动物学报 **49**(3) :362～369 , 2003
Acta Zoologica Sinica

Troponin T isoforms in rat fast skeletal muscles *

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Abstract The relaxation and contraction in vertebrate skeletal muscle are regulated by Ca^{2+} through troponin and tropomyosin , which are located in the thin filament. Troponin is composed of three components , troponins C , I and T. Rat troponin T isoforms were examined in fetal , neonatal and adult rat skeletal muscles using two-dimensional polyacrylamide gel electrophoresis and immunology. Ten kinds of isoforms were discovered in adult rat fast skeletal muscles. Seven kinds of isoforms were found in fetal and neonatal rat skeletal muscles. These isoforms of troponin T are valuable in the development of specific markers in different tissues in different development stages and in different animals. The new TnT isoforms in rat skeletal muscles described in this paper will be useful in future research on specific markers [*Acta Zoologica Sinica* **49** (3) : 362 - 369 , 2003].

Key words Rat , Skeletal muscle , Troponin T , Isoform , Two-dimensional polyacrylamide gel electrophoresis

大鼠骨骼肌快肌肌钙蛋白 T 的同工型 *

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摘 要 骨骼肌快肌的收缩主要是由钙离子通过肌钙蛋白所调节控制。这些肌钙蛋白位于肌纤维之中。肌蛋白包括肌钙蛋白 T、肌钙蛋白 C、肌钙蛋白 I。采用双向聚丙烯酰胺凝胶电泳和免疫学技术 , 对大鼠胚胎、新生大鼠和成年大鼠的骨骼肌快肌肌钙蛋白 T 的同工型进行了研究。在成年大鼠的骨骼肌快肌中 , 发现了 10 种肌钙蛋白 T 同工型。在大鼠胚胎和新生大鼠的骨骼肌中 , 发现了 7 种肌钙蛋白 T 同工型。作为不同动物、不同发育阶段和不同组织发育的特殊标记 , 这些肌钙蛋白 T 同工型具有重要意义 [动物学报 **49** (3) : 362～369 , 2003]。

关键词 大鼠 骨骼肌 肌钙蛋白 T 同工型 双向聚丙烯酰胺凝胶电泳

Troponin is an important component of the thin filaments which give calcium sensitivity to muscle fibers (Ebashi , 1984; Morimoto *et al.* , 1998). Troponin is composed of troponin I (TnI) , troponin C (TnC) , and troponin T (TnT). These subunits , and especially TnT have been shown to have many different isoforms in different muscles. For example , the rabbit has five and the chicken has at least 70 isoforms of TnT in fast skeletal muscle (Briggs *et al.* , 1987; Imai , 1986). In rat fast skeletal muscles , SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with a monoclonal antibody against fast skeletal muscle TnT (Sabry *et al.* ,1991) has detected five isoforms (AF1-AF5) in adults , two isoforms (FF1-FF2) in the fetus , and two isoforms (NF1 and NF2) in neonatal animals. However , the study of the rat fast skeletal muscle TnT gene has shown a possibility that the gene could express the maximum of 64 isoforms through differential alternative splicing of exons (Breitbart *et al.* , 1985). Therefore , the number of isoforms which were detected so far is much smaller than that predicted from

Received Mar. 15 ,2002 ;revised Mar. 08 ,2003
* This research was funded by Japan Science and Technology Corporation
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Brief introduction to the first author : Female , Ph. D. Research interest :culture of bone marrow stem cells.

gene organization. The present study was undertaken to comprehensively enumerate the TnT isoforms in rat fast skeletal muscles by two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE) and immunoblotting with polyclonal antibody against rat skeletal muscle TnT. Ten isoforms in the adult and seven isoforms during the late fetal to neonatal stages of development were detected. The expression patterns of these TnT isoforms was found to vary among different muscles. Chicken isoforms have been classified into two types, breast-fast-muscle-type and leg-fast-muscle-type, based on differences in expression patterns of the isoforms between breast and leg muscles (Yao *et al*., 1992). However, the rat TnT isoforms in fast skeletal muscle are not so many and can not be classified into types.

In this study, the troponin T isoform patterns of rat fast skeletal muscles were investigated to detect all isoforms expressed in rat muscles and to understand changes in their expression through development.

1 Materials and methods

1.1 Materials

Rat were purchased from CLER JAPAN, INC. Fetal and postnatal skeletal muscles were dissected out from rats, cut into small blocks and stored at - 20 ℃ until used.

