Cardiac troponin T in developing, regenerating and denervated rat skeletal muscle

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Summary

Fetal rat skeletal muscles express a troponin T (TnT) isoform similar to the TnT isoform expressed in the embryonic heart with respect to electrophoretic mobility and immunoreactivity with cardiac TnT-specific monoclonal antibodies. Immunoblotting analyses reveal that both the embryonic and the adult isoforms of cardiac TnT are transiently expressed during the neonatal stages. In addition, other TnT species, different from both cardiac TnTs and from the TnT isoforms expressed in adult muscles, are present in skeletal muscles during the first two postnatal weeks. By immunocytochemistry, cardiac TnT is detectable at the somitic stage and throughout embryonic and fetal development, and disappears during the first weeks after birth, persisting exclusively in the bag fibers of the muscle spindles. Cardiac TnT is re-expressed in regenerating muscle fibers following a cold injury and in mature muscle fibers after denervation. Developmental regulation of this TnT variant is not coordinated with that of the embryonic myosin heavy chain with respect to timing of disappearance and cellular distribution. No obligatory correlation between the two proteins is likewise found in regenerating and denervated muscles.

Key words: cardiac troponin T, muscle differentiation, muscle regeneration, muscle denervation, rat embryo, skeletal muscle.

Introduction

Troponin T (TnT) is the tropomyosin-binding subunit of the troponin complex which is involved in the calcium-dependent regulation of striated muscle contraction (Ebashi et al. 1973). Cardiac and skeletal muscles contain multiple molecular isoforms of TnT (Dhoot et al. 1979) derived from different genes and from differential RNA splicing (Breibart et al. 1985; Cooper and Ordahl, 1985; Gahlmann et al. 1987). In both mammalian and avian muscles, alternative pre-mRNA splicing of exons in the 5' and 3' regions of the fast skeletal TnT transcripts generates a large number of mRNAs, which are differentially expressed in embryonic and adult skeletal muscles (Breibart et al. 1985; Bucher et al. 1989). In the chicken heart, a single gene generates two developmentally regulated mRNAs that code for an embryonic and an adult isoform of TnT (Cooper and Ordahl, 1985). The embryonic cardiac isoform is also transiently expressed during development in chicken skeletal muscle (Toyota and Shimada, 1981; Cooper and Ordahl, 1985; Ogasawara et al. 1987). Two distinct cardiac TnT isoforms, presumably derived from a single gene by alternative RNA splicing (Jin and Lin, 1989) are expressed in the fetal and adult rat heart (Jin and Lin, 1988; Dhoot, 1988; Saggin et al. 1988).

In the present work, we show that cardiac TnT is present in fetal and neonatal rat skeletal muscle and is re-expressed in regenerating and denervated muscle. We also show that the expression of cardiac TnT is not coordinated with the expression of the embryonic skeletal myosin heavy chain (MHC).

Materials and methods

Animals

Heart and skeletal muscles from normal and experimentally treated Wistar rats of different ages were used in this study. Muscle regeneration was induced by a cold injury as previously described (Sartore et al. 1982). In brief, 3-month-old rats were anesthetized with ether, the tibialis anterior and the soleus muscles were exposed and a copper rod, precooled in liquid nitrogen, was applied to the muscle surface for 5 s. Specimens from injured muscles were removed at different time intervals (3, 7, 11 days) and frozen in liquid nitrogen. Denervation experiments were performed in 3-month-old rats by cutting out a segment of the right sciatic nerve in the thigh under ether anesthesia. The soleus and tibialis anterior muscle from the operated and controlateral side were removed after 7 and 15 days and frozen in liquid nitrogen.

Monoclonal antibodies

Two anti-TnT and one anti-MHC monoclonal antibodies (mAbs) were used in this study. RV-C2 is a cardiac TnT-specific mAb obtained from mice immunized with myofibrillar proteins from hypothyroid rabbit (Saggin *et al.* 1988). T-59, which reacts with both cardiac and skeletal TnT isoforms, was obtained from mice immunized with a myofibrillar preparation from bovine heart, as previously described (Saggin *et al.* 1988 and 1989). BF-G6 is an anti-embryonic MHC obtained from mice immunized with bovine fetal skeletal myosin (Schiaffino *et al.* 1986; Gorza *et al.* 1988; Schiaffino *et al.* 1988).

