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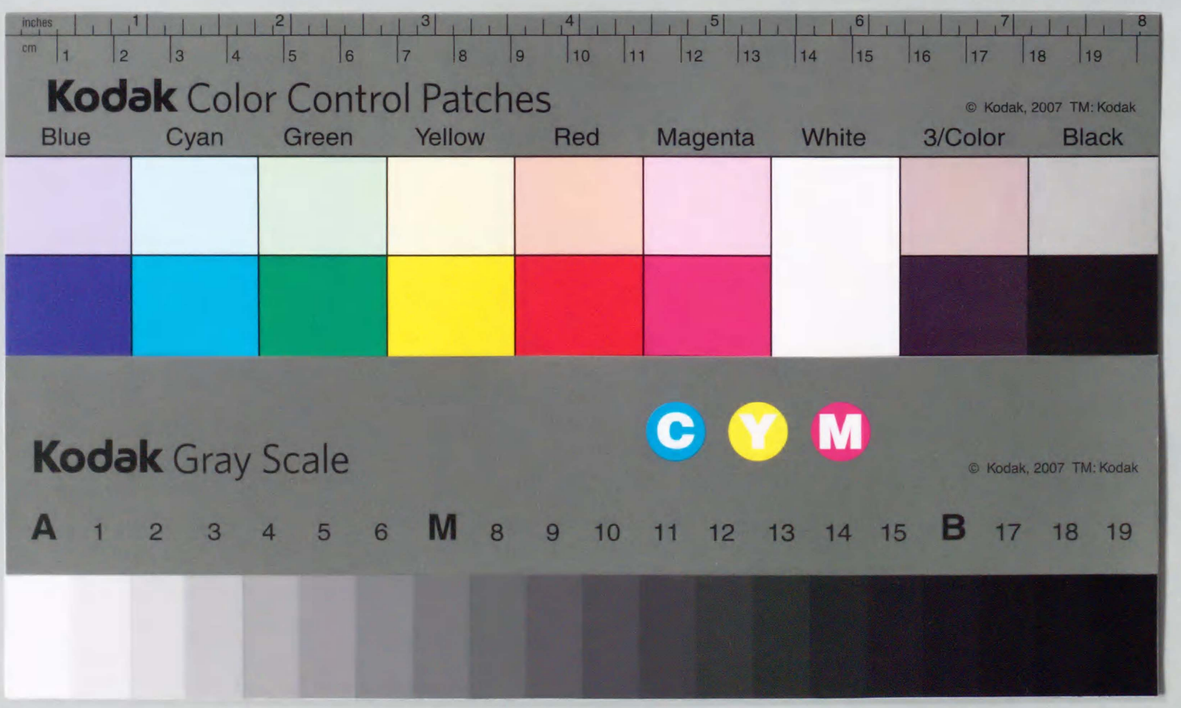

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January, 1997

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Science, in the Doctoral Program in Biological Sciences, University of Tsukuba.

Contents

Abstract

Cells prepared from chicken skeletal muscles of different developmental stages were cultured to study their troponin T isoform expression, using antisera specific to fast- and slow-muscle-type isoforms. It was found that cultured myogenic cells from chicken and chick embryos could be classified into two types, fast type and fast/slow type in which fast- and slow-muscle-type isoforms were coexpressed. Cells expressing only slow-muscle-type troponin T isoforms could not be found. Most cells prepared from *pectoralis major* (fast muscle) and *gastrocnemius* (mixed muscle) of embryos earlier than the 11th day of incubation belonged to the latter, with only a small fraction belonging to the former. The percentage of fast type cells in the cells prepared from *pectoralis major* increased along development to over 90% by the 17th day of incubation, while, in the cells prepared from *gastrocnemius*, it reached a plateau of 30-40% by the 13th day of incubation. All the cells from *anterior latissimus dorsi* (slow muscle) belonged to the fast/slow type. The *in vitro* expression of troponin T isoforms was different from the *in vivo* expression, and each muscle seems to be determined differently in the composition of cell types during the developmental course. Since two distinct populations of cells committed to myogenic cell lineages were supposed to give rise to the two types of myotubes, the serial subcloning were performed to investigate the intrinsic stability of troponin T expression of the cultured myogenic cells. The results of clonal analysis suggested that the expression pattern of troponin T isoform in cultured muscle cells is stable and that myogenic cell lineages play an important role in giving rise to different muscle types.

Introduction

One of the ultimate aims in studying the development of multicellular animals is to elucidate the mechanisms that are involved in the transformation of the fertilized egg into a new individual. This process of cell multiplication determine the phenotypes of differentiated cells. One approach to understanding the complex regulation underlying the development is to elucidate the steps that commit a cell to a specialized function in a particular tissue.

The generation of multinucleated skeletal muscle fiber by the fusion of myoblasts (myogenic progenitor cells) is increasingly proving to be an attractive system for studying the mechanisms that govern tissue differentiation and maturation. That is, the formation of skeletal muscle *in vivo* is easily followed; myoblasts are mesodermal in origin; skeletal muscle fibers are formed by temporally discrete phases of myoblast fusion to form multinucleated muscle cells, termed myotubes that mature into myofibers. In the limbs of birds and rodents, the first phase of myoblast fusion (primary myogenesis) gives rise to primary myotubes which begin to form before the cleavage of the muscle mass into discrete muscles. The second phase of myoblast fusion (secondary myogenesis) starts after all the primary myotubes have formed and these secondary myotubes is formed closely contacted to the primary myotubes (Condon *et al.*, 1990). In humans the third phase of myoblast fusion may give rise to what have been termed tertiary myotubes (Draeger *et al.*, 1987).

The *in vitro* formation of muscle fibers also can be followed. Myoblasts, prepared by trypsinization of muscle tissue can be grown as monolayers and fuse together. Since single muscle cells isolated are capable of forming in culture a muscle colony within which the differentiation of multinucleated cross-striated muscle fibers occurs (Konigsberg, 1963), this culture system is very useful for studying the molecular and cellular bases of commitment and identifying factors that regulate the commitment to a

particular cell lineage. The idetification and characterization of the family of muscle regulatory factors MyoD (Davis *et al.*, 1987), myogenin (Wright *et al.*, 1989), myf-5 (Braun *et al.*, 1989), and MRF4 (Rhodes and Konieczny, 1989) have greatly contributed to our understanding of myogenesis during the last decade. Thus, our understanding of the factors regulating muscle cell commitment and differentiation has been advanced, but little is known about the mechanisms controlling the diversity among muscle fibers.

One crucial problem of skeletal muscle development is the mechanism by which muscle fiber diversity is generated. In skeletal muscles, proteins specific to myofibril and sarcoplasmic reticulum and enzymes essential for metabolism are uniquely expressed. Among them, contractile proteins have families consisting of very similar isoforms. The term isoform has been restricted to proteins with the same biological activity or the same role with different structure but originating from the same genome. The generation of isoform diversity has been accomplished predominantly by two mechanisms: The differential expression of multigene families and the alternative splicing of a single gene or use of alternative promoters. The isoforms of a given myofibrillar proteins can be considered as interchangeable components of sarcomeric machinery. Isoform switching occurs in relation to fiber type diversification during muscle development and regeneration, and different sets of those families of isoforms are considered to be coordinately regulated within developmentally and functionally distinct types. A major open issue in muscle development is the origin of muscle diversity (Bandman, 1992; Olson, 1992; Schiaffino and Reggiani, 1996).

In some cases, the heterogeneity of muscle fibers is usually explained on the basis of functional demands, innervation and activity pattern, and to a minor extent, response to hormonal signal. Until recently, the predominant view was that myoblasts are in a fairly homogenous population, implying that muscle fibers diversify as or after they form. Several sets of experiments supporting this view are discussed further

below. A normally 'fast' muscle became 'slow' if it was reinnervated by a nerve that normally supplied a 'slow' muscle (Buller *et al.*, 1960). Moreover, grafting experiments in birds showed that cells in particular somites are not irreversibly committed to specific muscles; for example, somites transplanted from a cervical to a lumbar position gave rise to normal hindlimb muscles that became innervated by motor axons from appropriate lumbar spinal segments (Lance-Jones, 1988). Indeed, it seems likely that connective tissue cells contribute to the patterning of the muscles and that extracellular cues play important roles in guiding axons to appropriate destinations (Lance-Jones and Dias, 1991). Pette and colleagues studied the influence of electrically induced contractile activity on myosin heavy chain (MyHC) and myosin light chain isoform patterns in the cultured chicken muscle cells (Dusterhoft and Pette, 1990), on MyHC and troponin subunits isoform patterns in the rabbit muscles (Leeuw and Pette, 1993), and on MyHC isoform pattern in the cultured rat muscle cells (Wehrle *et al.*, 1994). The results of these experiment suggest that enhanced contractile activity promotes the expression of the slow phenotype. Izumo *et al.* (1986) observed that expression of MyHC isoforms in adult rat is sensitive to thyroid hormone. Taken together, these and other studies suggest that initially similar myoblasts are diversified by extracellular factors that act at relative late stages of myogenesis.

