RX film at -70° C for 1 day.

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