1.2 Reagents

Agarose (agarose IEF) and ampholytes (pharmalyte) were made by pharmalia fine chemicals. Coomassie brilliant blue R and bovine serum albumin were made by BDH chemicals. Rhodomine (TRITC)-conjugated goat anti-rabbit IgG (H + L) was made by Jackson Immunoresearch Laboratories, Inc., bromophenol blue and haematoxylin crystals was made by Merck. Eosin was made by Chroma-Gesellschaft. The other regents were produced by Wako Pure Chemical Industries.

1.3 Electrophoresis

Two-dimensional SDS-polyacrylamide gel electrophoresis (2D SDS-PAGE) was carried out according to the improved method of Hirabayashi with some previously described modification (Hirabayashi, 1981; Xie, 1992). One percent nonidet P-40 was in-

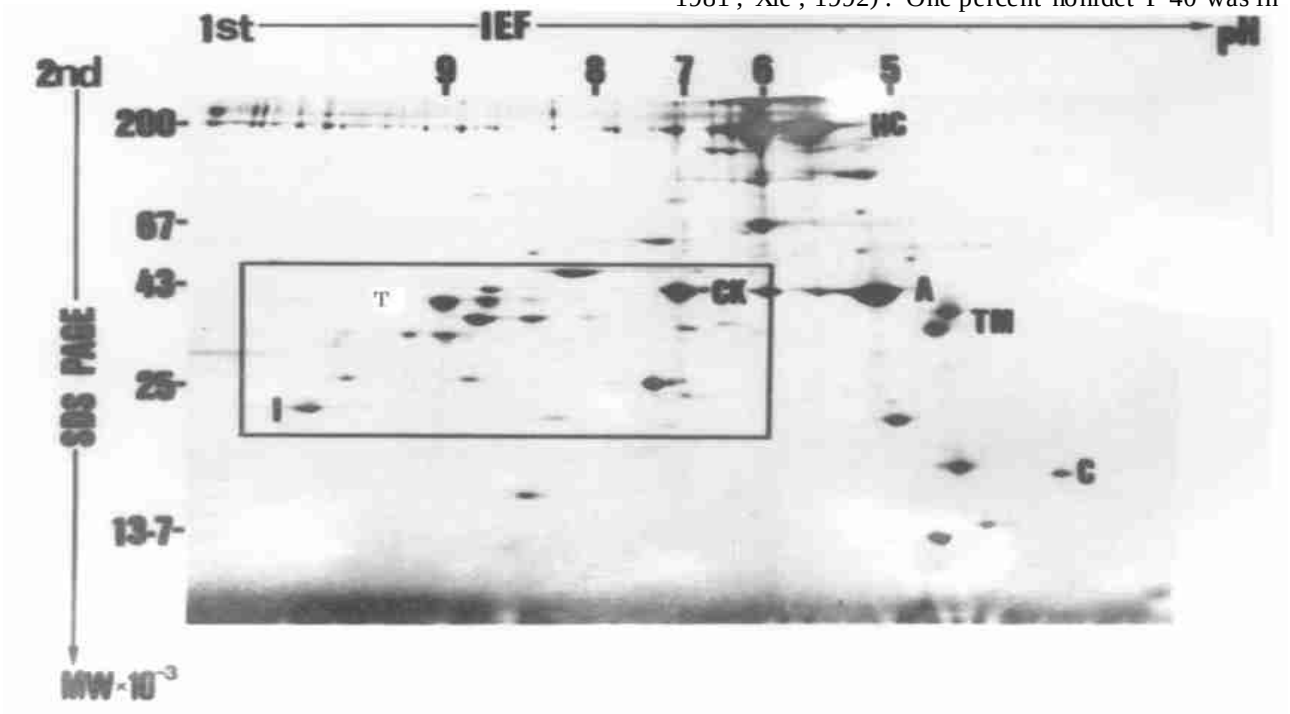

Fig. 1 SDS-PAGE protein map of adult rat muscle

2D SDS-PAGE pattern of adult rat *pectoralis major muscle*. First dimension isoelectric focusing was performed with 40 μl of the muscle extract about (200 μg protein). The pH range was approximately from 3.0 to 9.5. The second dimension SDS-PAGE was performed with a concentration gradient of 14 % acrylamide. A: Actin HC: Myosin heavy chain CK: Creatine kinase T: Troponin T I: Troponin I C: Troponin C TM: Tropomyosin

cluded in both the extraction medium, for muscle homogenization, and the agarose gel for isoelectric focusing.

