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Developmental Changes of Cardiac and Slow Skeletal Muscle Troponin T Expression in Chicken Cardiac and Skeletal Muscles

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ABSTRACT—Numerous troponin T (TnT) isoforms are produced by alternative splicing from three genes characteristic of cardiac, fast skeletal, and slow skeletal muscles. Apart from the developmental transition of fast skeletal muscle TnT isoforms, switching of TnT expression during muscle development is poorly understood. In this study, we investigated precisely and comprehensively developmental changes in chicken cardiac and slow skeletal muscle TnT isoforms by two-dimensional gel electrophoresis and immunoblotting with specific antisera. Four major isoforms composed of two each of higher and lower molecular weights were found in cardiac TnT (cTnT). Expression of cTnT changed from high- to low-molecular-weight isoforms during cardiac muscle development. On the other hand, such a transition was not found and only high-molecular-weight isoforms were expressed in the early stages of chicken skeletal muscle development. Two major and three minor isoforms of slow skeletal muscle TnT (sTnT), three of which were newly found in this study, were expressed in chicken skeletal muscles. The major sTnT isoforms were commonly detected throughout development in slow and mixed skeletal muscles, and at developmental stages until hatching-out in fast skeletal muscles. The expression of minor sTnT isoforms varied from muscle to muscle and during development.

Key words: muscle development, fiber type, isoform, alternative splicing, troponin T

INTRODUCTION

Vertebrate striated muscles are grouped into cardiac, fast skeletal, and slow skeletal muscles based on differences in their contractile properties and structures. Protein compositions are also peculiar to each group of muscles and the expression of those proteins is specifically regulated during muscle development (for reviews, see Obinata, 1985; Shiaffino and Reggiani, 1996).

Troponin, the key protein for Ca^{2+}-sensitive molecular switching of contraction in the vertebrate striated muscle, consists of three components, troponin T (TnT), troponin I, and troponin C (for reviews, see Shiaffino and Reggiani, 1996; Zot and Potter, 1987). TnT has isoforms which are encoded by three genes characteristic of cardiac, fast skeletal, and slow skeletal muscles (Breitbart *et al*., 1985; Cooper and Ordahl, 1985; Gahlmann *et al*., 1987; Smillie *et al*., 1988; Mesnard *et al*., 1993; Yonemura *et al*., 1996). Each gene can generate a variety of transcripts and protein isoforms by alternative splicing (for review, see Shiaffino and Reggiani, 1996).

Miyazaki *et al*. (1999) determined the structure of the chicken fast skeletal muscle troponin T (fTnT) gene and indicated that 2^{15} transcripts were potentially produced by splicing of 16 alternative exons in the gene. Actually far fewer transcripts have been detected. Smillie *et al*. (1988) and Schachat *et al*. (1995) found four variants of chicken fTnT cDNA and 16 variants of chicken fTnT 5'-cDNA, respectively. Imai *et al*. (1986) and Yao *et al*. (1992) reported that the chicken leg muscle had 40 kinds of fTnT isoforms and the chicken wing muscles had 80 kinds of fTnT isoforms including phosphorylated forms. Developmental changes of fTnT isoform expression have been examined in detail (Matsuda *et al*., 1981; Abe *et al*., 1986). In the breast muscle, transition from L-type (low-molecular-weight isoforms) to B-type (high-molecular-weight isoforms) fTnT occurs during development, while only L-type fTnT is expressed through-

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out development in the leg muscle. B-type isoforms are classified into B_N, B_C, and B_A subtypes according to the order of their appearance during breast muscle development. B_N-subtype fTnT appears around hatching-out and is replaced by B_C-subtype fTnT, which is subsequently superseded by B_A-subtype fTnT. Thus, the adult breast muscle has only B_A-subtype isoforms. Similar transition of fTnT expression is seen during development of wing muscles (Yao *et al*., 1992). However, L-type and B_N- and B_C-subtype isoforms do not disappear and their expression is continued even in the adult.

Compared with fTnT, sufficient information is not available on expression of cardiac TnT (cTnT) and slow skeletal muscle TnT (sTnT), although there are several studies on their expression. Ogasawara *et al*. (1987) and Saggin *et al*. (1988, 1990) reported developmental changes of chicken and rat cTnT expression, respectively. Sabry and Dhoot (1991) showed tissue-specifically and developmentally regulated expression of rat and human cTnT as well as sTnT. Avian and mammalian cTnT switches from high-molecular-weight to low-molecular-weight isoforms during the heart development (Ogasawara *et al*., 1987; Saggin *et al*., 1988; Jin, 1996). The chicken cTnT gene generates one embryonic and one adult transcript by inclusion and exclusion of the exon 5, respectively (Cooper and Ordahl, 1985). The mouse cTnT gene generates one embryonic and three adult transcripts by alternative splicing involved in exons 3a, 4, and 12 (Jin *et al*., 1996). cTnT is expressed not only in the cardiac muscle but also in skeletal muscles at earlier stages of development. Genetic programming of rat and mouse cTnT synchronized in developing cardiac and skeletal muscles was proposed in spite of their differences in the structure, function and developmental process (Jin, 1996). On the other hand, Ogasawara *et al*. (1987) suggested that chicken cTnT expression was regulated differentially between cardiac and skeletal muscles. More extensive studies are necessary to show definitely whether synchronized genetic programming exists or not, because in those studies limited skeletal muscle samples were analyzed.