However, none of these results shows directly that myoblasts are homogeneous, and several studies have provided evidence for a diversity of myoblasts, and some differences among myoblasts are now known to be heritable and thus may reflect distinct myogenic sublineages. The work of Hauschka and colleagues in the mid 1970s provided the first evidence that myoblasts were not equivalent. Using different cell culture conditions, they demonstrated that chicken embryonic and fetal myoblasts could be subdivided into different classes, based on their dependence on the presence of conditioned medium. The conditioned medium-dependent group could be further

subdivided into at least two classes based on the morphology of myotubes they formed in culture (White *et al.*, 1975). More recently, Stockdale and colleagues have convincingly demonstrated that chicken embryonic myoblasts can be subdivided into two classes based on the type of MyHC that they express when differentiated into myotubes. One class expresses only fast MyHC isoforms whereas the second expresses both fast and slow MyHC isoforms. In contrast, fetal myoblasts were found to initially express fast MyHC isoforms, and with time also expressed slow MyHC isoforms (Miller and Stockdale, 1989; Stockdale and Miller, 1987). Up to date, the predominant view is that the sequential generations of muscle fibers and the expression of unique proteins depend on the developmental programs of myogenic cells and are diversified by extracellular cues (Cossu and Molinaro, 1987; Donoghue and Sanes, 1994; Gunning and Hardeman, 1991; Miller, 1992; Stockdale, 1992).

Though many proteins are responsible for the functions of muscle, it is the ATPase activity of the MyHC that appears to determine the contraction velocity of a particular muscle fiber and it is also the basis for the standard histochemical classification of fast and slow muscle fiber type (Bandman, 1992; Schiaffino and Reggiani, 1996). In fact, MyHC is considered useful for studying myogenic cell lineage. The many isoforms of MyHC are grouped into fast and slow muscle types and the particular developmental program for sequential expression of MyHC isoforms available to a muscle fiber is dependent on the commitment of myoblasts to distinct myogenic cell lineage (Cho *et al.*, 1993; DiMario *et al.*, 1993; Edom *et al.*, 1994; Feldman and Stockdale, 1991; Miller *et al.*, 1985; Stockdale, 1992). However, the compositions of the multigene families of muscle specific proteins are so different from one another (Gunning and Hardeman, 1991; Schiaffino and Reggiani, 1996) that the regulatory mechanisms to express developmentally and functionally distinct types of isoforms of these proteins seemed to be different from one another. The expression of contractile protein isoforms in the developing rat and human hindlimb has recently been

examined. Sutherland *et al.* (1991) analyzed the extent to which fast and slow isoform mRNA levels are quantitatively coordinated during early development. This analysis indicates that each contractile protein gene has its own unique determinants of mRNA accumulation.

MyHC is not a sole marker of muscle fiber types, and other components of contractile apparatus also can be good markers. Among these are myosin light chain, tropomyosin, troponin C, troponin I, and troponin T (TnT). For example, TnT, one of troponin complex, is uniquely expressed. TnT has three classes of isoforms specific to different fiber types of striated muscle: fast-muscle-type (F-type) TnT, slow-muscle-type (S-type) TnT, and cardiac-muscle-type (C-type) TnT (Bandman, 1992; Perry, 1985; Schiaffino and Reggiani, 1996). The three classes of isoforms are encoded by three different genes (Cooper and Ordahl, 1984; Smillie *et al.*, 1988; Yonemura *et al.*, 1996). F-type TnT isoforms are one of the most representative products generated from the same gene through alternative splicing. Numerous F-type TnT isoforms have been observed in both adult and developing muscles. Alternative splicing of the rat F-type TnT gene may generate up to 128 different mRNAs (Breitbart and Nadal-Ginard, 1986; Breitbart and Nadal-Ginard, 1987; Morgan *et al.*, 1993). Similar results have been reported for rabbit and quail genes (Briggs and Schachat, 1993; Bucher *et al.*, 1989; Hastings *et al.*, 1985). Especially, in chicken F-type TnT gene, many alternative exons have been found and they may yield as many as two thousands isoforms (Schachat *et al.*, 1995; Smillie *et al.*, 1988). At the protein level, as many as 80 variants of F-type TnT have been observed in adult and developing chicken muscles, and classified into breast-muscle-type (B-type) and leg-muscle-type (L-type) with respect to their molecular weights and their isoelectric points (Nakamura *et al.*, 1989; Obinata, 1985; Perry and Cole, 1974). The expression of these isoforms are regulated by tissue- and stage-specific manner. TnT isoforms were reported to change from L-type to B-type during development of chicken breast

muscle. In leg muscles only the L-type isoform continued to be expressed for life. While, in wing muscles L-type TnT appeared firstly and then B-type followed the L-type during development in a manner similar to that in breast muscle, but almost all isoforms of both types were remained even at the adult stage when L-type isoform and a part of B-type isoform disappeared in breast muscle (Yao *et al.*, 1992). Since B-type TnT isoforms appear to include the specific amino acid sequences generated from the several specific exons (Jin and Smillie, 1994; Schachat *et al.*, 1995; Smillie *et al.*, 1988), the expression of B-type and L-type TnT isoforms is considered to be a useful marker of tissue- and stage-specific regulation of alternative splicing.

Using TnT as a marker, Yao and colleagues showed by tissue transplantation experiments that expression patterns of chicken TnT isoforms seemed to be fixed in cell lineage. They transplanted breast muscle into leg muscle, leg muscle into breast muscle, and slow muscle into breast and leg muscles, and found persistent expression of TnT isoforms specific to the donor tissue after the transplantation (Yao *et al.*, 1994a; Yao *et al.*, 1994b; Yao *et al.*, 1992). These results suggest that the expression of TnT isoforms are considered to be a useful marker of myogenic cell lineage.

In this study, the expression of TnT isoforms has been investigated by immunohistochemical techniques in cultured cells prepared from chicken skeletal muscles at various developmental stages in order to investigate the stability of TnT expression pattern *in vitro*. I have shown that the cultured myogenic cells from chickens and chick embryos are classified into two types, fast type and fast/slow type and that each muscle seems to be determined differently in the composition of cell types during the developmental course. Furthermore, I have performed clonal analysis of cultured myogenic cells prepared from *pectoralis major* of 13-day old chick embryos and 1-day old chicks and shown that the *in vitro* expression patterns of F-type and S-type TnT isoforms are stable without any change of cell types detected in colonies repeatedly subcloned. Additionally, to investigate whether B-type or L-type

TnT isoform is expressed in the cultured muscle cells, two-dimensional gel electrophoresis and immunoblotting are performed. Then both B-type and L-type TnT isoforms are found in cultured muscle cells prepared from both breast and leg muscles of chick embryos and adult chickens.

Materials and Methods

Animals

White leghorn chickens (*Gallus domesticus* (L)) and their fertilized eggs were obtained from commercial sources.

Preparation and Characterization of Antisera

To prepare an antiserum against F-type TnT, the immunogen was prepared by the method of Ebashi *et al.* (1971), further purified by SDS-PAGE, and cut out from the gels. The gels were dialyzed at 4°C against phosphate buffered saline (PBS) for one day, mixed with Freund's complete adjuvant, and injected into the back of a guinea pig. The anti-F-type TnT (guinea pig serum) reacted with F-type isoforms of TnT (Fig. 1). The anti-S-type TnT (rabbit serum) has been described by Yao *et al.* (1992). The anti-C-type TnT (rabbit serum) is a personal gift from Dr. M. Oishi, Kitasato University.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially according to Laemmli's method (Laemmli, 1970) with 12% acrylamide gels.

Extracts of *pectoralis major*, *triceps brachii*, *gastrocnemius*, *anterior latissimus dorsi* (ALD) and *ventricle* of adult chicken were used for characterization of antisera. After SDS-PAGE, proteins in gels were transferred to a nitrocellulose membrane by a combination of the methods of Towbin *et al.* (1979) and Franke *et al.* (1981) at 300 mA for 3 hr at 0°C.

Immunoblotting was performed using anti-F-type TnT, anti-S-type TnT, and anti-C-type TnT as the first antibodies. The second antibodies were rhodamine-conjugated anti-rabbit IgG goat serum and rhodamine-conjugated anti-guinea pig IgG goat serum.