1.4 Making anti-rat troponin T antibody

Polyclonal antibody against rat fast skeletal muscle TnT was elicited by injecting TnT subcutaneously into a rabbit. Using polyclonal antibody against chicken fast skeletal muscle TnT, one spot of fast skeletal muscle TnT was detected on 2D SDS-PAGE gels of rat *pectoralis major* muscle and was cut out and emulsified with Freund's complete adjuvant (Difco). An antiserum of reasonably high titer was obtained after the second weekly injection. This antibody strongly reacted with rat troponin T, and has cross reaction with chicken troponin T.

1.5 Immunoblotting

Immunoblotting was performed according to the procedure of Franke *et al*. with some modification (Franke *et al*., 1981; Hirai, 1983). Protein in polyacrylamide gels were transferred onto nitrocellulose paper sheets (Sartorius; pore size 0.2 μm) by electrophoresis for 3 h at 300 mA at 0 ℃. The sheets were then incubated with 2% bovine serum albumin with polyclonal antibody against rat fast skeletal muscle TnT, and then with rhodamine-conjugated goat anti-rabbit IgG, and washed after each incubation. The dried sheets were photographed under long wavelength UV light.

2 Results

When we analyzed the rat *pectoralis major* skeletal muscle with the improved 2D SDS-PAGE (Hiralbayashi, 1981; Nakamura *et al*., 1989), many scattered protein spots with a wide range of molecular weights (10～200 kDa) and isoelectric points (pH 3.0～9.5) could be observed (Fig. 1). Therefore, it was difficult to identify TnT within a constellation of protein spots. This area is boxed in Fig. 1.

To detect TnT isoforms, we prepared polyclonal antibody against rat fast skeletal muscle TnT and examined the expression of TnT isoforms of 21 kinds of adult rat skeletal muscles on 2D SDS-PAGE patterns by immunoblotting with the polyclonal antibody. Seven representative patterns showing the expression of TnT isoforms are presented in Fig. 2. We found ten kinds of TnT isoforms in rat adult skeletal muscles and determined molecular weight and isoelectric points of these isoforms. The adult isoforms were termed as AF with the numbers according to their molecular weights and isoelectric points. The results are shown in Table 1. The distribution of TnT isoforms in 21 kinds of adult rat skeletal muscles are summarized in Table 2. AF5.3, AF5.2 and AF5.1 were found in many kinds of muscles, but a few muscles expressed AF4.4.

We examined developmental changes of TnT isoforms using *pectoralis major* and *gastrocnemius*.