Electrophoresis and immunoblotting

Skeletal and cardiac muscles from fetuses, newborns and adult rats were directly homogenized in Laemmli's buffer (Laemmli, 1970) to minimize proteolytic degradation. Proteins were separated by SDS–PAGE using 8 % gels (Anderson and Oakeley, 1989) and stained with Coomassie blue. Duplicate gels were electrophoretically transferred to nitrocellulose sheets essentially as described by Piperno and Fuller (1985). By this method, we observed a complete elution of all the proteins having an apparent relative molecular mass less than 100×10^3 without a significant transfer to a second sheet of nitrocellulose. The transfer was monitored by Coomassie blue staining of the gel and Ponceau Red staining of the nitrocellulose. The blots were first incubated with anti-TnT mAbs and then with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts) and developed with 3,3'-diaminobenzidine (Sigma)· in the presence of imidazole, as described elsewhere (Saggin *et al.* 1988).

Immunocytochemistry

Immunoperoxidase staining was performed using previously described procedures (Gorza *et al.* 1988). Briefly, cryosections of unfixed tissues were incubated with appropriate dilutions of anti-TnT or anti-MHC mAbs for 30 min at 37 °C. Unbound antibodies were removed by repeated rinses in phosphate-buffered saline (PBS). Sections were then incubated with appropriate dilutions of anti-mouse immunoglobulins conjugated with peroxidase (Dakopatts) for 30 min at 37 °C. After several rinses in PBS, the sections were incubated in 1 mg ml^{-1} of *p*-phenylendiamine-pyrocathecol (Polysciences) in Tris–HCl 0.05 M, pH 7.6 in the presence of 0.01 % hydrogen peroxide, to reveal peroxidase activity. Correlation of TnT and MHC expression was studied using double immunofluorescence. Sections were first stained for TnT using unlabelled RV-C2 followed by rhodamine-conjugated anti-mouse immunoglobulins (Dakopatts), then treated with non-immune mouse immunoglobulins to block anti-mouse immunoglobulins free binding sites and subsequently stained for MHC using mAb BF-G6 directly conjugated with fluorescein isothiocyanate (Sigma) as previously described (Azzarello *et al.* 1987).

Results

Immunoblotting analysis of TnT isoforms expressed in developing skeletal muscle

Immunoblotting analysis of mAbs RV-C2 and T-59 with

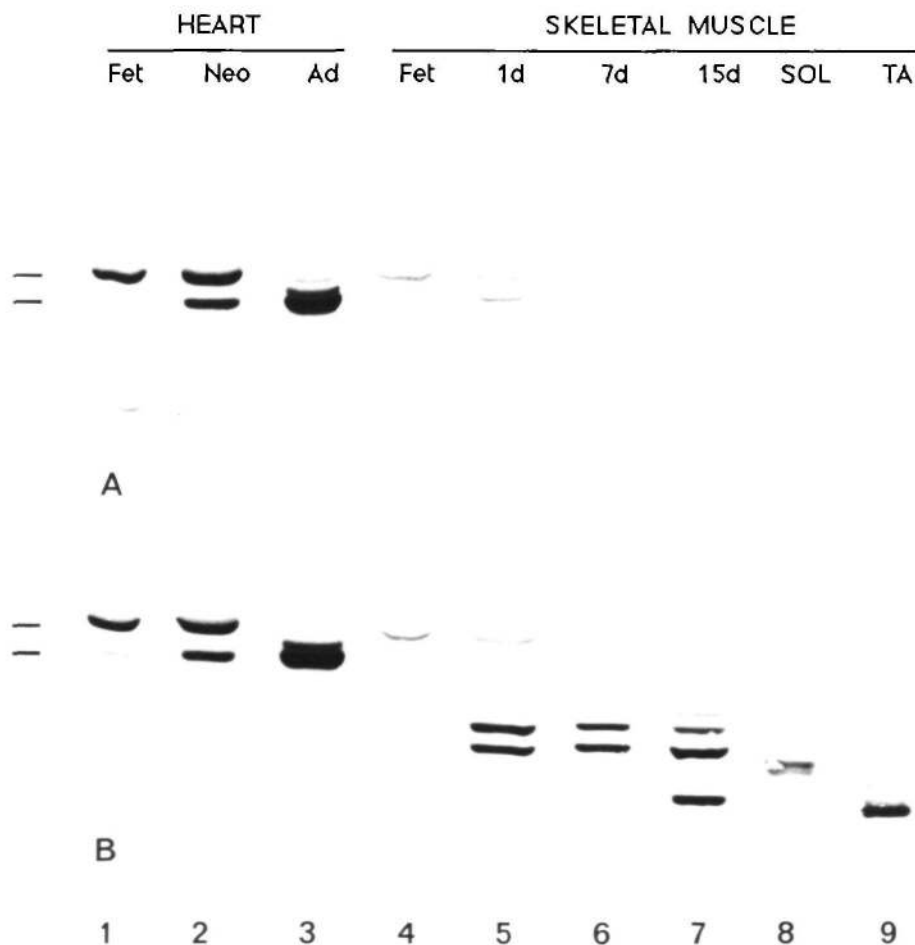|	HEART			SKELETAL MUSCLE					
Fet	Neo	Ad	Fet	1d	7d	15d	SOL	TA