Cell Isolation

Muscle tissue (2-5 g) was minced with sharp scissors into 1-2 mm fragments. The fragments were washed twice with Ca^{++}- and Mg^{++}-free Tyrode's solution (CMF) and treated with trypsin at a final concentration of 0.1% in CMF for 30 min at 37°C with constant shaking. The ratio of the trypsin solution to muscle tissue was kept at 4-5 ml solution/g tissue. After the treatment, the suspension was centrifuged at approximately 100xg for 5 min to collect liberated single cells. The pellet was resuspended in 2 ml of a growth medium (37.5% Dulbecco's modified Eagle's minimum essential medium (DMEM), 37.5% Ham's F12, 20% horse serum, 5% chick embryo extract, and Gentamicin at 4.0 mg/liter of medium). Cells were then counted with a hemocytometer. The chick embryo extract was prepared essentially according to the method of Hauschka and Konigsberg (1966).

Differential Trypsinization

To obtain purer populations of myoblasts, the method by Kaighn *et al.* (1966) was used with some modifications as follows.

Two ml suspension of the cells (10^2-10^3 cells) was incubated in a 60 mm collagen-coated plastic dish for 3 days. Before the myoblasts fused, the medium was removed and the plate washed twice with CMF to remove unattached cells and debris. After 2 ml of 0.01% trypsin in CMF was added, the plate was incubated at 37°C in 5% CO_2. After about 3-5 min bipolar processes of myoblasts retracted and the cells rounded up, while flattened fibroblasts remained unaffected. Then, the plate was swirled to suspend cells and the suspension was transferred to a centrifuge tube. The cells in the solution were collected by centrifugation at approximately 100xg for 5 min and resuspended in 3 ml of the growth medium.

Differential Cell Adhesion

This preparation procedure were performed essentially according to the method of Yaffe (1968) to obtain purer populations of myoblasts.

The cell suspension was plated on non-collagen-coated plastic dishes and incubated at 37°C in 5% CO_2. After 30 min of incubation, the medium was collected. The plating was repeated once more, and the floating cells were collected.

Cell Culture

The collected cells were counted and plated at a concentration of 1 x 10^3 cells/well with 0.3 ml of the growth medium on a collagen-coated Celltight C-1 Celldesk (Sumilon) in one well of a 24-well plate to culture at 37°C in 5% CO_2. When myoblasts began to fuse in culture, after about 2 days, the growth medium was exchanged with a differentiation medium (47.5% DMEM, 47.5% Ham's F12, 4% horse serum, 1% chick embryo extract, and Gentamicin at 4.0 mg/liter of medium). Thereafter, the differentiation medium was changed every other day. The cells adhering to Celldesks were examined on the 6th day after plating.

Cell Culture for Clonal Analysis

All cultures at a clonal density were grown in an equal mixture of fresh and conditioned growth media. The conditioned growth medium was prepared by the method of White *et al.* (1975). The isolated cells were counted and plated at a clonal density (approximately 100 cells) on a collagen-coated 60 mm dish with 2 ml of the medium and cultured at 37°C in 5% CO_2. After 5 days in culture, subcloning of chicken myogenic cell colonies was carried out as described by Rutz and Hauschka (1982) with some modifications as follows. A muscle colony was located and marked on the dish. An open-ended stainless cylinder which had silicone grease applied to one rim was placed over the colony, so that the cylinder encircled the colony and

formed a greased seal with the dish. Then, the colony was rinsed and dissociated with 0.05% trypsin. The dissociated cells from a single colony were divided into two groups: One group was plated on a new collagen-coated 60 mm dish again for the next colony formation, the other was in one well of a 24-well plate with 0.3 ml of the medium on a collagen-coated Celltight C-1 Celldesk (Sumilon). The latter cells adhering to Celldesks were used for immunocytochemical studies on the 10th day after plating. The former cells on the 60 mm dish were grown for 5 days to form the next colonies and the colonies were subcloned once more.

The method of the serial subcloning analysis were summarized in Fig. 6. Primary (1°) colonies were those formed by muscle cells isolated directly from tissues; secondary (2°) colonies were formed from dissociated muscle cells of subcloned primary colonies, tertiary (3°) colonies designated #1.1 and #1.2 in Table 5, for example, were formed from secondary colonies designated #1 in Table 5, and quaternary (4°) colonies formed, for example, from the colony #1.1 were designated #1.1.1 and #1.1.2 in Table 5. Then TnT expression in the progeny of the colonies was investigated as mentioned above. In this way, the sequentially subcloned colonies were numbered to indicate their ancestry in Tables 5 and 6.

Indirect Immunofluorescence Microscopy

Immunohistochemistry was carried out according to the method used by Yao *et al.* (1994a). Muscle blocks prepared from *pectoralis major*, *gastrocnemius*, ALD, and *ventricle* of chickens of different ages were fixed in Bouin's solution. After 12 hr, they were washed 1 day with several changes of 70% ethyl alcohol to remove the picrate. The blocks were dehydrated, cleared, impregnated with normal butyl alcohol, and embedded in paraffin. Serial longitudinal sections (7 μm thick) of the tissues were immunostained with the anti-F-type TnT, anti-S-type TnT, and anti-C-type TnT.

Immunocytochemistry was carried out by the method of Hirai and Hirabayashi (1986) with some modifications as follows. The cultured cells adhering to Celldesk were fixed with 3% paraformaldehyde in PBS for 20 min at 0°C, permeabilized with 0.1% Triton X-100 in PBS for 3 min at 0°C, and treated with 0.1 M glycine in PBS for 20 min at room temperature. The cells were firstly incubated with the anti-S-type TnT for 1 hr at room temperature, and washed 5 times with PBS for 2 min and secondly incubated with the fluorescein-conjugate anti-rabbit IgG goat serum for 1 hr. The same process as this was repeated with the anti-F-type TnT and the rhodamine-conjugated anti-guinea pig IgG goat serum.

Pictures were taken with a combination of a Nikon Optiphot microscope and a Nikon FX-35WA camera.

Two-Dimensional Gel Electrophoresis and Immunoblotting of Cultured Muscle Cells

The collected cells were counted and plated at a concentration of 1 x 10^5 cells/dish with 2 ml of the growth medium on a collagen-coated 60 mm dish to culture at 37°C in 5% CO_2. When myoblasts began to fuse in culture, after about 3 days, the growth medium was exchanged with a differentiation medium. Thereafter, the differentiation medium was changed every other day. After 10 days in culture, the cells were used for electrophoresis.

Two-dimensional gel electrophoresis (2D-GE) was carried out by the method of Hirabayashi (1981) with some modifications as follows. The cultured cells were washed twice with CMF and treated with trypsin at a final concentration of 0.5% in CMF for 5 min at 37°C. After the treatment, the suspension was centrifuged at approximately 100xg for 5 min to collect liberated cells. The pellet was resuspended in 10 vol (v/w) of a digestion buffer consisting of 500 U/ml Benzon nuclease (Merck), 5 M urea, 2 M thiourea, 1 mM phenylmethylsurfonyl fluoride (PMSF), and 0.13% ß-mercaptoethanol, and then centrifuged at 20,000 xg for 30 min. The supernatant was

used for electrophoretic analysis. After centrifugation, 30-50 μl of the supernatant was loaded onto a cylindrical agarose gel. First-dimension isoelectric focusing was carried out at 500 V for 25 hr at 4°C. Second-dimension SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using a 12% polyacrylamide gel in the presense of 3M urea.

After electrophoresis, proteins in gels were transferred onto immobilon (Millipore; pore size, 0.45 μm) PVDF sheets electrophoretically by a combination of the methods of Towbin *et al.* (1979) and Franke *et al.* (1981) at 300 mA for 3 hrs at 0°C.

Immunoblotting was performed according to Takai *et al.* (1994) with some modifications as follows: The sheets were incubated in a blocking solution (2% bovine serum albumin in PBS) at 37°C in the anti F-type TnT guinea pig serum and then in biotinyled anti-guinea pig IgG goat serum (Funakoshi), with washing after each incubation. Then, the sheets were incubated in an ABC solution (ABC kit, Vector) and proteins were visualized with a Konica immunostain HRP-1000 solution (Konica).

Results

Specificity of Antisera

SDS-PAGE and immunoblotting patterns of tissue extracts prepared from adult muscles of chicken are presented in Fig. 1. The immunoblotting patterns were obtained with three kinds of antisera: anti-F-type TnT, anti-S-type TnT, and anti-C-type TnT.

Reacting with the anti-F-type TnT, extracts of *pectoralis major*, *triceps brachii*, and *gastrocnemius* gave 2 bands (Fig. 1b, lane 1), 7 bands (Fig. 1b, lane 2), and 4 bands (Fig. 1b, lane 3), respectively. *Triceps brachii* was used here only to monitor the variety of F-type TnT isoforms. These bands corresponded to the F-type isoform compositions of TnT as described previously (Yao *et al.*, 1992).

In the reaction with the anti-S-type TnT, extracts of *gastrocnemius* and ALD gave one band (Fig. 1c, lanes 3 and 4) corresponding to the S-type isoform composition as reported previously (Yao *et al.*, 1992).