Yonemura *et al*. (2000) found variants of chicken sTnT cDNA, which differed from one another by inclusion or exclusion of one codon encoding an alanine residue and single base substitutions at two positions in the 5'-region and at four positions in the 3'-region. The heterogeneity of chicken sTnT transcripts is generated from 3.5 kb of the sTnT gene (Yonemura *et al*., 2000), although the chicken fTnT gene is about 35 kb (Miyazaki *et al*., 1999) and the chicken cTnT gene is about 9 kb (Cooper and Ordahl, 1985). Only two kinds of sTnT isoforms were detected in the adult chicken slow muscle, *anterior latissimus dorsi* (ALD) (Mikawa *et al*., 1981). One and another additional exons are alternatively spliced in mouse and human sTnT genes, respectively (Gahlmann *et al*., 1987; Huang *et al*., 1999). Developmental transition of several isoforms of rat sTnT was reported (Sabry and Dhoot, 1991).

As mentioned above, the heterogeneities of sTnT and cTnT appear to be less pronounced than that of fTnT. However, precise and detailed information on expression of cTnT and sTnT isoforms in cardiac and skeletal muscles during development is very important in order to shed light on mechanisms of diversification of muscle fiber types. In this study, we investigate expression patterns of chicken cTnT and sTnT isoforms by two-dimensional gel electrophoresis (2D SDS-PAGE) and immunoblotting and those of sTnT transcripts by S1 nuclease mapping. Most studies carried out so far relied on one-dimensional resolution of TnT isoforms which could detect only their molecular weight differences, but herein we take advantage of two-dimensional separation of isoforms which have often different pI values. We report isoform switching of cTnT in the avian and mammalian hearts, but not in the chicken skeletal muscles. We also report differences in expression patterns of sTnT isoforms including those newly found during development.

MATERIALS AND METHODS

Materials

White leghorn chickens (*Gallus domesticus* (L)) and their fertilized eggs were obtained from commercial sources. The chicken slow skeletal muscle (ALD), mixed skeletal muscles (*gastrocnemius*, *complexus*, and *biventer cervicalis*), fast skeletal muscles (*pectoralis major* and *biceps femoris*), and cardiac muscle, and mouse cardiac and leg skeletal muscles were dissected out and stored frozen at –80°C until preparation of samples for 2D SDS-PAGE. Chicken ALD, *gastrocnemius*, *biventer cervicalis*, and *pectoralis major* were used for RNA preparation.

Electrophoresis and immunoblotting

2D SDS-PAGE was carried out according to the improved method of Hirabayashi (1981) with 12% acrylamide/3M urea gels in the second dimension. Nonidet P-40 (1%) was included in agarose gels for isoelectric focusing and the extraction medium for sample preparation in order to achieve better separation of TnT isoforms. After 2D SDS-PAGE, proteins in gels were transferred onto nitrocellulose membranes at 300 mA for 3 hr according to the methods of Towbin *et al*. (1979) and Franke *et al*. (1981). Immunoblotting was performed by using the rabbit specific antisera against chicken sTnT, fTnT, and cTnT as the first antibody, and rhodamine-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) as the second antibody. Preparations and specificities of the three specific antisera were described previously (Yao *et al*., 1992; Mashima *et al*., 1996). Semi-quantitative estimation of TnT spots on immunoblotting patterns was performed using BAS5000 (FUJI FILM) and the ratios of low-molecular-weight to high-molecular-weight cTnT isoforms were calculated.

Antisense DNA probes and S1 nuclease mapping

Using chicken sTnT cDNA clones (Yonemura *et al*., 1996), the probe for S1 nuclease mapping, namely probe A,

was constructed in the previous study (Yonemura *et al.*, 2000). The probe A, labeled with [^{32}P] ATP using T4 polynucleotide kinase (Nippon gene), is a 690 nt fragment (position 1 to 690 of chicken sTnT cDNA) lacking three bases (135–137) for alanine. Total RNA (10 or 20 μg) was precipitated with 10^5 cpm of the probe A. The pellet was dissolved in 20 μl of the hybridization buffer. Hybridization was performed overnight at 55°C before digestion with S1 nuclease (Gibco BRL). Protected fragments were separated on 6% acrylamide/8M urea gels, which were exposed to X-ray film (Kodak) with an intensifying screen.