Fig. 1. Immunoblots of mAbs RV-C2 (A) and T-59 (B) on whole muscle homogenates after SDS–PAGE. Lane 1: ventricles from 18-day fetus; lane 2: ventricles from 1-day newborn; lane 3: ventricles from adult rat; lane 4: hindlimb muscles from 18-day fetus; lane 5: hindlimb muscles from 1-day newborn; lane 6: hindlimb muscles from 7-day rat; lane 7: hindlimb muscles from 15-day rat; lane 8: soleus muscle from adult rat; lane 9: tibialis anterior muscle from adult rat. The position of the embryonic and adult cardiac TnT isoforms expressed in fetal and neonatal skeletal muscle is indicated on the left. Note in B that that skeletal muscles from 1- and 7-day-old rats contain also two major TnT isoforms with electrophoretic mobility intermediate between that of cardiac TnTs and that of adult skeletal TnTs.
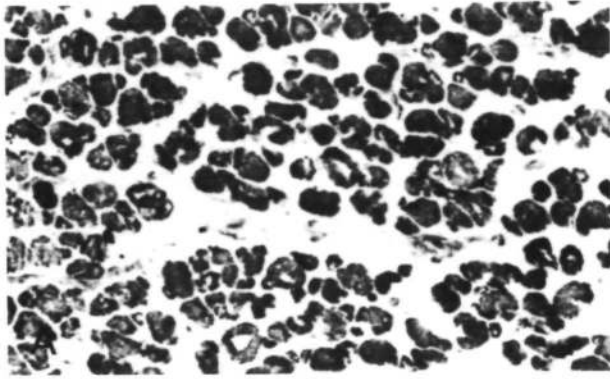
B

heart, the adult isoform showing a slightly higher electrophoretic mobility (Saggin *et al.* 1988). As shown in Fig. 1A, the two isoforms are better resolved using the SDS–PAGE procedure described by Anderson and Oakeley (1989). In addition, this procedure reveals another band with intermediate mobility, which is absent in fetal heart and progressively increases from neonatal to adult stages, and a higher mobility band, whose nature remains to be determined.

Adult skeletal muscle is completely unreactive with RV-C2; by contrast, skeletal muscle from 18-day-old fetus shows a reactive band having the same electrophoretic mobility as the major embryonic cardiac TnT isoform. Both the embryonic and the major adult cardiac TnT isoforms are present in skeletal muscle from 1- and 7-day-old rats, while only a trace amount of the adult form is barely detectable by 15 days.

To determine whether other TnT species are present in developing skeletal muscle, we used a mAb (T-59) that reacts with both cardiac and skeletal muscle TnTs in rat tissue homogenates (Fig. 1B). Immunoblotting analysis shows that TnT switching in developing rat skeletal muscle involves the sequential expression of distinct fetal, neonatal and adult TnT isoforms. In fetal muscle, only the embryonic cardiac TnT isoform is