Reacting with the anti-C-type TnT only the extract of *ventricle* gave a single band (Fig. 1d, lane 5).

Expression of TnT Isoforms in Adult Skeletal Muscles

Longitudinal serial sections of *pectoralis major* (Fig. 2a-c),*gastrocnemius*, (Fig. 2d-f), ALD (Fig. 2g-i), and *ventricle* (Fig. 2j-l) muscles from adult chickens were stained with the three kinds of antisera. All fibers in the adult *pectoralis major* were stained with anti-F-type TnT (Fig. 2a). Fibers in adult *gastrocnemius* were of three types; many fibers were stained only with anti-F-type TnT, some stained with both the antisera and occasional fibers stained only with anti-S-type TnT (Fig. 2d and 2e). All fibers in adult ALD were stained only with anti-S-type TnT (Fig. 2h) and all fibers in *ventricle* were stained with only anti-C-type TnT (Fig. 2l). These

immunohistochemical observations were compatible with the results of immunoblotting of these adult muscle extracts (Fig. 1).

Expression of TnT Isoforms in Developing Skeletal Muscles

Immunohistochemical studies of serial sections of *pectoralis major*, *gastrocnemius*, and ALD of different stages were performed (Table 1). In some reports, tissue specific expression pattern of TnT isoforms has been established in chicken and other vertebrate species (Dhoot and Perry, 1979; Obinata, 1985; Reiser *et al.*, 1992). But so far, no information was reported about the distribution of F-type and S-type TnT isoforms in the developing muscles of chicken.

All fibers of *pectoralis major* from 15-day old embryos (E15) and older ones were stained only with anti-F-type TnT, but those from 11- and 13-day old embryos (E11 and E13) were stained with all of the three kinds of antisera. In contrast to these, all fibers of ALD from 17-day old embryos and older ones were stained only with anti-S-type TnT, but those from 13- and 15-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT, and all fibers of ALD from 11-day old embryos were stained with the three kinds of antisera. While, in *gastrocnemius* from 15-day old embryos and older ones, fibers were stained with either anti-F-type TnT or anti-S-type TnT, or with both the antisera, and all fibers from 11- and 13-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT.

As the representative results, the longitudinal serial sections of *pectoralis major* (Fig. 3a-c), *gastrocnemius* (Fig. 3d-f), and ALD (Fig. 3g-i) from 1-day old chicks were presented. All fibers in the *pectoralis major* were stained with anti-F-type TnT (Fig. 3a). Fibers in the *gastrocnemius* were of three types; most fibers were stained with either anti-F-type TnT or anti-S-type TnT, and some with both the antisera (Fig. 3d and e). All fibers in ALD from the chicks were stained only with anti-S-type TnT (Fig. 3h).

Sections from *pectoralis major* (Fig. 4a-c) *gastrocnemius* (Fig. 4d-f), and ALD (Fig. 4g-i) from 11-day old embryos were also presented. Both *pectoralis major* and ALD muscles were stained with the three kinds of the antisera. All fibers of *gastrocnemius* were stained with both anti-F-type TnT and anti-S-type TnT, but not with the anti-C-type TnT.

Expression of TnT Isoforms in Cultured Muscle Cells

The expression pattern of TnT isoforms in chicken skeletal muscles seemed to be changed on around the 15th day of incubation (Table 1). In addition, the previous studies (Yao *et al.*, 1994a; Yao *et al.*, 1994b; Yao *et al.*, 1992) suggested that the expression pattern of TnT isoforms was fixed in cell lineage. Therefore, I thought that characteristics of TnT isoform expression in myogenic cells might have been fixed on around the 13th day of incubation, and changes in the isoform expression might be detected in cultured cells prepared from the embryos of around the 13th day of incubation.

Myogenic cells were isolated from *pectoralis major*, *gastrocnemius*, and ALD of chickens of different stages, using differential trypsinization and cell adhesion techniques, cultured for 9 days, and double-immunostained with the anti-F-type TnT and anti-S-type TnT to examine which type of TnT isoforms was expressed in each cell.

Representative results were presented in Fig. 5. Cells from *pectoralis major* of 11-day old embryos (Fig. 5a-d), *gastrocnemius* of 13-day old embryos (Fig. 5e-h), and ALD of 1-day old chicks (Fig. 5i and j) were stained. Cells stained with both anti-F-type TnT and anti-S-type TnT were in the majority at these stages (Fig. 5c-f, i, and j) There were, however, some cells stained only with the anti-F-type TnT among those from *pectoralis major* and *gastrocnemius* (Fig. 5a, b, g, and h).

The results of double staining of cultured muscle with the anti-F-type TnT and S-type TnT are summarized in Table 2. Most cells from *pectoralis major* and *gastrocnemius* of the youngest embryos (E11) were stained with both anti-F-type TnT and anti-S-type TnT ("Fast/Slow" in Table 2), leaving only small fractions (7.2% and 1.0%, respectively) stained only with the anti-F-type TnT ("Fast" in Table 2). Differences in the percentages of stained cells along development were found between *pectoralis major* and *gastrocnemius*: In the former the percentages of cells stained only with anti-F-type TnT increased to over 90% by the 17th day of incubation, while those in the latter reached a plateau of 30-40% by the 13th day of incubation.

ALD presented a clearcut result: All cells from ALD were stained with both anti-F-type TnT and anti-S-type TnT irrespective of the stage of source materials. Preparation of the muscle cells from the embryos was so difficult that the types of the cells from ALD of 17-day old embryos or younger one could not be determined (Table 2).

Another clearcut result in Table 2 was that no cell was stained only with the anti-S-type TnT ("Slow" in Table 2).

Expression of TnT Isoforms in Cultured Muscle Cells from Chick Embryos of Early Stages

It was showed that cultured myogenic cells from chickens and chick embryos were classified into two types with respect to TnT isoform expression, fast type and fast/slow type. Since the ratio of fast type to fast/slow type cells in culture is low at the earlier stages, it is interesting when the fast type cells appeared in the early development.

Myogenic cells were isolated from somites and breast and lower leg muscles of chickens of early stages, using differential trypsinization and cell adhesion techniques,

cultured for 10 days, and double-immunostained with the anti-F-type TnT and anti-S-type TnT to examine which type of TnT isoforms was expressed in each cell.

The results of double staining of cultured muscle cells with the anti-F-type TnT and S-type TnT were summarized in Table 3. All cells from somites of 3.5-day old embryos and breast and lower leg muscles of 7-day old embryos (E7) were stained with both anti-F-type TnT and anti-S-type TnT ("Fast/Slow" in Table 3). Approximately 90% of cells prepared from breast and lower leg muscles of the 8- and 9-day old embryos (E8 and E9) were stained with both anti-F-type TnT and anti-S-type TnT, leaving small fractions (approximately 10%) stained only with the anti-F-type TnT ("Fast" in Table 3). These results suggested that all myogenic cells were fast/slow type until the 7th day of incubation and that fast type cells appeared on the 8th day of incubation. No cells were stained only with the anti-S-type TnT ("Slow" in Table 3).

Clonal Analysis of Chicken Muscle Cells

The changing ratio of fast type cells to fast/slow type cells depending on developmental stages and different muscles raises doubts on the stability of troponin T isoform expression in cultured cells, which was investigated by clonal analysis.

Firstly, clonal colonies of the myogenic cells prepared from breast and lower leg muscles at various stages were stained with anti-F-type and anti-S-type TnT sera. These muscle colonies were secondary ones subcloned from single primary colonies. So it was statistically expected that these colonies were the progeny of a single muscle cell. The results of this experiment are summarized in Table 4. All colonies from breast muscles of 9-day old embryos and lower leg muscles of 9-day old and 11-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT. There were both fast type and fast/slow type colonies from breast muscles of 11-day old and 13-day old embryos and lower leg muscles of 13-day old and 17-day old embryos. All

colonies from breast muscles of 17-day old embryos were stained only with anti-F-type TnT. No colonies were stained only with anti-S-type TnT ("Slow" in Table 4).

Since the colonies were uniform with respect to reactivity with the antisera and no colonies were intermingled with fast and fast/slow types of cells, it was strongly suggested that TnT expression of the cultured myogenic cells was intrinsically stable.