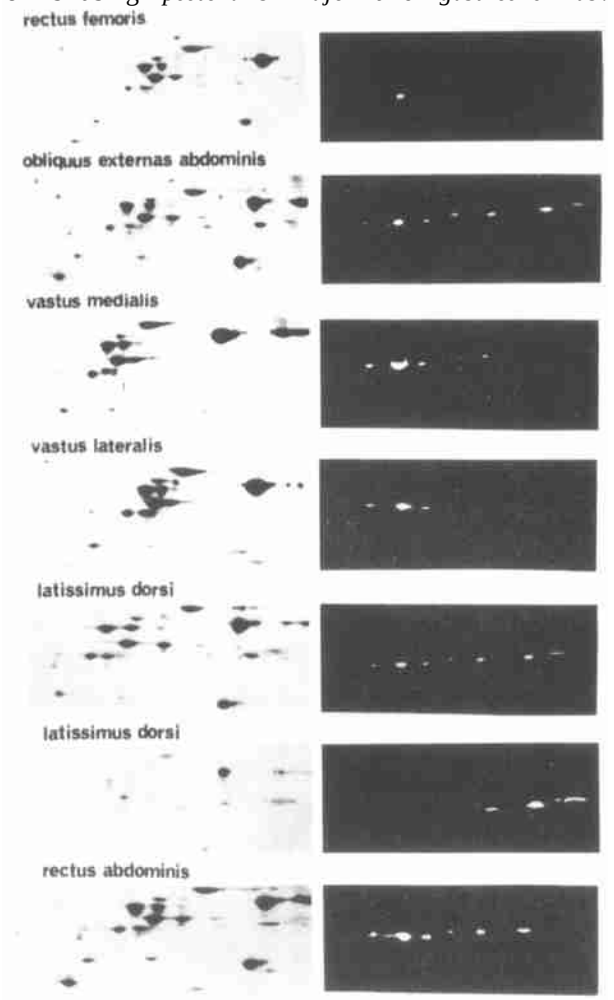

Fig. 2 Fast Tn T isoforms of adult rat muscles

TnT isoforms in seven rat skeletal muscles. 2D SDS-PAGE gels were stained with Coomassie brilliant blue R (left), concentration of acrylamide of the second dimension gel was 14%. Immunoblotting of 2D SDS-PAGE patterns was carried out with polyclonal antibody against rat fast skeletal muscle TnT (right).

The results are shown in Fig. 3 and 4. At the fetal stage (18 days post-conception), four TnT isoforms were detected and their molecular weights and isoelectric points were determined in Table 3. At the neonatal stage, three other isoforms were detected and their molecular weights and isoelectric points were determined (Table 4). The fetal and neonatal isoforms were termed FF and NF, respectively, and numbered according to their molecular weights and isoelectric points. Developmental changes of TnT isoforms in *pectoralis major* and gastrocnemius are schematically represented in Fig. 5 and 6. We found that there were some differences in the developmental changes of TnT isoforms between *pectoralis major* and *gastrocnemius*. Fetal isoforms were expressed at developmental stages from 18-day-old embryos to 21-day-old rats, but the expression of FF1.2 in *pectoralis major* ended when rats were born. However, stages of NF1.1 were not different, and the stages when the expression of NF1.2 and NF2.3 started were different between *pectoralis major* and *gastrocnemius*. Furthermore, there were large differences in developmental changes of adult isoforms.

Table 1　Molecular weights and isoelectric points of adult rat troponnin T isoforms

	pH9.5	9.2	9.0	8.5	8.0	7.0	6.5
33.0 kD	-	-	-	-	-	-	AF1.7
31.0 kD	-	-	-	-	-	AF2.6	AF2.7
30.5 kD	-	-	-	AF3.4	AF3.5	-	-
29.5 kD	-	-	-	AF4.4	AF4.5	-	-
29.0 kD	AF5.1	AF5.2	AF5.3	-	-	-	-

All isoforms found in 21 kinds of rat adult fast skeletal muscles and developing rat *pectoralis major* and *gastrocnemius* muscle are schematically represented in Fig. 7.

Table 2　Distribution of troponin T in adult rats

Isoelectric points	AF1.8	AF2.7	AF2.6	AF3.5	AF3.4	AF4.5	AF4.4	AF5.3	AF5.2	AF5.1
Rectus femoris	+	-	+	-	-	+	-	+	+	-
Vastus intermedius	+	-	+	+	-	+	-	+	+	+
Vastus medialis	-	-	-	+	+	-	-	+	+	+
Vastus lateralis	-	-	-	-	+	-	-	+	+	+
Sartorius	+	-	+	+	+	+	-	+	+	+
Rhomboideus	-	+	+	-	-	-	-	+	+	+
Biceps femoris	+	-	+	+	-	+	-	+	+	+
Pectoralis major	+	+	+	+	+	+	-	+	+	+
Rectus abdominis	+	+	+	+	+	+	+	+	+	+
Obliquus externas abdominis	+	+	+	+	+	+	-	+	+	+
Latissimus dorsi	+	+	+	+	+	+	+	+	+	+
Deltoideus	+	+	+	+	+	+	-	+	+	+
Biceps brachii	+	+	+	+	+	+	-	+	+	+
Extensor carpi radialis	+	+	+	+	+	+	-	+	+	+
Triceps brachii	-	-	-	-	-	-	-	-	+	+
Digastricus	+	-	+	+	+	+	-	+	+	+
Flexor digitorum congus	+	+	+	+	+	-	-	+	-	-
Tibialis anterior	+	-	+	+	+	+	-	+	+	+
Gastrocnemius	+	-	+	-	-	-	-	+	+	+
Tibialis posterior	+	+	+	+	+	+	+	+	+	+