skeletal and cardiac muscle is illustrated in Fig. 1. We have previously reported that RV-C2 reacts specifically with two TnT isoforms expressed in fetal and adult rat
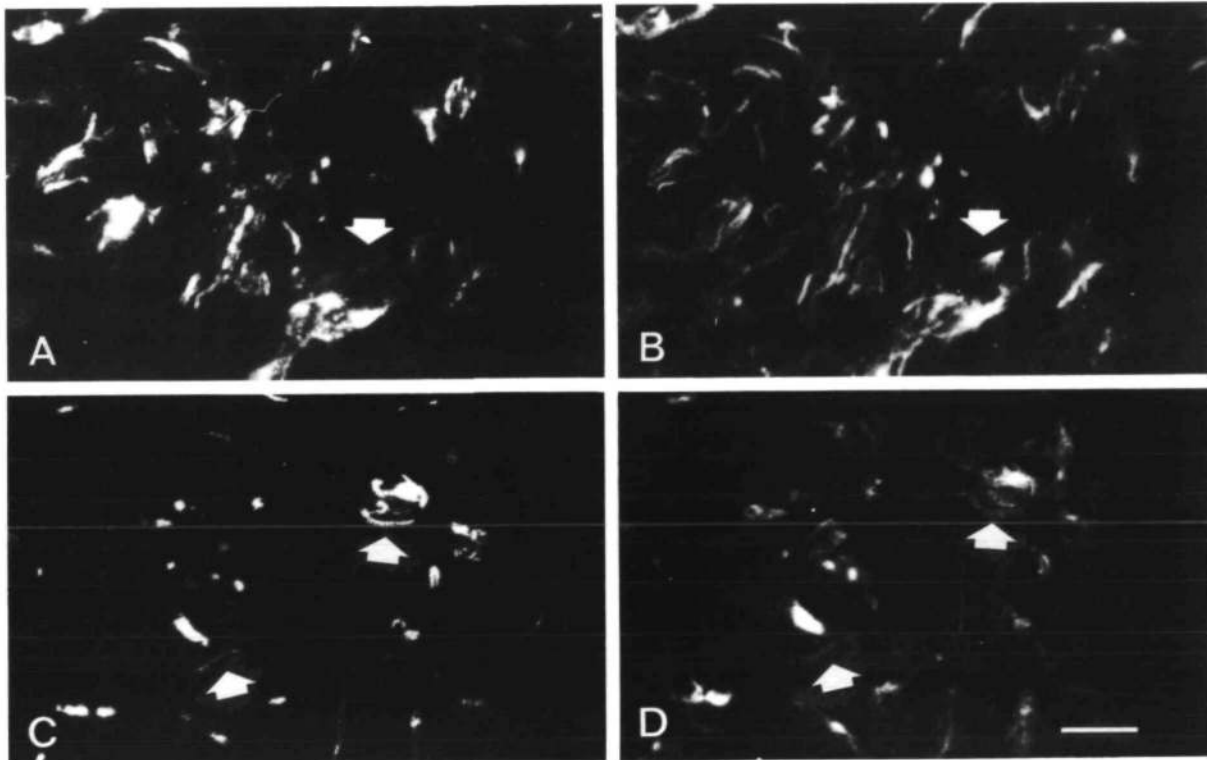