Secondly, serial subclonal analysis was performed to get a more precise idea of the stability of the commitment of TnT expression of cultured muscle cells. In Fig. 6, the method of the serial subcloning analysis were summarized. The cells isolated from *pectoralis major* of 13-day old embryos and 1-day old chicks were cultured at a clonal density and incubated for 5 days to form primary (1°) muscle colonies. Secondary (2°) muscle colonies were formed from subcloned myogenic cells isolated from single primary muscle colonies, tertiary (3°) muscle colonies were from cells of single secondary muscle colonies, in this subcloning procedure. At each stage of subcloning, a part of those colonies was analyzed by double immunofluorescence staining with anti-F-type and anti-S-type TnT sera to determine the type of TnT expressed in each colony. The results of serial subclonal analysis are summarized in Tables 5 and 6. This sequential clonal analysis was a painstaking experiment since numbers of colony to be analyzed increased exponentially in every generation. With the limited capacity of my culture facility I could follow 14 tertiary colonies as summarized in Tables 5 and 6. The important results were that, without exception all of the fibers in a colony formed from a single muscle cell were of the same type and that no change of the immunoreaction was observed in muscle colonies formed from a myogenic cell progeny repeatedly subcloned.

Colonies from *pectoralis major* of 13-day old embryos were stained with the antisera and the representative results are presented in Fig. 7. One colony, a progeny of the colony designated #4.1.1 in Table 5, was stained with both anti-F-type TnT and

anti-S-type TnT (Fig. 7a and b), and the other, a progeny of the colony designated #8.2.1 in Table 5, was stained only with the anti-F-type TnT (Fig. 7c and d).

B-type and L-type TnT Isoforms Expressed in Cultured Muscle Cells

The cells prepared from *pectoralis major* and *gastrocnemius* of 13-day old embryos and adult chickens were cultured for 10 days. To investigate the expression patterns of F-type TnT isoforms precisely, the cultured muscle cells were analyzed by 2D-GE in the presence of 3 M urea followed by immunoblotting with anti-F-type TnT serum. Both B-type and L-type TnT isoforms were found irrespective of the origin of the cells, i.e., embryonic or adult, or breast or leg. Representative result with *pectoralis major* and *gastrocnemius* of 13-day old embryos was presented in Fig. 8.

Discussion

In Vivo Expression of TnT Isoforms

Muscle fiber types could be distinguished on the basis of the expression of TnT isoforms (Dhoot and Perry, 1979; Perry, 1985). In the chicken, it was functionally defined by Swynghedauw (1986) that *pectoralis major* was a fast muscle, ALD a slow one, and *gastrocnemius* a mixed one, and these were in good agreement with results of this study (Figs. 1 and 2). Therefore, TnT isoforms are reasonably considered to be good markers of chicken muscle fiber types.

All fibers of *pectoralis major* from 15-day old embryos (E15) and older ones were stained only with the anti-F-type TnT, but those from 11- and 13-day old embryos (E11 and E13) were stained with all the three kinds of antisera. Therefore, differential expression of TnT isoforms occurred during myogenesis, supporting Obinata's results that showed transition of TnT isoforms from cardiac-muscle-type to fast-muscle-type in myogenesis of chicken breast muscle (Obinata, 1985). However, no information was so far reported about the expression of S-type TnT isoforms. In *gastrocnemius* and ALD from 17-day old embryos and older ones, fibers expressed the same TnT isoforms as adult fibers do, while, fibers of 13- or 15-day old embryos and younger ones did not (Figs. 2, 3, 4, and Table 1).

To make sure of the results of the tissue immunostaining of *pectoralis major* from 11- and 13-day old embryos, immunoblotting was performed and it was confirmed that the fibers of these muscles expressed all three kinds of TnT (data not shown).

Our results also showed changes in expression of TnT isoforms in *gastrocnemius* and ALD. Taking these results together, it can be concluded that the expression pattern of TnT isoforms in chicken skeletal muscles transits in the embryonic muscle tissues, and that muscle fiber type is probably determined on around the 15th day of incubation.

In Vitro Expression of TnT Isoforms

On the basis of the expression of TnT isoforms, cultured muscle cells were classified into two types: One is fast type cells stained only with anti-F-type TnT and the other is fast/slow type cells stained with both anti-F-type TnT and anti-S-type TnT. No cells could be found which were stained only with anti-S-type TnT even in the muscle cells prepared from *gastrocnemius* and ALD (Table 2 and Fig. 5). However, the fibers stained only with anti-S-type TnT were present in the tissues of these muscles (Figs. 3, and 4, and Table 1). Why were slow type cells (the cells expressing only S-type TnT) absent in culture?

I can not remove completely the possibility that the discrepancy in S-type TnT isoform expression between cultured cells and muscle tissues was caused by cell selection during cell preparation. I do not think, however, that cell selection was a major reason for this discrepancy, because I could not find slow type cells among the cells prepared by a method in which processes of differential trypsinization and differential cell adhesion were omitted (data not shown). Then, how do the cells in muscle tissue express only S-type TnT isoform? I think there are two possibilities: One is that the intrinsic potential of slow type cells to express F-type TnT isoforms is suppressed *in vivo*. This possibility was supported by similar experiments with S-type MyHC isoforms in cultured cells from human fast muscle (Cho *et al.*, 1993) and with C-type TnT isoforms in cultured cells from chicken fast muscle (Toyota and Shimada, 1983). The other possibility is that muscle cell differentiation was delayed in cultured cells. Cells cultured in the differentiation medium for 6 days might have been still immature to express specific isoforms, and expressed both F-type and S-type isoforms like *in vivo* muscle cells at earlier developmental stages. In the previous study, Yao *et al.* (1994b) transplanted ALD into *pectoralis major*, and detected the fibers expressing only S-type TnT on the 58th day after the operation. Therefore, a

long-time culture as in their experiments might have made it possible for the cells to express only S-type TnT.

Since I think the two cases are equally possible and have no further experimental bases to remove either one, I cannot determine which is more likely, or rather I would like to say that both are the cases.

Ratios of fast/slow type to fast type cells varied depending on the stage and origin of the muscle examined. In the cultured cells from 11-day old embryos, most cells belonged to the fast/slow type, while, in the cells from 13-day old embryos, the ratio of fast/slow type to fast type was 3:1. The ratios in the cultured cells prepared from embryos older than the 17th day of incubation were seemed to be fixed depending on the cell sources. The developmental change in the ratio seemed to reflect the appearance of satellite cells (Feldman and Stockdale, 1992) rather than the phase of myogenesis, such as primary or secondary myogenesis (Miller *et al.*, 1985).

MyHC isoforms have been used as good markers of muscle fiber types, and their expressions have been described in detail (Stockdale and Miller, 1987). However, the results reported here are not in good agreement with those works based on the expression of MyHC isoform. With respect to MyHC, most cultured cells prepared from *pectoralis major* of 11-day old embryos were fast type (Miller *et al.*, 1985), all cultured cells from adult *pectoralis major* were fast type, and those from adult ALD were a mixture of fast type and fast/slow type (Feldman and Stockdale, 1991; Miller *et al.*, 1985).

The reason for the discrepancy between the results with TnT and MyHC is not clear, but probably it would be due to the fiber type markers used in the two experiments. On the basis of TnT expression, there were only slow type fibers in adult ALD (Fig 2g-i), but on the basis of MyHC expression, there were fast type and slow type fibers (Feldman and Stockdale, 1991; Miller *et al.*, 1985). It is conceivable that regulatory mechanisms of expressions are different between TnT and MyHC.

As far as I know, this is the first report in which the preparation of muscle cells from ALD of 1-day old chicks was succeeded. In fact, preparation of muscle cells from the young ALD was very difficult, so that no one uses these young tissues to prepare cells for culture.

All cells from 3.5- and 7-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT ("Fast/Slow" in Table 3). Approximately 90% of cells from breast and lower leg muscles of the 8- and 9-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT, but small fractions (approximately 10%) were stained only with the anti-F-type TnT ("Fast" in Table 3). These results suggested that all myogenic cells were fast/slow type until the 7th day of incubation and that fast type cells appeared at the 8th day of incubation. Since the transition from primary myogenesis to secondary myogenesis occurs during days 6~8 *in ovo* (Miller and Stockdale, 1986), I think that the developmental change in the ratio of fast type cells to fast/slow type cells reflects the phase transition from the primary myogenesis to the secondary one.

The origin of fast type cells is not clear. These results and our previous study showed that most muscle cells were in a lineage of fast/slow type during early development (~11-day old embryo) and the ratio of fast type cells to fast/slow type cells increased in the later developmental period. I suppose that this developmental change reflects the emergence of new subpopulations caused by extracellular cues such as nerve control and hormonal signals.

Stability of Chicken TnT Expression in Vitro

The results in Table 4 were in good agreement with those in Tables 2 and 3. Because of the small number of secondary colonies selected from the primary ones, ratios of fast type to fast/slow type clonal colonies did not closely reflect the ratios in original tissues and stages, but the ratios showed similar tendencies as those in Table 3

and our previous results: Most muscle cells are of fast/slow type at the early stage of development, and the ratios in the cultured cells prepared from embryos older than the 17th day of incubation seemed to be fixed depending on the cell sources.