+ : indicates the presence of isoforms　　- : indicates the absence of isoforms

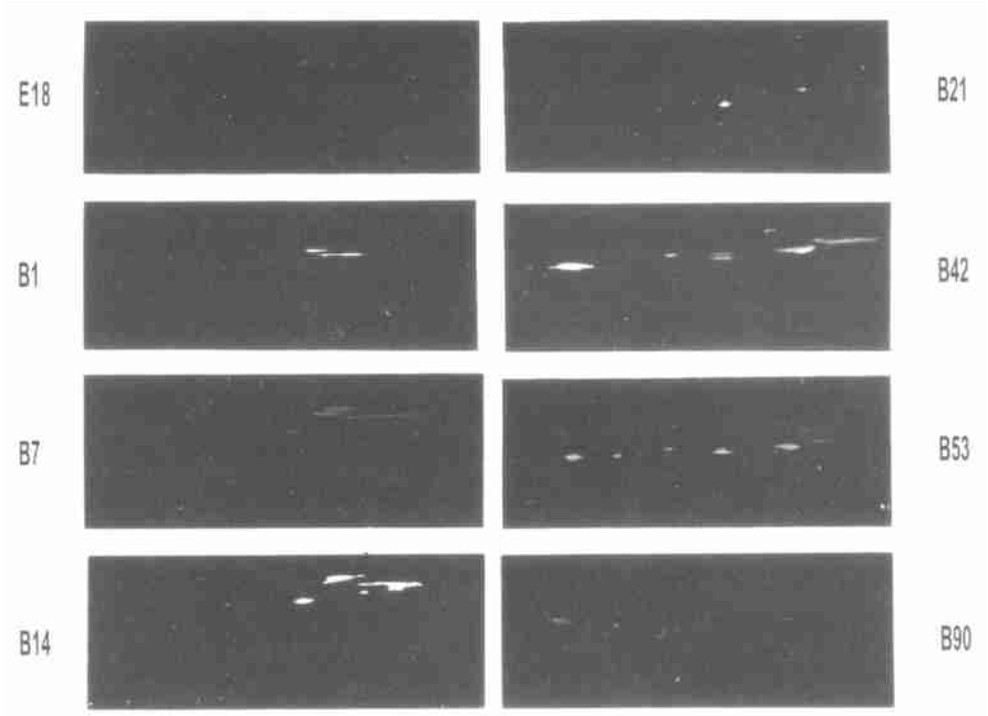

Fig. 3 Developmental change of Tn T isoform composition (rat Pectorails major**)**

The concentration of acrylamide in the second dimension gel was 14 %. Immunoblotting of 2D SDS-PAGE patterns was carried out with polyclonal antibody against rat fast skeletal muscle TnT. E18: 18-day-old embryo B1: 1-day-old rat B7: 7-day-old rat B14: 14-day-old rat B21: 21-day-old rat B42: 42-day-old rat B53: 53-day-old rat B90: 90-day-old rat