Fig. 3. Double immunofluorescence staining of muscle fibers from 11-day-old (A and B) and 13.5-day-old (C and D) rat embryos with mAbs RV-C2, specific for cardiac TnT (A and C) and BF-G6, specific for embryonic MHC (B and D). Most of the fibers are labeled by both antibodies, except for some fibers that stain selectively for cardiac TnT or embryonic MHC (arrows). Bar: 18 μm.
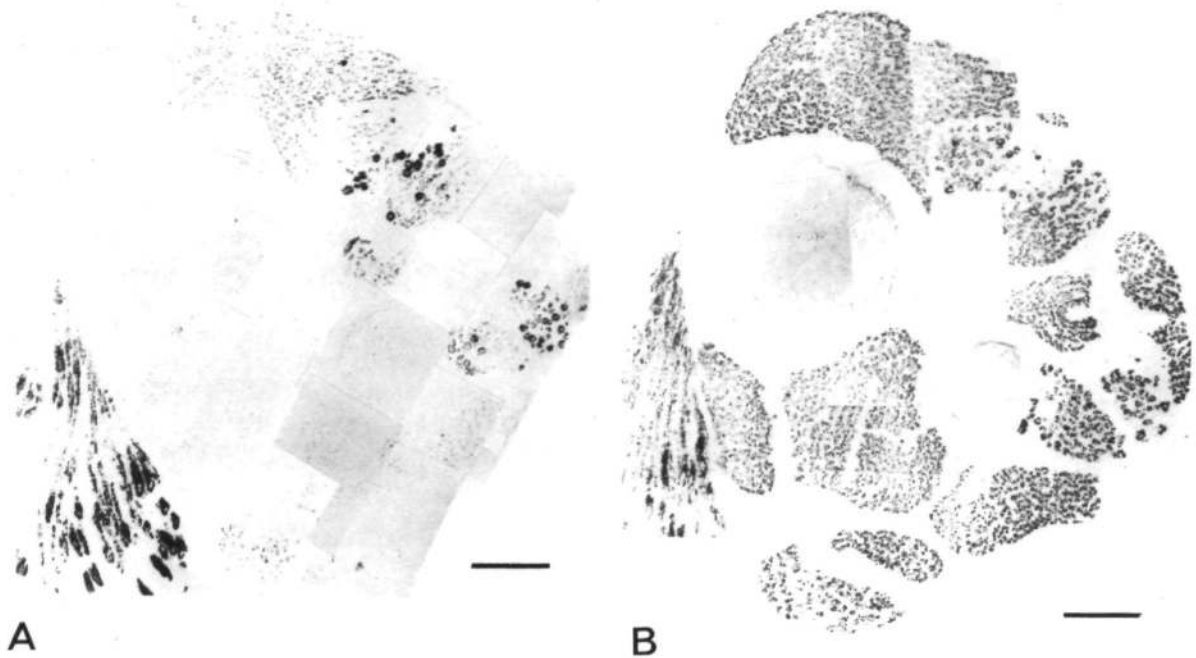
Fig. 4. Serial cross-sections of the whole leg from 18-day-old rat fetus stained for cardiac TnT (A) and for embryonic MHC (B). Immunoperoxidase staining. Bar: 320 μm.

identified. In 1- and 7-day-old rats, T-59 stains two major neonatal-specific bands, in addition to the two cardiac TnTs: neonatal bands have higher electrophoretic mobility than cardiac TnTs but lower mobility than the TnT species present in fast and slow muscles from adult rats. A complex TnT profile is apparent by 15 days: in addition to the two neonatal bands, T-59 stains a slower migrating component and faster migrating bands similar to those seen in adult muscle.

Distribution of embryonic cardiac TnT and embryonic MHC in developing skeletal muscle