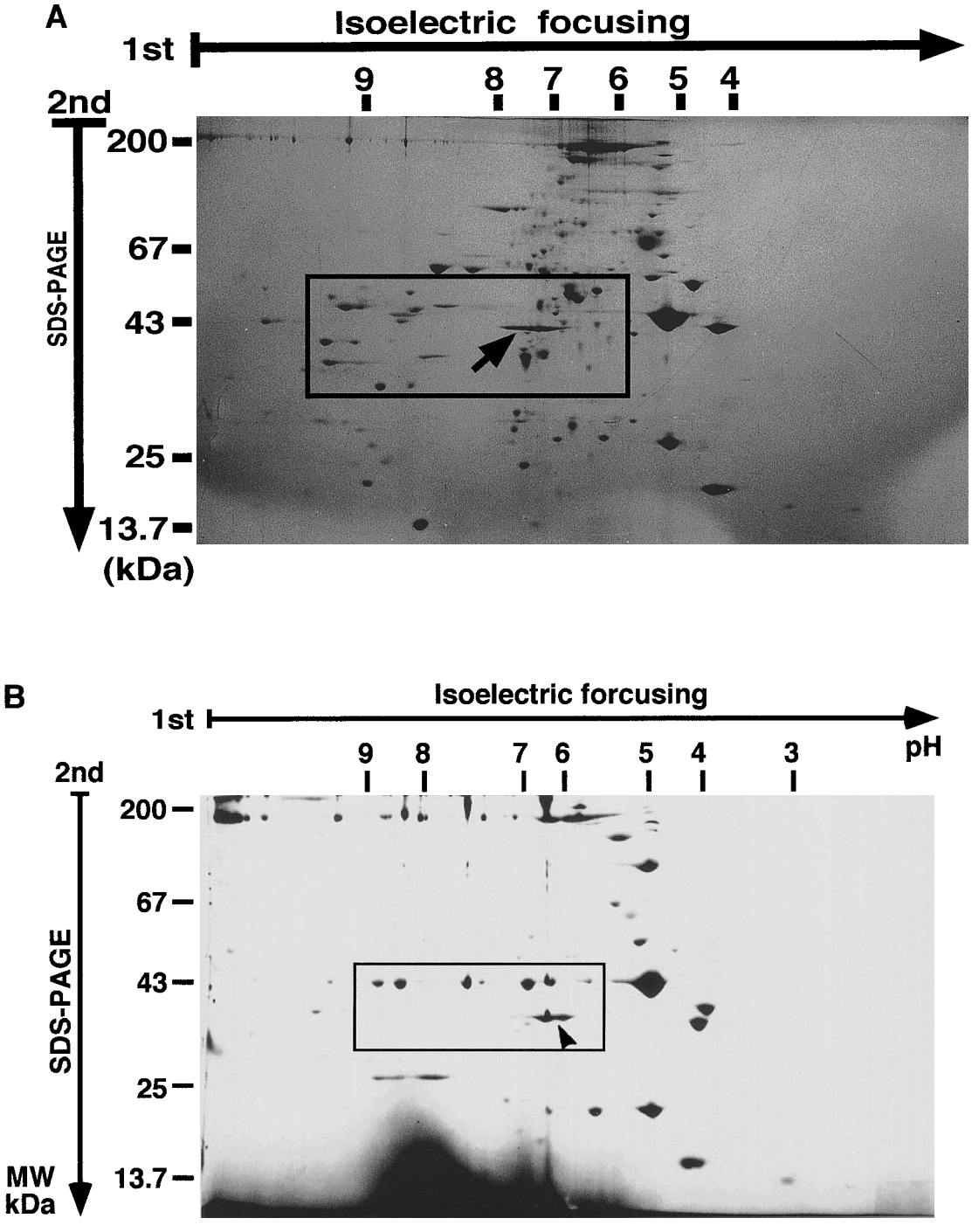

Fig. 1. Two-dimensional gel electrophoreses of adult chicken heart (A) and ALD (B) extracts. The first dimension isoelectric focusing was performed in the direction basic to acidic. Gels were stained with Coomassie brilliant blue R-250. The arrow (A) and arrowhead (B) indicate cTnT and sTnT, respectively. The expression patterns of cTnT (A) and sTnT (B) isoforms were precisely examined by immunoblotting of the boxed areas.

RESULTS

Developmental changes of cTnT expression in the cardiac and skeletal muscles

Chicken cTnT gave the molecular weight of about 43 kDa and the isoelectric point (pI) of 7–8 on the 2D SDS-PAGE pattern of the adult chicken heart (Fig. 1A, arrow). To investigate developmental changes of chicken cTnT isoforms in the cardiac muscle, we examined their expression by immunoblotting of areas including TnT isoforms (box in Fig. 1 A) with the rabbit anti-cTnT antiserum (Mashima *et al*., 1996). The expression patterns of cTnT are shown for 7, 9, 10, 13, 16, and 19-day-old embryos, and 1, 5, 10, 15, 20, 30-day-old chicks, and the adult (a in Fig. 2A) and also schematically represented (b in Fig. 2A). Two major high-molecular-weight isoforms were expressed in the hearts during embryonic stages (red and green). The relative amounts of those isoforms gradually decreased and finally solely the acidic one was present in the adult. The relative amounts of one or two low-molecular-weight isoforms (blue) were very low at earlier embryonic stages and increased gradually during heart development and became predominant after hatching-out. The lower-molecular-weight polypeptide (yellow) began to be detected around hatching-