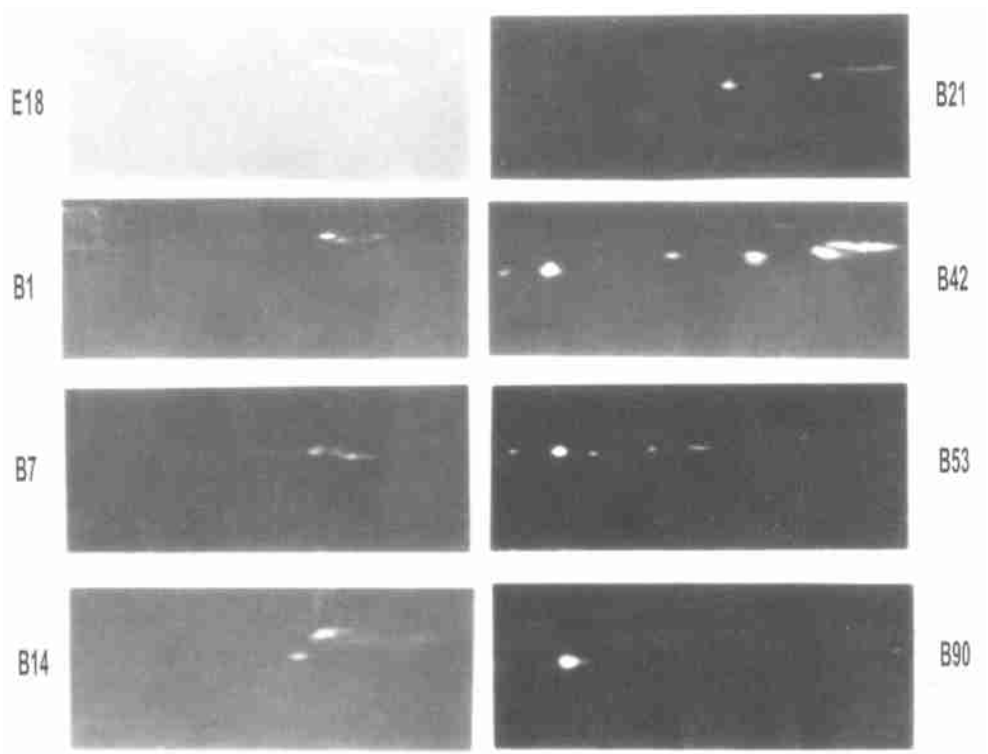

Fig. 4 Developmental change of Tn T isoforms composition (rat gastrocnemius**)**

The concentration of acrylamide in the second dimension gel was 14 %. Immunoblotting of 2D SDS PAGE patterns was carried out with polyclonal antibody against rat fast skeletal muscle TnT. E18: 18-day-old embryo B1: 1-day-old rat B7: 7-day-old rat B14: 14-day-old rat B21: 21-day-old rat B42: 42-day-old rat B53: 53-day-old rat B90: 90-day-old rat

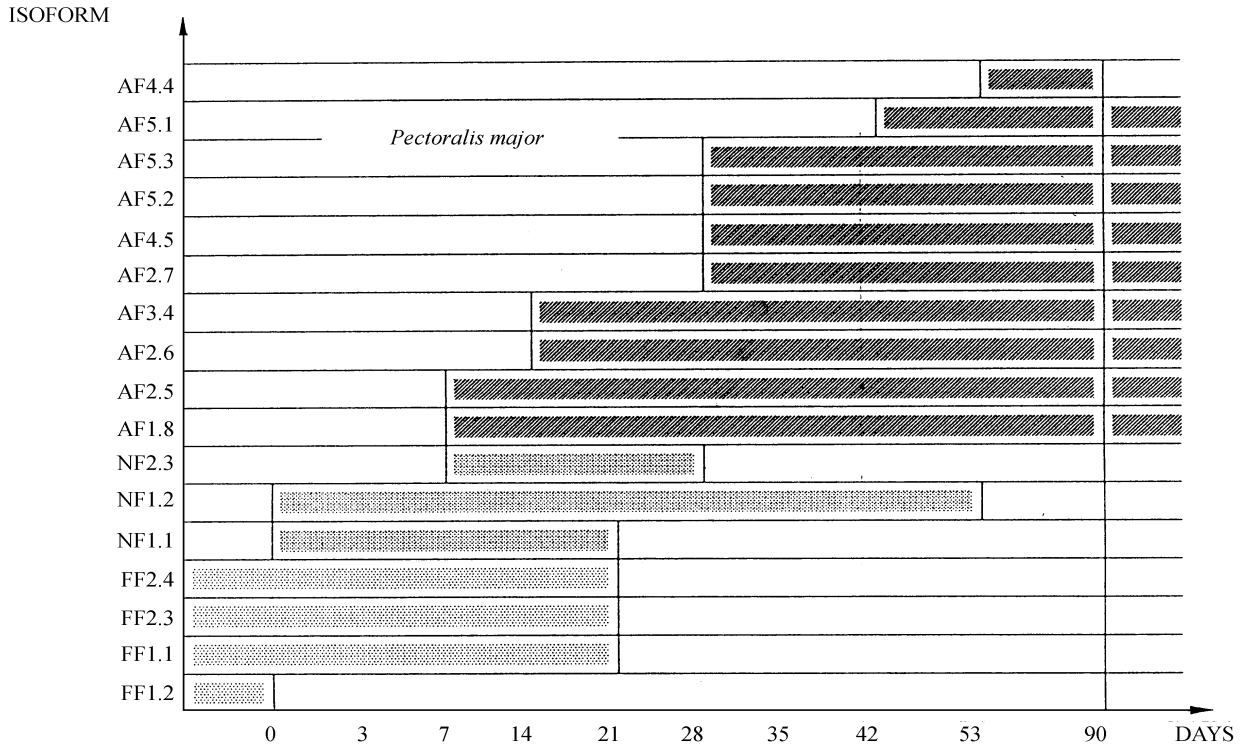

Fig. 5 Developmental change of Tn T isoform in rat pectorails major **(schematic presentation)**