The results of serial subclonal analysis in Tables 5 and 6 suggested that TnT expression of the cultured muscle cells was intrinsically stable, since there were no change of the cell types in colonies formed from a muscle cell progeny repeatedly subcloned. Studies on the expression of MyHC in myotubes derived from clonal cultures of chicken and rat muscle were consistent with the existence of two lineages of satellite cells that serve as the origin of slow and fast fibers (Cossu and Molinaro, 1987; Dusterhoft and Pette, 1993; Feldman and Stockdale, 1991; Hartley *et al.*, 1992; Hughes and Blau, 1992). On the other hand, slow and fast cell lineages were not revealed from similar studies in satellite cells of human postnatal muscle (Cho *et al.*, 1993; Edom *et al.*, 1994), but fusing or nonfusing muscle-colony-forming myoblasts were found in the human fetal limb (Hauschka, 1974). Serial subclonal study of quail muscle showed that the initial cell progeny of an individual fetal myogenic progenitor cell were of a fast cell lineage, whereas later progeny were a fast/slow cell lineage on the basis of MyHC expression (Schafer *et al.*, 1987). The clonal progeny of human satellite cell were found to be heterogeneous, using desmin and actins as markers (Baroffio *et al.*, 1995). The apparent contradiction among these different studies including this report may be due to the use of different markers, but it may also reflect differences in species and developmental stages.

Expression of B-type and L-type TnT Isoforms in Cultured Muscle Cells

Previously, only L-type TnT isoforms were detectable in cultured muscle cells irrespective of the origin of the cells (Matsuda *et al.*, 1981). However, both B-type and L-type TnT isoforms were expressed in cultured muscle cells (Fig. 8). It seemed that the apparent difference was due to the use of methods to detect the TnT isoforms.

In this study, the immunoblotting method amplified with avidin and biotin (ABC kit described in Materials and Methods) was used, whereas, the method used in previous study was simple immunoelectrophoresis. So, the difference of sensitivities yielded the apparent difference.

In the previous study, Yao and colleagues showed by tissue transplantation experiments that expression patterns of chicken TnT isoforms seemed to be fixed in cell lineage. (Yao *et al.*, 1994a; Yao *et al.*, 1994b; Yao *et al.*, 1992). So, the result of this study appeared to be in disagreement with the result of the tissue transplantation. Why did the cultured cells express both B-type and L-type TnT isoforms irrespective of the origin of the cells? There are no further experimental bases to explain this contradiction of the two experiments. But, I think tissue- and stage-specific mechanism generating B-type and L-type TnT isoforms is too intricate to maintain its stability in the culture system.

Finally, only two cell lineages, fast and fast/slow types, were found in chicken skeletal muscle in respect to TnT isoform expression, which reflects the developmental stages and origins of the muscle cells and is intrinsically stable and reproducible, although the expression pattern apparently deviates *in vitro* from that *in vivo*. TnT isoforms can be useful markers of myogenic cell lineage, as well as MyHC isoforms. I think that the regulatory mechanism of TnT gene family is a good model of the mechanisms controlling the diversity among muscle fibers of fast muscle and slow muscle and that this culture system is helpful to study the regulatory mechanisms of F-type and S-type TnT genes. In further studies, the investigation of transcriptional control of F-type and S-type TnT genes will be performed.

Acknowledgments

I wish to express my sincere thanks to Professor Tamio Hirabayashi and Assistant Professor Jun-Ichi Miyazaki for their advices and encouragement given throughout the course of this work.

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Tables

Table 1. Reactivity of muscle tissues with antisera.

The results from immunofluorescence analysis of serial sections of *pectoralis major*, *gastrocnemius*, and *anterior latissimus dorsi* muscles at various stages are summarized. The serial sections were stained separately with anti-fast-muscle-type troponin T, anti-slow-muscle-type troponin T or anti-cardiac-muscle-type troponin T. F, anti-fast-muscle-type troponin T positive; S, anti-slow-muscle-type troponin T positive; C, anti-cardiac-muscle-type troponin T positive; Slashes mean that single fibers are positive with 2 or 3 antisera; E11~19, 11~19-day old embryos; H1 and H60, 1- and 60-day old chickens.

Stage	*Pectoralis major*	*Gastrocnemius*	*Anterior latissimus dorsi*
E11	F/S/C	F/S	F/S/C
E13	F/S/C	F/S	F/S
E15	F	F,F/S,S	F/S
E17	F	F,F/S,S	S
E19	F	F,F/S,S	S
H1	F	F,F/S,S	S
H60	F	F,F/S,S	S
Adult	F	F,F/S,S	S

Table 2. Reactivity of cultured muscle cells with antisera.

The results from double immunofluorescence analysis of cultured cells prepared from *pectoralis major*, *gastrocnemius* and *anterior latissimus dorsi* are summarized. The cells stained with the antisera were counted. Fast, only anti-fast-muscle-type troponin T positive; Fast/Slow, both anti-fast- and anti-slow-muscle-type troponin T positive; Slow, only anti-slow-muscle-type troponin T positive; E11~17, 11~17-day old embryos; H1 and H60, 1- and 60-day old chickens.

Source of Cells		Percentage (Numbers) of Cells		
Muscle	Stage	Fast	Fast/Slow	Slow
Pectoralis	E11	7.2(15)	92.8(194)	0(0)
major	E13	34.0(223)	66.0(432)	0(0)
	E17	93.8(436)	6.2(29)	0(0)
	H1	93.4(99)	6.6(7)	0(0)
	H60	95.0(165)	5.0(9)	0(0)
Gastrocnemius	E11	1.0(4)	99.0(380)	0(0)
	E13	33.0(148)	67.0(301)	0(0)
	E17	43.1(260)	56.9(343)	0(0)
	H1	37.4(43)	62.6(72)	0(0)
	H60	37.6(74)	62.4(123)	0(0)
Anterior				
latissimus	H1	0(0)	100(82)	0(0)
dorsi	H60	0(0)	100(75)	0(0)

Table 3. Reactivity of cultured muscle cells with antisera.

The results from double immunofluorescence analysis of cultured cells prepared from somites and breast and lower leg muscles are summarized. The cells stained with the antisera were counted. Fast, only anti-fast-muscle-type troponin T positive; Fast/Slow, both anti-fast- and anti-slow-muscle-type troponin T positive; Slow, only anti-slow-muscle-type troponin T positive; E3.5~9, 3.5~9-day old embryos.

Source of Cells		Percentage (Numbers) of Cells		
Muscle	Stage	Fast	Fast/Slow	Slow
Somite	E3.5	0(0)	100(128)	0(0)
Breast	E7	0(0)	100(262)	0(0)
	E8	3(6)	97(166)	0(0)
	E9	11(6)	89(48)	0(0)
Lower leg	E7	0(0)	100(227)	0(0)
	E8	4(9)	96(210)	0(0)
	E9	14(9)	87(57)	0(0)

Table 4. Reactivity of cultured muscle cell colonies with antisera.

The results from double immunofluorescence analysis of cultured colonies prepared from breast and lower leg muscles are summarized. Each colony was subcloned from a primary colony, so these secondary colonies were statistically expected to be the progenies of single myoblasts. The colonies stained with the antisera were counted. There were no colonies in which fast and fast/slow types of cells intermingled. Fast, only anti-fast-muscle-type troponin T positive; Fast/Slow, both anti-fast- and anti-slow-muscle-type troponin T positive; Slow, only anti-slow-muscle-type troponin T positive; E9~17, 9~17-day old embryos.

Source of Cells		Numbers of Colonies		
Muscle	Stage	Fast	Fast/Slow	Slow
Breast	E9	0	28	0
	E11	15	25	0
	E13	21	36	0
	E17	53	0	0
Lower leg	E9	0	19	0
	E11	0	18	0
	E13	14	25	0
	E17	42	27	0

Table 5. Effect of sequential subcloning on the troponin T expression in muscle colonies from *pectoralis major* of 13-day old chick embryos.

The results from double immunofluorescence analysis of the sequentially subcloned colonies prepared from *pectoralis major* of 13-day old embryos are summarized. Myogenic cells were dissociated from secondary muscle colonies designated #1~10. Tertiary (3°) colonies (#1.1~10.2) formed by progeny of each secondary colony were likewise subcloned to form 4° colonies (#1.1.1~10.2.1), and the troponin T expression of the myotubes of the colonies in the remaining 3° subclones was determined by double immunofluorescence analysis with anti-fast-muscle-type and anti-slow-muscle-type troponin T. Sequential subcloning and numbering to indicate ancestry were repeated until 4° or 5° subclones were analyzed. F, only anti-fast-muscle-type troponin T positive; F/S, both anti-fast- and anti-slow-muscle-type troponin T positive; S, only anti-slow-muscle-type troponin T positive; -, no muscle colonies were formed.