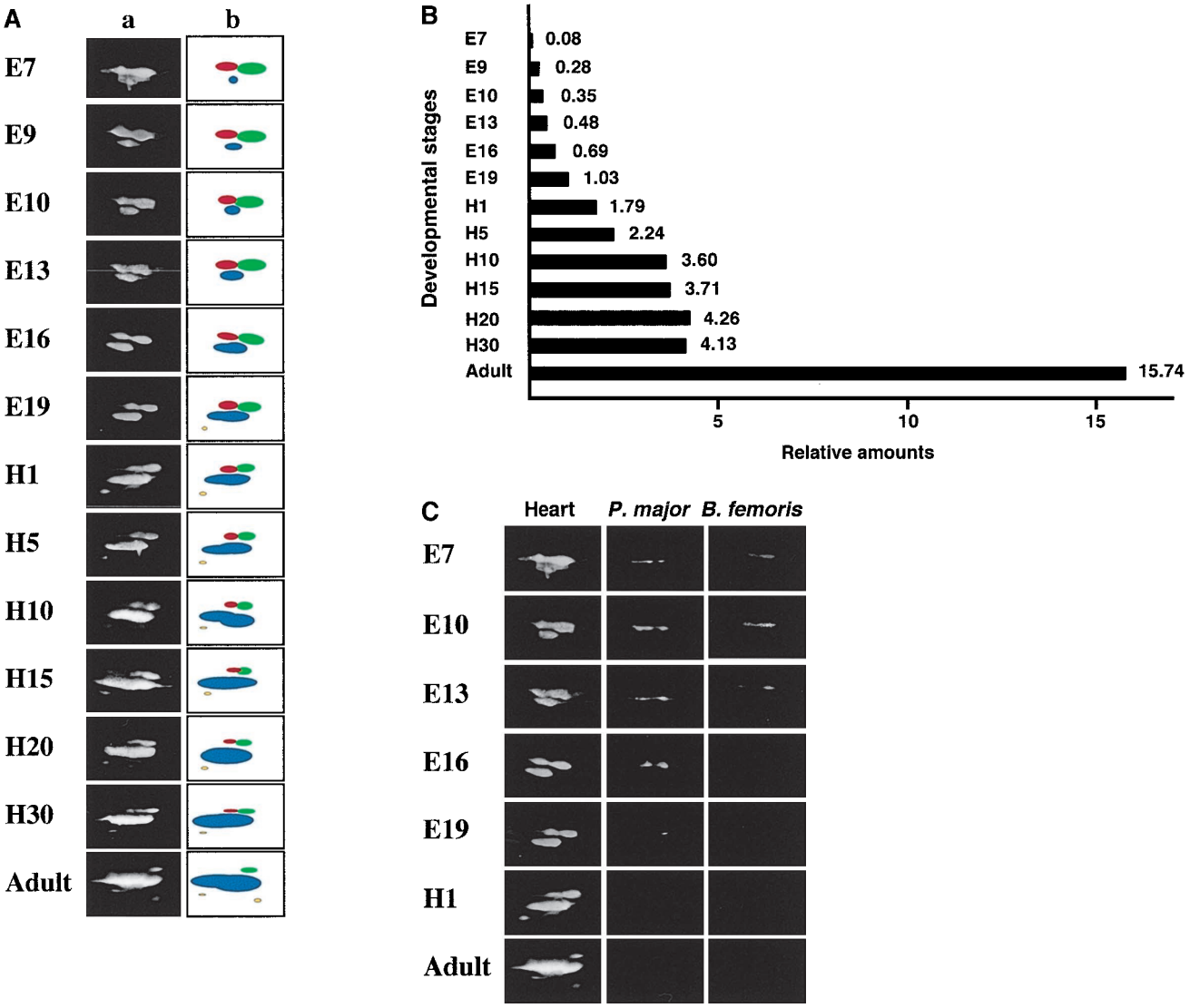

Fig. 2. Developmental changes of chicken cTnT isoforms. A, cardiac muscle extracts of 7 (E7), 9 (E9), 10 (E10), 13 (E13), 16 (E16), and 19-day-old (E19) embryos and 1 (H1), 5 (H5), 10 (H10), 15 (H15), 20 (H20), 30-day-old (H30), and adult chickens were analyzed by 2D SDS-PAGE and immunoblotting with the anti-cTnT antiserum (a in A). Schematic representation of developmental changes in cTnT isoforms is also shown (b in A). Red and green spots indicate the high-molecular-weight isoforms, blue spots indicate the low-molecular-weight isoforms, and yellow spots indicate the lower- and lowest-molecular-weight minor isoforms. B, semi-quantitative estimation of TnT spots on immunoblotting patterns was performed by BAS5000 (FUJI FILM) and ratios of low- to high-molecular-weight isoforms at respective developmental stages were calculated. C, expression of cTnT was also examined by 2D SDS-PAGE of chicken *pectoralis major* and *biceps femoris* extracts of 7 (E7), 10 (E10), 13 (E13), 16 (E16), and 19-day-old (E19) embryos and 1-day-old (H1) and adult chickens and immunoblotting. The cTnT expression patterns of the cardiac muscle are shown for references.

out and the lowest-molecular-weight polypeptide (yellow) was found in the adult. Those polypeptides reacted with the anti-cTnT antiserum, but it remains to be shown whether they are new isoforms or degradation products. Since they were observed throughout heart maturation, the lower- and lowest-molecular-weight polypeptides may not be produced artificially. The results indicated that expression of cTnT isoforms switched from high-molecular-weight to low-molecular-weight isoforms during heart development. The ratios of low- to high-molecular-weight isoforms showed the predominance of the latter at earlier developmental stages and the predominance of the former at later stages with approximately 1:1 ratio at the 19-day-old embryo (Fig. 2B).

We examined cTnT expression in skeletal muscles (*pectoralis major* and *biceps femoris*) during development (Fig. 2C). The expression patterns of cTnT isoforms are shown for 7, 10, 13, 16, and 19-day-old embryos, and 1-day-old and adult chickens. Both skeletal muscles had only high-molecular-weight isoforms. The expression of cTnT isoforms was found in the embryo *pectoralis major* until 19-day old and in the embryo *biceps femoris* until 13-day old. The results indicated that cTnT isoforms did not disappear simultaneously in different skeletal muscles and that the expression patterns of cTnT isoforms in the cardiac muscle were different from those in the skeletal muscles. No transition from high-molecular-weight to low-molecular-weight isoforms was found in the skeletal muscles, suggesting that alternative splicing factors may be different between the cardiac and skeletal muscles.