Muscle fibers in fetal and neonatal rat skeletal muscle are labelled by anti-cardiac TnT, whereas adult muscle fibers are completely unreactive (Fig. 2). The distribution of cardiac TnT in developing skeletal muscle was compared to that of embryonic MHC using immunoperoxidase and double immunofluorescence procedures. In 11-day embryos, numerous cells in the myotomal region of the somites are labelled by both antibodies; at this stage, as well as in 13.5-day embryos, only rare fibers stain selectively with either one or the other antibody (Fig. 3). In the late fetal and neonatal stages, the reactivity for cardiac TnT varies in different muscles without any obvious relation to specific fiber types: thus a stronger reaction is seen in soleus and tibialis anterior compared to gastrocnemius muscles. In contrast, reactivity for embryonic MHC is homogeneously strong in all hindlimb muscles (Fig. 4). During postnatal development, the reactivity for cardiac TnT is progressively and homogeneously
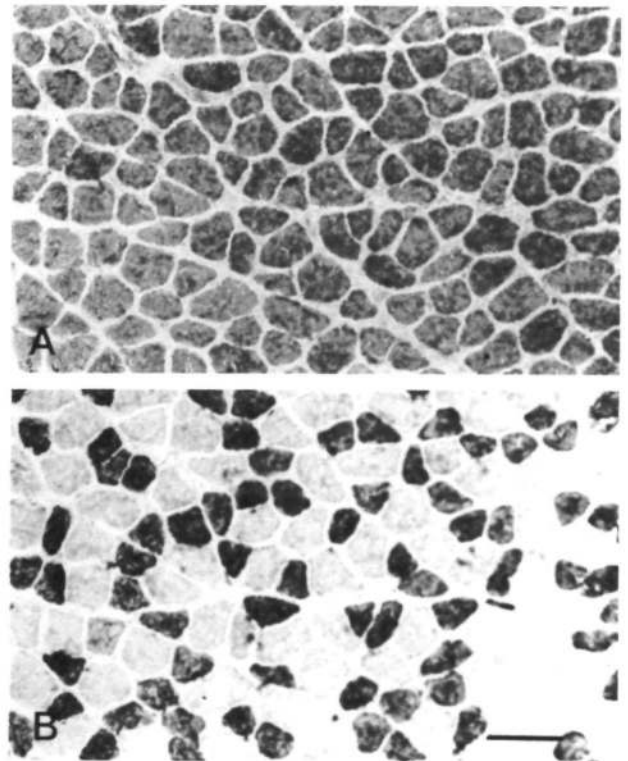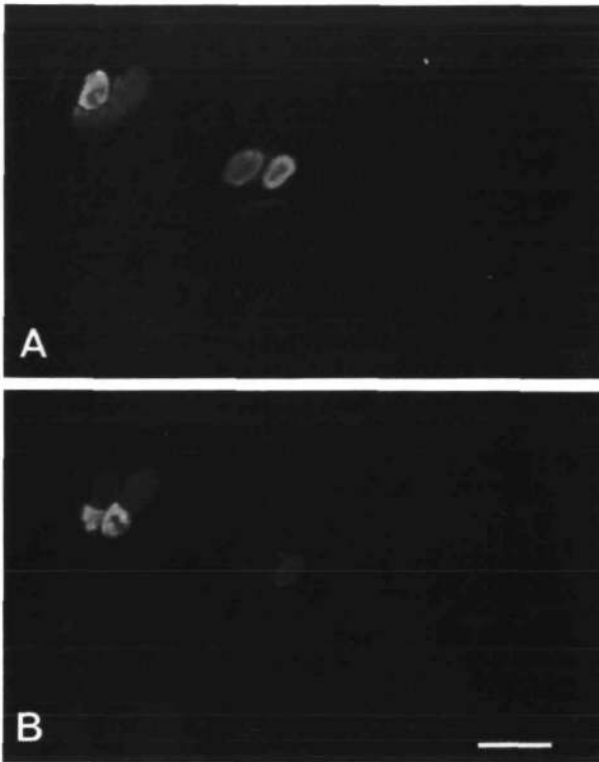

Fig. 5. Serial cross-sections of soleus muscle from 15-day-old rat stained for cardiac TnT (A) and for embryonic MHC (B). Immunoperoxidase staining. Bar: 36 μm.

Fig. 6. Double immunofluorescence of two muscle spindles in adult soleus muscle stained for cardiac TnT with mAb RV-C2 (A) and for embryonic MHC with mAb BF-G6 (B). RV-C2 reacts strongly with the nuclear bag fibers of the spindles, while BF-G6 stains brightly the nuclear chain fibers. Bar: 28 μm.

Fig. 7. Regenerating fibers in soleus muscle 3 days (A) and 11 days (B) after a cold injury. Immunoperoxidase staining with anti-cardiac TnT. Bar: 36 μm.
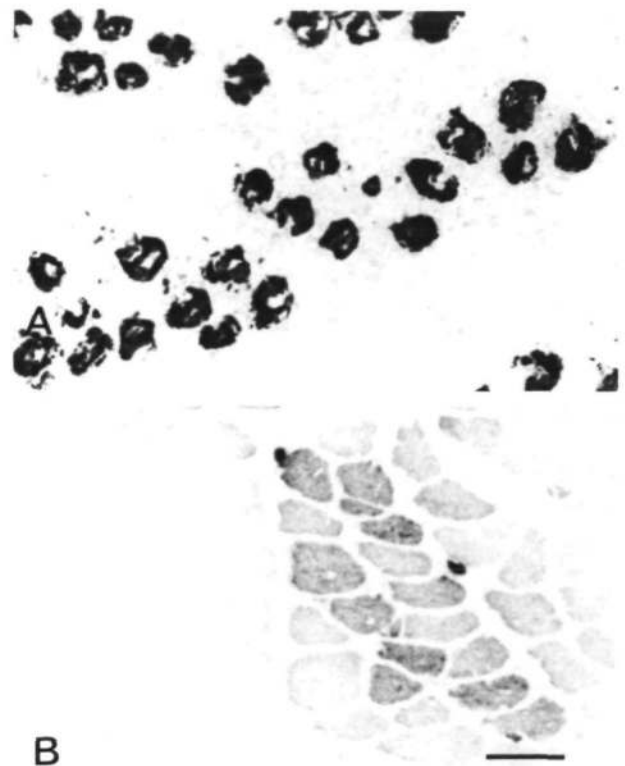
reduced in the different fiber types in soleus and tibialis muscles, while embryonic MHC persists for several weeks in type 2A fibers (Fig. 5).