3°	Numbers of Colonies			4°	Numbers of Colonies			5°	Numbers of Colonies		
	F	F/S	S		F	F/S	S		F	F/S	S
#1	0	3	0	#1.1	0	3	0	#1.1.1	-	-	-
								#1.1.2	0	2	0
				#1.2	0	4	0	#1.2.1	0	3	0
								#1.2.2	0	1	0
#2	0	2	0	#2.1	0	2	0	#2.1.1	-	-	-
								#2.1.2	-	-	-
				#2.2	0	6	0	#2.2.1	-	-	-
								#2.2.2	-	-	-
#3	0	2	0	#3.1	0	1	0				
				#3.2	0	4	0				
#4	0	8	0	#4.1	0	8	0	#4.1.1	0	10	0
								#4.1.2	-	-	-
				#4.2	0	5	0	#4.2.1	0	2	0
								#4.2.2	-	-	-
#5	0	2	0	#5.1	0	1	0	#5.1.1	-	-	-
								#5.1.2	-	-	-
				#5.2	0	1	0	#5.2.1	-	-	-
								#5.2.2	-	-	-
#6	0	3	0	#6.1	0	1	0				
				#6.2	0	1	0				
#7	18	0	0	#7.1	11	0	0				
				#7.2	8	0	0				
#8	17	0	0	#8.1	6	0	0	#8.1.1	3	0	0
				#8.2	7	0	0	#8.2.1	5	0	0
#9	-	-	-	#9.1	1	0	0				
				#9.2	5	0	0				
#10				#10.1	5	0	0	#10.1.1	-	-	-
				#10.2	8	0	0	#10.2.1	-	-	-

Table 6. Effect of sequential subcloning on the troponin T expression in muscle colonies from *pectoralis major* of 1-day old chicks.

The results from double immunofluorescence analysis of the sequentially subcloned colonies prepared from *pectoralis major* of 1-day old chicks are summarized. Myogenic cells were dissociated from secondary muscle colonies designated #1~4. Tertiary (3°) colonies (#1.1~4.2) formed by progeny of each secondary colony were likewise subcloned to form 4° colonies (#1.1.1~3.2.1), and the troponin T expression of the myotubes in the remaining 3° subclones was determined by double immunofluorescence analysis with anti-fast-muscle-type and anti-slow-muscle-type troponin T. Sequential subcloning and numbering to indicate ancestry were repeated until 4° or 5° subclones were analyzed. F, only anti-fast-muscle-type troponin T positive; F/S, both anti-fast- and anti-slow-muscle-type troponin T positive; S, only anti-slow-muscle-type troponin T positive; -, no muscle colonies were formed.

3°	Numbers of Colonies			4°	Numbers of Colonies			5°	Numbers of Colonies		
	F	F/S	S		F	F/S	S		F	F/S	S
#1	16	0	0	#1.1	3	0	0	#1.1.1	3	0	0
								#1.1.2	-	-	-
				#1.2	5	0	0	#1.2.1	-	-	-
#2	12	0	0	#2.1	2	0	0	#2.1.1	4	0	0
								#2.1.2	-	-	-
								#2.1.3	3	0	0
								#2.1.4	9	0	0
								#2.1.5	5	0	0
				#2.2	2	0	0				
#3	20	0	0	#3.1	6	0	0	#3.1.1	-	-	-
								#3.2.1	-	-	-
				#3.2	1	0	0				
#4	19	0	0	#4.1	5	0	0				
				#4.2	2	0	0				

Figures

Fig. 1. Specificity of antisera.

The specificity of anti-fast-muscle-type troponin T (b), anti-slow-muscle-type troponin T (c), and anti-cardiac-muscle-type troponin T (d) was investigated by immunoblotting of the extracts from *pectoralis major* (lane 1), *triceps brachii* (lane 2), *gastrocnemius* (lane 3), *anterior latissimus dorsi* (lane 4), and *ventricle* (lane 5). a, SDS-PAGE pattern stained with Coomassie brilliant blue R.

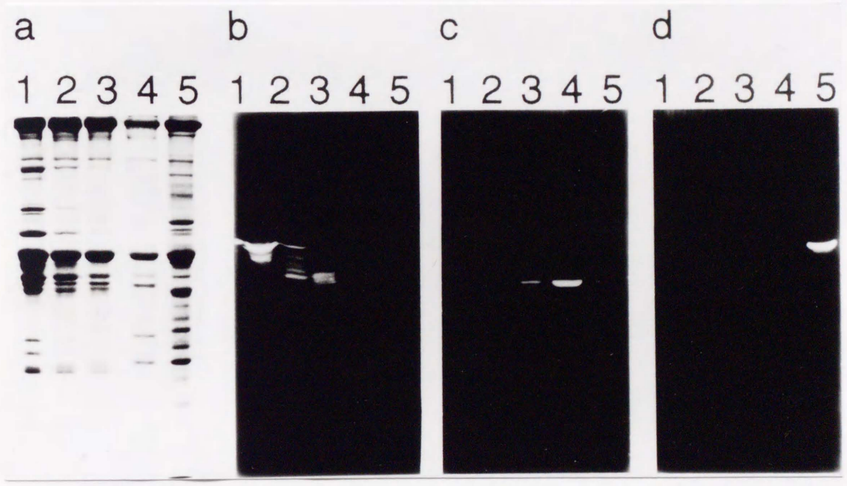

Fig. 2. Immunofluorescence microscopy of adult muscle tissues.

Serial sections of adult chicken *pectoralis major* (a-c), *gastrocnemius* (d-f), *anterior latissimus dorsi* (g-i), and *ventricle* (j-l) muscles were stained with anti-fast-muscle-type troponin T (a, d, g, and j), anti-slow-muscle-type troponin T (b, e, h, and k), and anti-cardiac-muscle-type troponin T (c, f, i, and l). Bar=10μm.

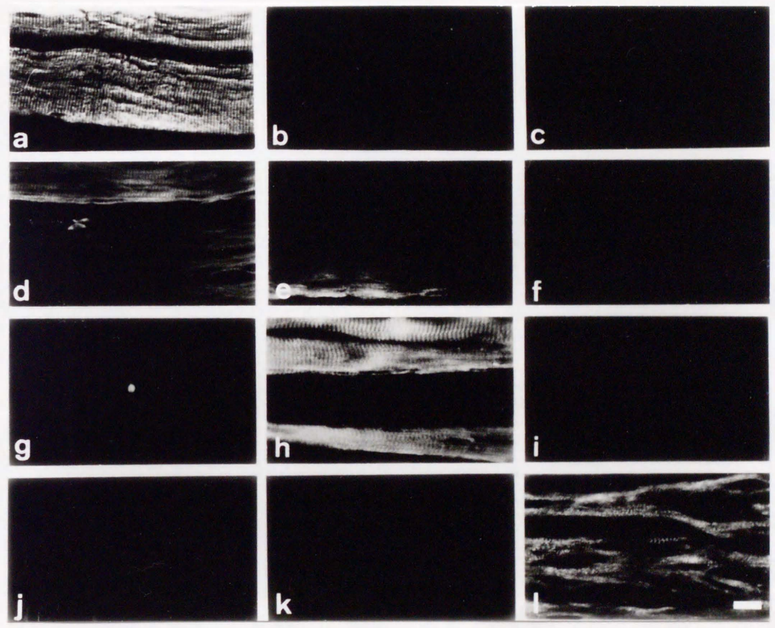

Fig. 3. Immunofluorescence microscopy of muscle tissues from 1-day old chicks.

Serial sections of 1-day old chick *pectoralis major* (a-c), *gastrocnemius* (d-f), and *anterior latissimus dorsi* (g-i) muscles were stained with anti-fast-muscle-type troponin T (a, d, and g), anti-slow-muscle-type troponin T (b, e, and h), and anti-cardiac-muscle-type troponin T (c, f, and i). Bar=10μm.

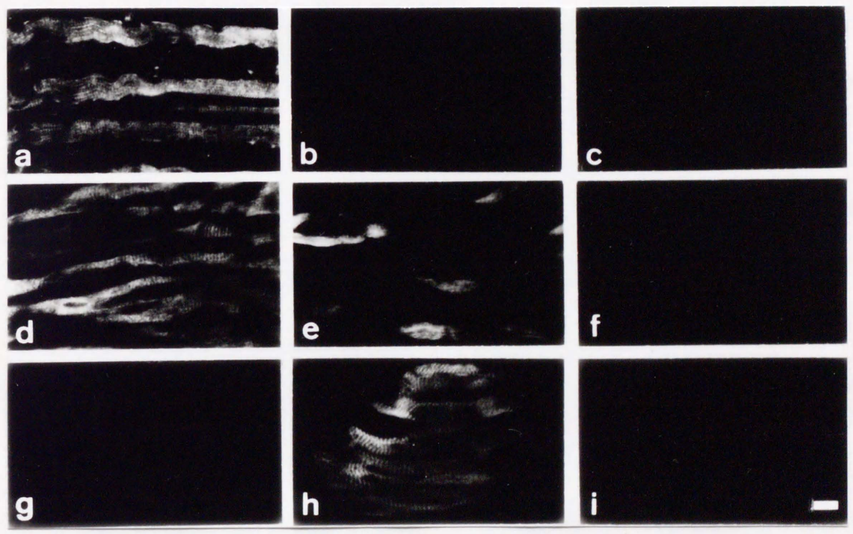

Fig. 4. Immunofluorescence microscopy of muscle tissues from 11-day old chick embryos.