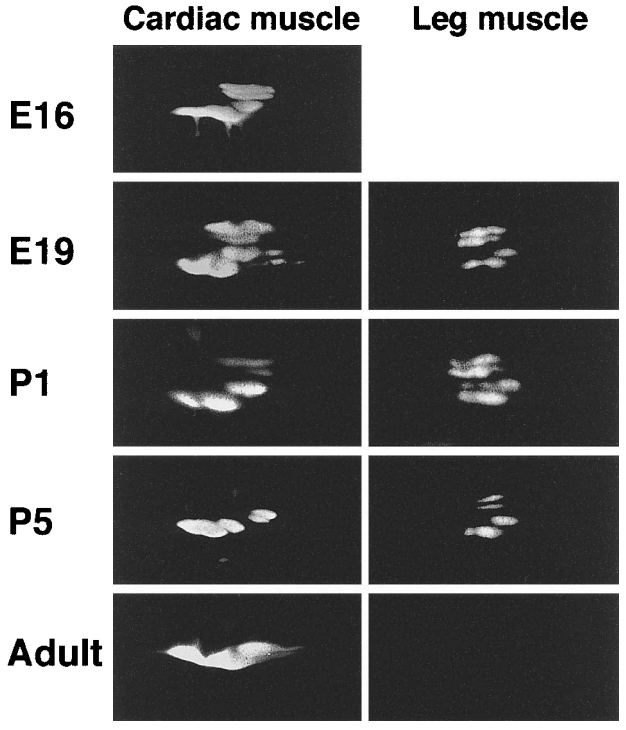

Fig. 3. Developmental changes of mouse cTnT isoforms. Mouse cardiac and leg muscle extracts of 16 (E16) and 19-day-old (E19) embryos, 1 (P1) and 5-day-old (P5) neonates, and the adult were analyzed by 2D SDS-PAGE and immunoblotting with the anti-cTnT antiserum. The leg muscle from the 16-day-old embryo was not used.

To investigate whether developmental changes of cTnT isoforms are also observed in mammalian cardiac and skeletal muscles, we examined cTnT expression in the mouse cardiac and skeletal (leg) muscles during development. The expression patterns of mouse cTnT isoforms are shown for 16 and 19-day-old embryos, 1 and 5-day-old neonates, and the adult (Fig. 3, the 16-day-old embryo was not used in the leg muscle). In the mouse cardiac muscle, transition from high-molecular-weight to low-molecular-weight isoforms was also observed, and only low-molecular-weight isoforms were found in the adult. In the leg muscle, transition from high-molecular-weight to low-molecular-weight isoforms was much less striking; e.g. considerable amounts of high-molecular-weight isoforms were detected in the 5-day-old neonate leg muscle, but not in the cardiac muscle at the same developmental stage. These results showed that cTnT expression was regulated similarly in avian and mammalian cardiac muscles but differently in skeletal muscles.

Developmental changes of sTnT expression in the skeletal muscles

Chicken sTnT gave the molecular weight of about 31 kDa and the pI of 6–7 on the 2D SDS-PAGE pattern of the adult chicken ALD (Fig. 1B, arrowhead). To investigate developmental changes of chicken sTnT isoforms in the skeletal muscles, we examined their expression by immunoblotting of areas including TnT isoforms (box in Fig. 1B) with the anti-sTnT antiserum (Yao *et al*., 1992). The expression patterns of sTnT isoforms are shown for 7, 9, 13, 16, and 19-day-old embryos, and 1-day-old and adult chickens in *pectoralis major* and *biceps femoris* (Fig. 4A). *Pectoralis major* and *biceps femoris* are typical fast skeletal muscles, and express the B_A-subtype and L-type fTnT isoforms in the adult, respectively (data not shown). However, both muscles expressed two sTnT isoforms until the chick reached 1-day old.

Pectoralis major contains a highly homogeneous population of white (fast-twitch) fibers, but it has a small red-colored region from the anterior edge of the sternum to the proximal end of the humerus, which is called the red strip region. The region is reported to have both fast- and slow-twitch fibers (Ganthier and Lowey, 1977) and express slow-type and fast-type myosin light chains, slow-type and embryonic fast-type myosin heavy chains, and α-slow, α-fast, and β-tropomyosins (Matsuda *et al*., 1983; Reiser *et al*., 1996). We also examined the expression of sTnT and fTnT in the red strip region of adult *pectoralis major* by immunoblotting using the antiserum against fTnT in addition to the anti-sTnT antiserum. Although the bulk white region of *pectoralis major* expressed B_A-subtype fTnT exclusively, the red strip region showed an expression pattern similar to those of wing muscles, where L-type and B_A-, B_C-, and B_N-subtype fTnT isoforms are expressed in the adult (L, B_A, B_C, and B_N