The abscissa shows age of rats after birth. The ordinate shows rat TnT isoforms. The shaded regions indicate the expression of the isoforms

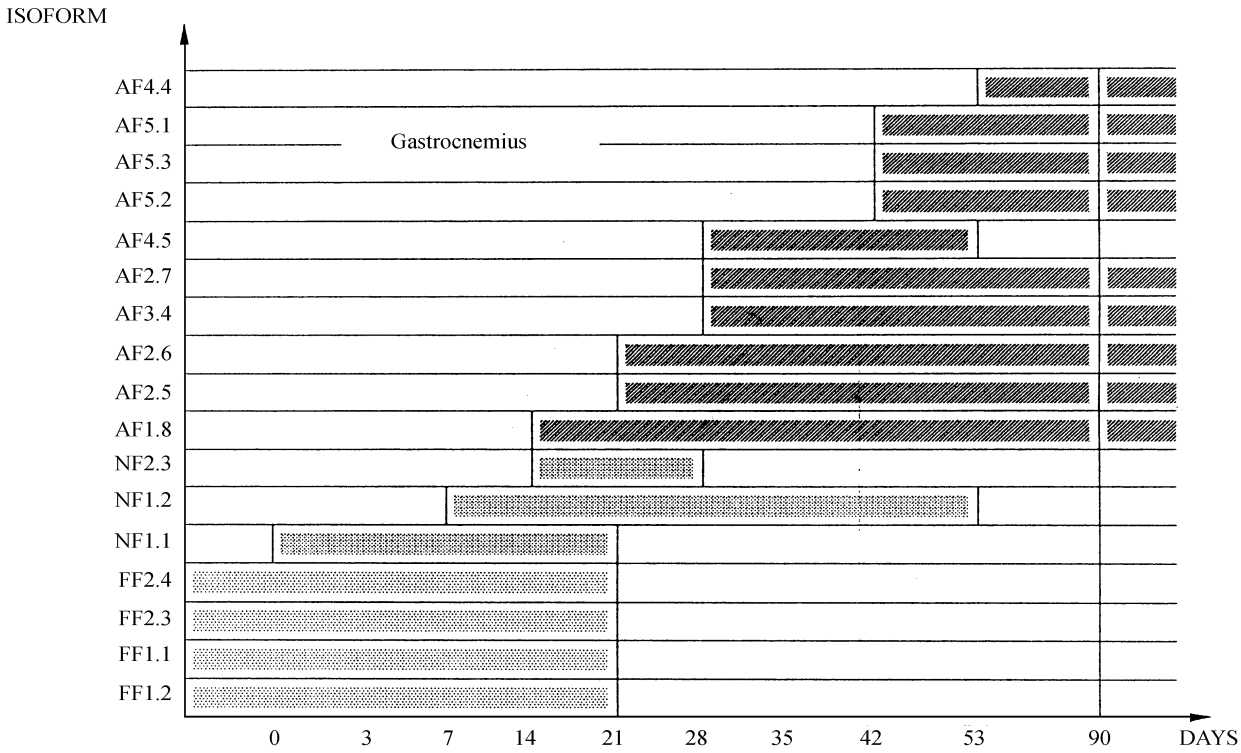

Fig. 6 Developmental change of Tn T isoform in rat gastrocnemius (schematic presentation)

The abscissa shows age of rats after birth. The ordinate shows rat TnT isoforms. The shaded regions indicate the expression of the isoforms