Serial sections of 11-day old chick embryo *pectoralis major* (a-c), *gastrocnemius* (d-f), and *anterior latissimus dorsi* (g-i) muscles were stained with anti-fast-muscle-type troponin T (a, d, and g), anti-slow-muscle-type troponin T (b, e, and h), and anti-cardiac-muscle-type troponin T (c, f, and i). Bar=10μm.

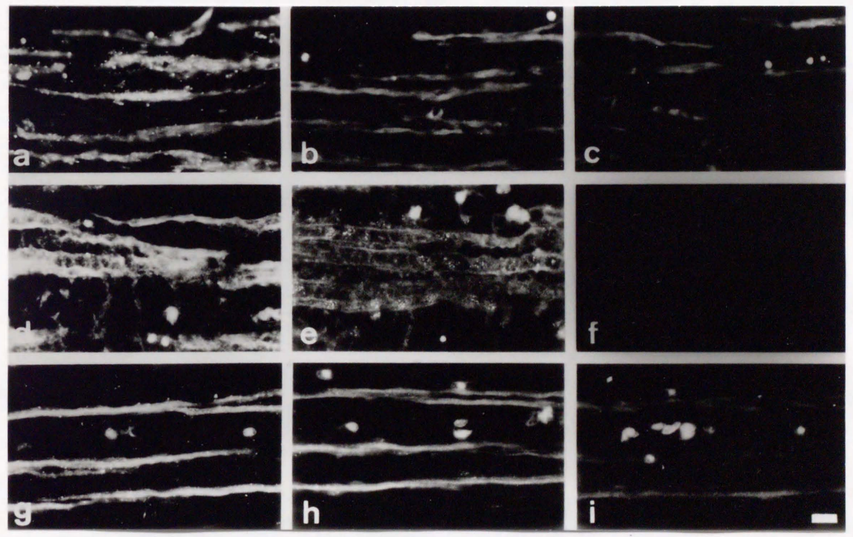

Fig. 5. Double immunofluorescence analysis of troponin T in cultured muscle cells.

Myoblasts were prepared from *pectoralis major* of 11-day old embryos (a-d), *gastrocnemius* of 13-day old embryos (e-h) and *anterior latissimus dorsi* of 1-day old chicks (i and j) and cultured for 9 days. Cells were fixed and processed for double immunofluorescence as described in Materials and Methods. Rhodamine (a, c, e, g, and i) and fluorescein (b, d, f, h, and j) fluorescence represents expression of fast-muscle-type troponin T and slow-muscle-type troponin T, respectively, in the same myotubes . Bar=10μm.

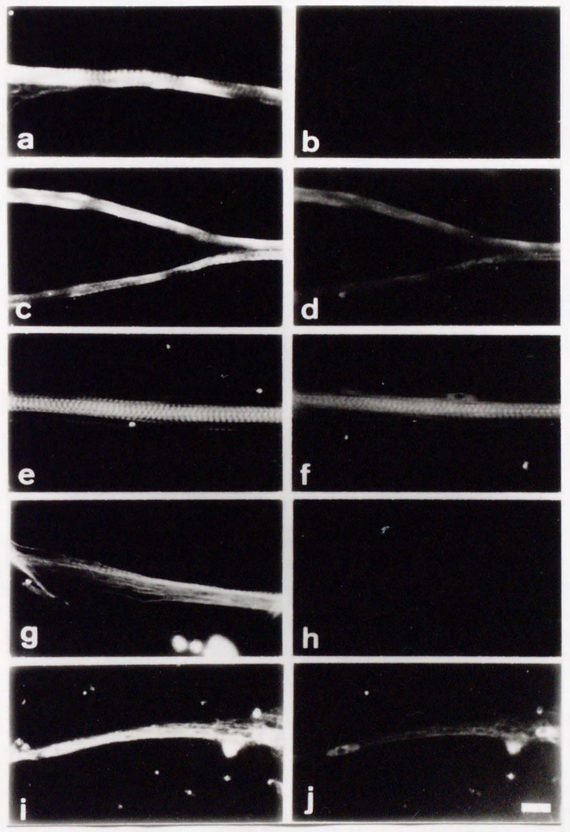

Fig. 6. Schematic representation of the method of the serial subclonal analysis.

Primary colonies (1°) were those formed by muscle cells isolated directly from tissues; secondary colonies (2°) were formed from dissociated muscle cells of subcloned primary colonies, 3° colonies designated #1.1 and #1.2, for example, were formed from the 2° colony designated #1, and 4° colonies formed, for example, from the colony #1.1 were designated #1.1.1 and #1.1.2. Type checking in TnT expression was carried by determing the type of the daughter colonies (Type Check). Open circle, cloned colony, Closed circle; colonies used for type checking.

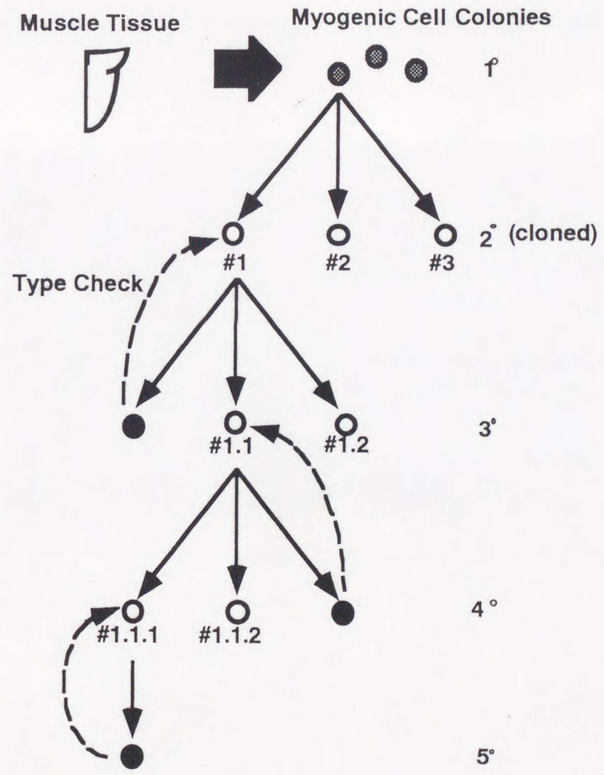

Fig. 7. Double immunofluorescence analysis of troponin T in cultured muscle cell colonies.

As described in Table 5, myogenic cells isolated from individual primary muscle colonies prepared from *pectoralis major* of 13-day old chick embryos were subjected to serial subcloning and colony formation. A portion of 5° muscle colonies composed entirely of fast/slow myotube (#4.1.1 in Table 5) is shown in a and b. A portion of 5° colonies composed entirely of fast myotube (#8.2.1 in Table 5) is shown in c and d. Cells were fixed and processed for double immunofluorescence analysis as described in Materials and Methods. Rhodamine (a and c) and fluorescein (b and d) fluorescence represents expression of fast-muscle-type troponin T and slow-muscle-type troponin T, respectively, in the same colonies. Bar=50μm.

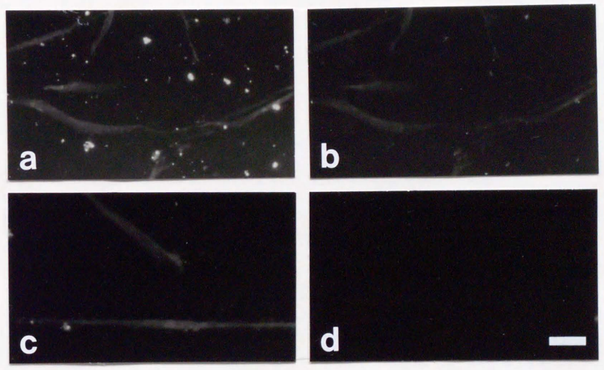

Fig. 8. Expression patterns of fast-muscle-type troponin T isoforms in cultured muscle cells demonstrated by two-dimensional gel electrophoresis and immunoblotting.

Extracts of the cells prepared from *pectoralis major* (a) and *gastrocnemius* (b) of 13-day old embryos were subjected to two-dimensional gel electrophoresis. The areas in which troponin T isoforms were included were analyzed by immunoblotting with the anti-fast-muscle-type troponin T serum. B, breast-muscle-type troponin T; L, leg-muscle-type troponin T (Nakamura *et al.*, 1989)