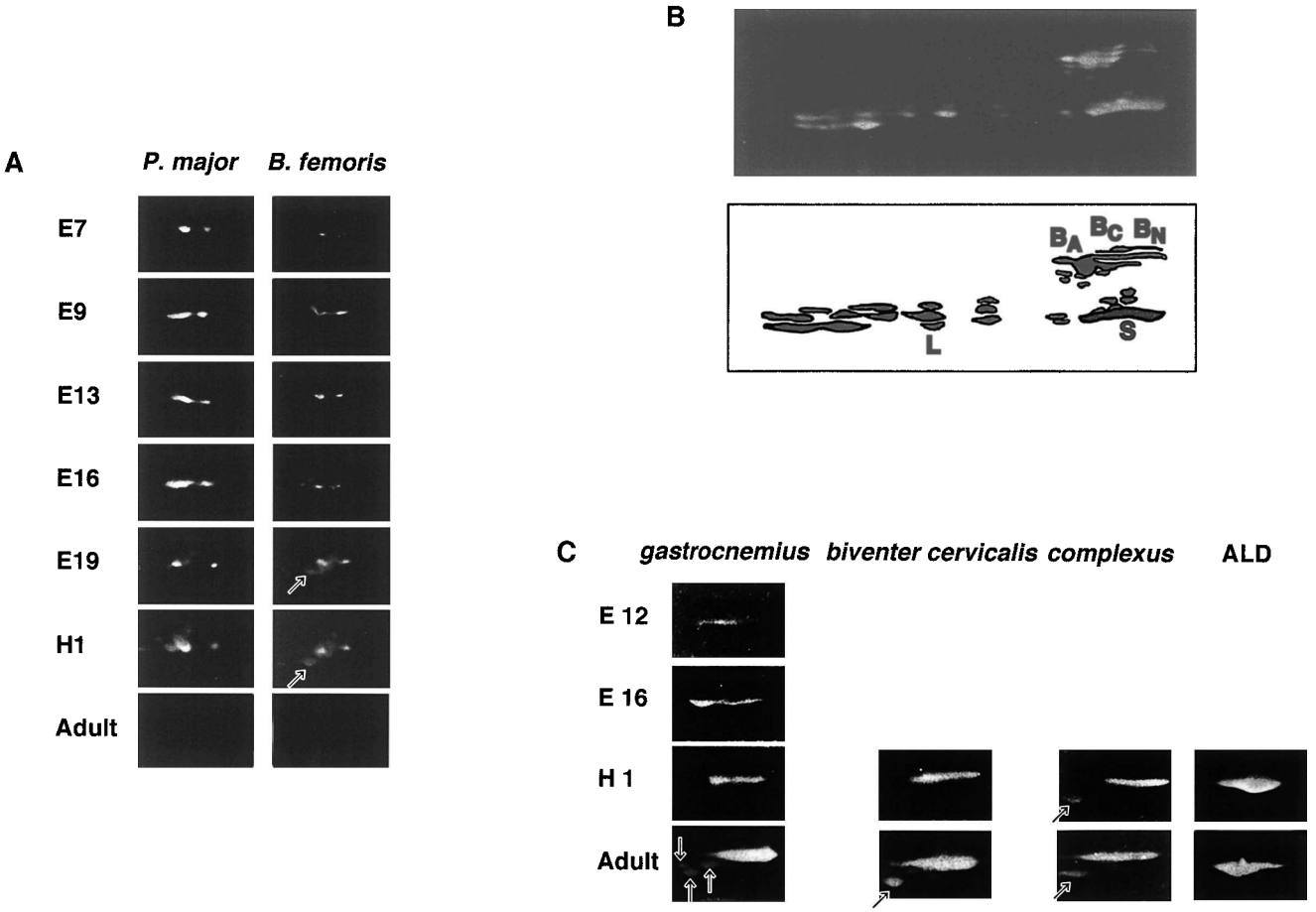

Fig. 4. Expression patterns of chicken sTnT isoforms. A, *pectoralis major* and *biceps femoris* (fast skeletal muscle) extracts of 7 (E7), 9 (E9), 13 (E13), 16 (E16), and 19-day-old (E19) embryos and 1-day-old (H1) and adult chickens were analyzed by 2D SDS-PAGE and immunoblotting with the anti-sTnT antiserum. Arrows indicate possible new sTnT isoforms. B, expression of sTnT and fTnT was examined by 2D SDS-PAGE of the extract from the red strip region of adult *pectoralis major* and immunoblotting with a mixture of antisera against sTnT and fTnT (upper panel). Schematic representation is also shown (lower panel). fTnT and sTnT are indicated by L, B_A, B_C, and B_N and S, respectively. C, expression of sTnT in ALD (slow skeletal muscle), *gastrocnemius*, *complexus* and *biventer cervicalis* (mixed skeletal muscles) of 1-day-old (H1) and adult chickens was examined as in A. For *gastrocnemius*, 12 (E12) and 16-day-old (E16) embryos were also used. Arrows indicate possible new sTnT isoforms.

in Fig. 4B). Additionally, the red strip region expressed sTnT isoforms (S in Fig. 4B). Thus, this is the only muscle which expresses simultaneously B-type and L-type fTnT and sTnT isoforms. No muscle has been found to express both B-type fTnT and sTnT concomitantly until the present study (Yao *et al*., 1992).

The expression patterns of sTnT isoforms are also shown for 12 and 16-day-old embryos in *gastrocnemius*, and 1-day-old and adult chickens in *gastrocnemius*, ALD, *complexus*, and *biventer cervicalis* (Fig. 4C). All the muscles expressed two major isoforms corresponding to those expressed in *pectoralis major* and *biceps femoris*. The result in adult ALD is consistent with that described by Mikawa *et al*. (1981). In addition, three minor isoforms were found in *gastrocnemius* of the adult chicken, and one minor isoform in *biventer cervicalis* of the adult chicken and in *complexus* of the 1-day-old and adult chickens (Fig. 4C, arrows). The minor isoform was also detected in *biceps femoris* of the 19-day-old embryo and 1-day-old chick, although it was very faint (Fig. 4A, arrows).

No expression of sTnT and fTnT was found in cardiac muscles of 7, 10, and 13-day-old embryos and 1 and 5-day-old chicks (data not shown).

Heterogeneity in sTnT mRNA

The minor isoforms were detected in the mixed skeletal muscles (*gastrocnemius*, *biventer cervicalis*, and *complexus*). Our previous study showed the heterogeneity in the 5'-region of sTnT transcripts which lead to three amino acid changes in sTnT proteins (Yonemura *et al*., 2000). Typical slow skeletal muscle, ALD, had two kinds of transcripts, one having the codon for alanine (position 135–137), C (258), and A (262) and the other lacking the codon and having T (258) and G (262) instead of C and A. To determine whether the minor sTnT isoforms can be explained by the heterogeneity in the 5'-region of sTnT transcripts, we performed S1 nuclease mapping of total RNA from six kinds of skeletal muscles. We used as a probe the 690 nt fragment (position