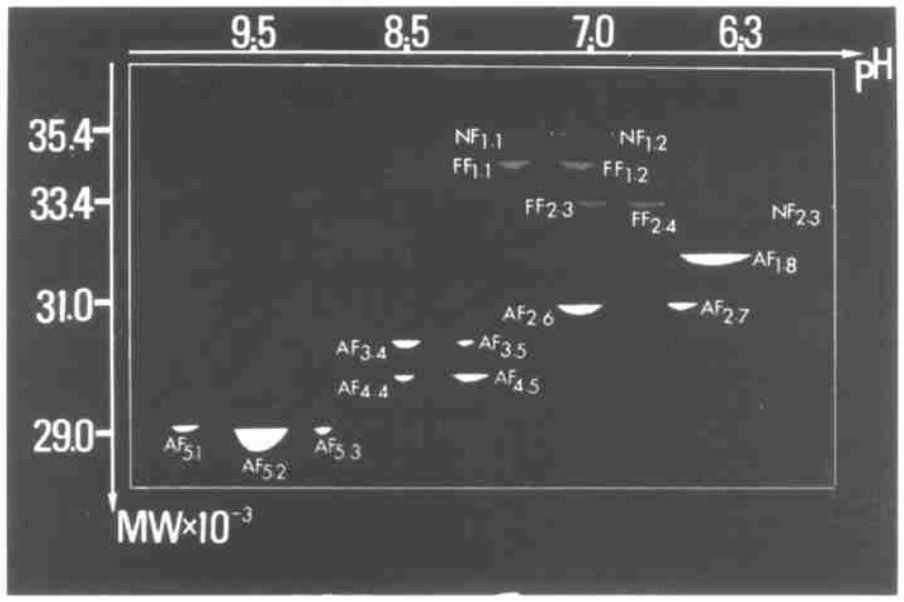

Fig. 7 Schematic presentation of rat fast muscle TnT

The range of molecular weights of the isoforms is from 29 to 35.4 kDa, and that of isoelectric points is from pH 6.3 to 9.7 FF: fetal isoforms NF: neonatal isoforms AF: adult isoforms

Table 3 Molecular weights and isoelectric points of fetal rat troponnin T isoforms

	pH7.4	7.2	7.0	6.7
35.0 kD	FF1.1	FF1.2	-	-
33.4 kD	-	-	FF2.3	FF2.4

Table 4 Molecular weights and isoelectric points of neonatal rat troponnin T is oforms

	pH7.3	7.1	6.3
35.4kD	NF1.1	NF1.2	-
33.4kD	-	-	NF2.3

3 Discussion

In this study, ten kinds of TnT isoforms were detected in 21 kinds of adult rat skeletal muscles. The adult isoforms in rat fast skeletal muscles did not usually appear in significant amounts until the early or late neonatal stage. The levels of expression of these isoforms varied among different skeletal muscles. In chickens, fast skeletal muscle TnT isoforms are classified into two types, breast-fast-muscle-type and leg-fast-muscle-type, because there are large differences in TnT isoform compositions between the leg and breast muscles (Yao *et al.*, 1992). However, we could not find sufficient differences among rat skeletal muscles to justify classifying TnT isoforms into different types.

Although chicken TnT has over 70 isoforms, rat TnT did not have so many isoforms. The study on the TnT gene of rat fast skeletal muscle has shown that the gene can potentially produce 64 different isoforms through alternative splicing of exons (Breibart *et al.*, 1985). Why have we found only four kinds of isoforms in the fetal stage, three kinds of isoforms in the neonatal stage, and ten kinds of isoforms in adults? We considered that there may be three cases as follows. 1. Additional isoforms are expressed in adult muscles other than the 21 kinds of adult muscles examined in this study. 2. Additional isoforms are expressed during development in muscles other than *pectoralis major* and *gastrocnemius* which were examined developmentally in this study. 3. Additional isoforms are expressed under special conditions, for example, hard training of rats. On the other hand, although the TnT gene of rat fast skeletal muscle may not express as many as 64 kinds of isoforms until now, only ten kinds of TnT mRNA had been detected in adult rat fast skeletal muscles (Breibart *et al.*, 1985; Morgan *et al.*, 1993).

We found that fetal, neonatal, and adult TnT

isoforms were selectively expressed in these developmental stages. However, we did not find fast skeletal muscle isoforms of TnT in early fetal (13-day-old embryo) muscle (data not shown). This is presumably because of the predominance of embryonic slow skeletal muscle-like isoforms of TnT at this stage of development (Sabry *et al*., 1991; Krishan *et al*., 2000). The developmental changes of TnT isoforms in rat fast skeletal muscle were very similar to those in chicken breast fast skeletal muscle. In chicken breast muscle, embryonic isoforms, neonatal chick isoforms and adult isoforms are expressed in the course of development. It is interesting that fast skeletal muscle TnT isoforms considerably change in isoform composition during development. These changes may be stimulated by innervation or the increase of thyroid hormones. In the next study, we will try to reproduce the developmental changes of isoforms in an *in vitro* cell culture system and try to investigate the mechanism of these changes.

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