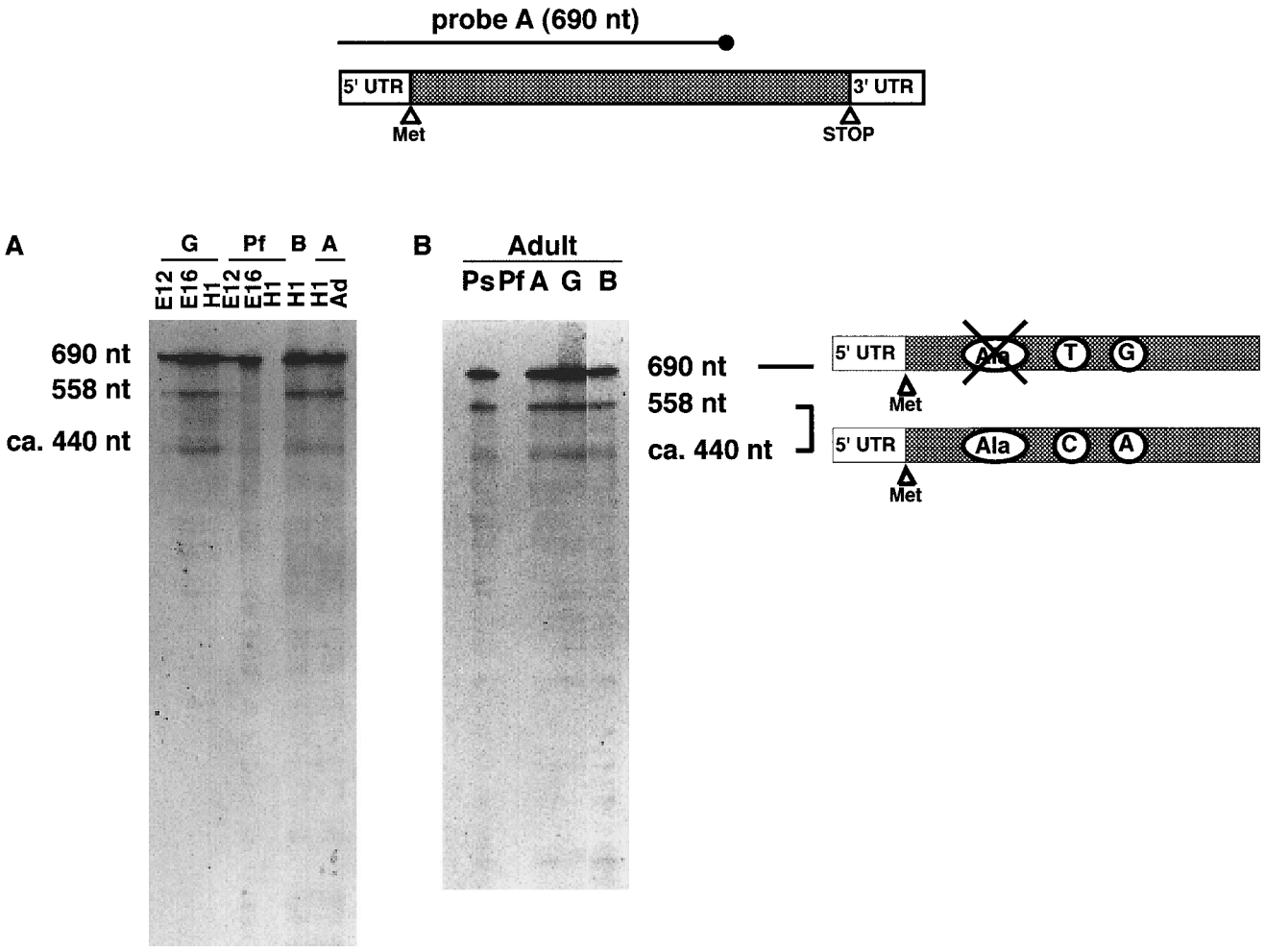

Fig. 5. S1 nuclease mapping of sTnT mRNA with an antisense probe from the 5'-region of sTnT cDNA. The upper panel shows schematically the structure of sTnT mRNA and the position of the 690 nt antisense probe (probe A) with the ^{32}P-labeled 3'-end (closed circle). Total RNA was isolated from *gastrocnemius* (G) and *pectoralis major* (Pf) of 12 (E12) and 16-day-old (E16) embryos and the 1-day-old (H1) chick and from *biventer cervicalis* (B) and ALD (A) of the 1-day-old chick in A. Total RNA was also isolated from the red strip (Ps) and white (Pf) regions of adult *pectoralis major* and from adult *gastrocnemius* (G) and *biventer cervicalis* (B) in B. Total RNA from adult ALD (Ad) was used for a reference in A. RNA was hybridized to the probe A and digested with S1 nuclease. Three bands of 690 nt, 558 nt, and approximately 440 nt were produced by RNA schematically shown on the right.

1 to 690 of chicken sTnT cDNA) which lacks the codon coding for alanine (position at 135–137). When total RNA from *gastrocnemius* (G) of 12 and 16-day-old embryos and the 1-day-old chick and from *biventer cervicalis* (B) and ALD (A) of the 1-day-old chick was examined by S1 nuclease mapping, three bands of 690 nt, 558 nt, and about 440 nt were detected (Fig. 5A). The band of 690 nt corresponds with the transcript lacking the codon for alanine, and the bands of 558 nt and about 440 nt with the transcript containing the codon for alanine (Yonemura *et al*., 2000). No difference in the expression patterns of sTnT transcripts was found during development. On the other hand, when total RNA from *pectoralis major* (Pf) of 12 and 16-day-old embryos and the 1-day-old chick was examined, only the band of 690 nt was detected in 12 and 16-day-old embryos and no band in the 1-day-old chick (Fig. 5A), indicating that the transcript containing the codon for alanine was not expressed in *pectoralis major*.

Three protected bands of 690 nt, 558 nt, and about 440 nt were also detected in the red strip region of adult *pectoralis major* (Ps), ALD (A), *gastrocnemius* (G), and *biventer cervicalis* (B) (Fig. 5B), but not in the white region of adult *pectoralis major* (Pf) as expected. Differences in the expression patterns were not discernible among the four adult muscles which expressed sTnT transcripts, suggesting that the presence of the minor isoforms can not be attributable to the heterogeneity in the 5'-region of sTnT transcripts.

DISCUSSION

In this study, we investigated the developmental changes of cTnT and sTnT isoforms by taking advantage of 2D SDS-PAGE and immunoblotting with specific antisera. The cTnT isoforms were expressed at all the developmental

stages examined herein in the chicken heart, and at the stages before hatching-out in the skeletal muscles. The results indicated differential regulation of cTnT gene expression between cardiac and skeletal muscles. It is supported by the previous studies. Development of the cardiac muscle is triggered by transcriptional regulatory factors, dHAND, eHAND, and Nkx 2.5 (Schwartz and Olson, 1999), but skeletal muscle development by the MyoD family (Olson, 1992). The 247 bp upstream sequence from the transcription initiation site in the chicken cTnT gene is necessary for the gene expression in the cardiac muscle, while only 129 bp is needed for its expression in the skeletal muscle (Mar *et al*., 1988).

Our results also showed cTnT transition from high-molecular-weight to low-molecular-weight isoforms in the avian and mammalian hearts, but not at all in the chicken skeletal muscles and less strikingly in the mouse leg muscle. Genomic programming of cTnT synchronized in cardiac and skeletal muscles was proposed by analysis of limited developmental samples (Jin, 1996). The present results clearly did not support synchronized genomic programming of cTnT, because expression of cTnT is regulated differentially between cardiac and skeletal muscles. We found 4 major and 2 minor isoforms in the chicken and at least 8 isoforms in the mouse. Cooper and Ordahl (1985) reported two variants of chicken cTnT cDNA, and Jin *et al*. (1996) four variants of mouse cTnT cDNA. Since the chicken cTnT gene has only one alternatively spliced exon, it is likely that two of 4 major isoforms are phosphorylated forms of respective counterparts, giving two pairs of protein spots with slightly different pIs as can be seen on immunoblotting patterns. The first amino acid serine is known as the primary phosphorylated site in cTnT. The two minor isoforms appear not to be degradation products, because they were reproducibly observed after embryonic day 19. However, the origin of the isoforms is not known at the present time.

The sTnT isoforms were expressed not only in the slow and mixed skeletal muscles, but also in the fast skeletal muscles until around hatching-out. The regulation and structure of the chicken sTnT gene have not been elucidated, but our results indicated differential regulation of the gene expression between the slow and fast skeletal muscles. This is supported by previous studies (Hughes *et al*., 1993; Blagden *et al*., 1997). We found two major isoforms commonly expressed in all the skeletal muscles examined in this study and three minor isoforms in some muscles and at the particular developmental stages. One of the major isoforms is assumed to be a phosphorylated form of the other, because at least one amino acid is phosphorylated in cTnT and fTnT although the post-translational modifications of sTnT have not been studied yet. It seems unlikely that the minor isoforms are degradation products or proteins which nonspecifically reacted with the anti-sTnT antiserum, because they were observed reproducibly after embryonic day 19. Also ALD and *pectoralis major* which were prepared for electrophoresis in the same way as the other muscles gave no minor isoforms. Furthermore, the muscles at younger stages which are possibly more susceptible to protein degradation due to their immature labile structure did not present the minor isoforms.

We investigated the heterogeneity of sTnT transcripts by S1 nuclease mapping to search the background for the minor isoforms. Our previous study showed two variants of sTnT transcripts, one having the codon for alanine (135-137) and C (258) and A (262) and the other lacking the codon and having T (258) and C (262) in the 5' region (Yonemura *et al*., 1996; 2000). The differences lead to three amino acid substitutions. Our results showed that all the muscles expressing sTnT gave the protected bands of 690 nt, 558 nt, and about 440 nt except for embryonic *pectoralis major*, where only the band of 690 nt was detected. Therefore, the minor isoforms were not produced by the heterogeneity in the 5' region of sTnT, because their expression did not correlate with expression of transcripts indicated by protected bands. However, since the gene structure, alternative splicing pathways, and post-translational modifications of sTnT are poorly understood at present, it is possible that the minor isoforms are genuine sTnT isoforms produced by different pathways of alternative splicing and/or differential post-translational modifications.

In the progress of this study, Krishan *et al*. (2000) reported that sTnT mRNA was detected in the mouse fetal cardiac muscle. However, our study at the protein level showed no expression of sTnT in the chicken cardiac muscle. Barton *et al*. (1999) also showed no sTnT mRNA expression in human fetal and adult cardiac muscles. We do not know whether the discrepancy was caused by differences in maturation of cardiac muscles or by differences in examined animals.

The red strip region of *pectoralis major* is unique, because it expressed L-type and B_N-, B_C-, and B_A-subtype fTnT isoforms, and concomitantly sTnT isoforms. The extensive study of distributions of fTnT and sTnT isoforms in the chicken muscles (Yao *et al*., 1992) did not show any muscle which expressed simultaneously B-type fTnT and sTnT isoforms. The red strip region of *pectoralis major* has type II fibers expressing fast-skeletal-muscle-type isoforms and type I fibers expressing slow-skeletal-muscle-type isoforms (Matsuda *et al*., 1983; Edman *et al*., 1988; Reiser *et al*., 1996), although the bulk white region has type II fibers (Obinata, 1985). Therefore, the region is a suitable area to search for fiber-type-specific gene expression and expressional regulatory factors of fTnT and sTnT genes as well as alternative splicing factors for the fTnT gene.

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