The intrafusal fibers of muscle spindles are the only fibers reacting for cardiac TnT and MHC in adult hindlimb muscles: however, as shown in Fig. 6, the bag fibers are more reactive for cardiac TnT, whereas the chain fibers are more reactive for embryonic MHC (see also Rowlerson *et al.* 1985). Embryonic MHC is known to be expressed also in extraocular muscles of adult rats (Wieczorek *et al.* 1985; Sartore *et al.* 1987); in contrast, no reactivity for cardiac TnT is observed in extraocular muscles (data not shown).

Re-expression of cardiac TnT and embryonic MHC in regenerating and denervated muscle

3 to 5 days following a cold injury numerous regenerating fibers in the injured area were found to stain for cardiac TnT (Fig. 7A). Reactivity persists in subsequent days and begin to disappear 11 days after the lesion (Fig. 7B). Double-labelling experiments showed that most regenerating fibers reacting for cardiac TnT are also reactive for embryonic MHC. However, the relative intensity of staining with the two antibodies is not closely correlated in the different fibers and some fibers stain only with one or the other antibody (Fig. 8).

Cardiac TnT was also detected in a number of denervated muscle fibers like embryonic MHC (Schiaf-

fino *et al.* 1988). As shown in Fig. 9, many fibers staining for cardiac TnT are often unreactive for embryonic MHC and the opposite is also true. We have previously reported that re-expression of embryonic MHC after denervation is confined to a specific fiber type, the type 2A fibers (Schiaffino *et al.* 1988). In contrast, as determined by analysis of serial sections with fiber type-specific anti-MHC mAbs (Schiaffino *et al.* 1989), cardiac TnT immunoreactivity can be detected in different fiber types (not shown).

Discussion

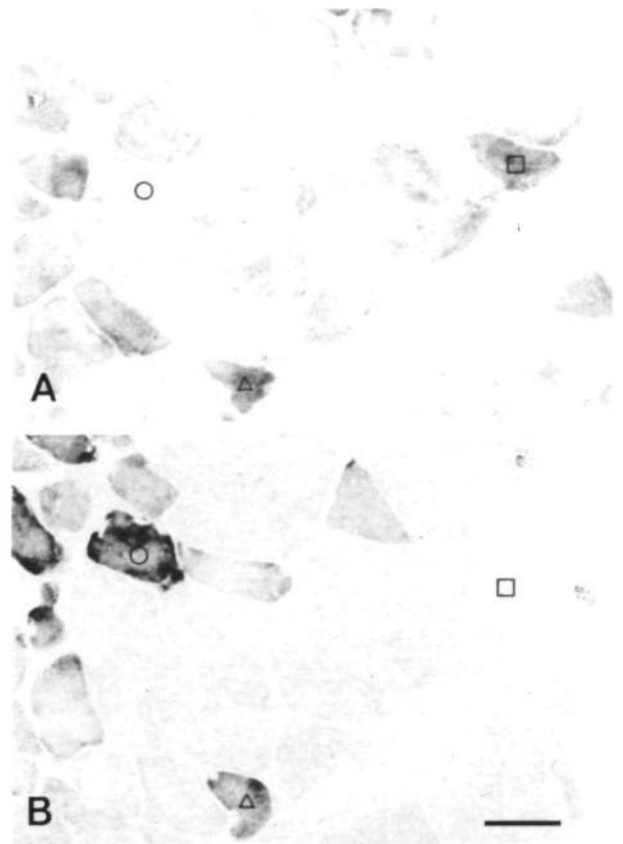*TnT isoforms in fetal and neonatal skeletal muscle*

This study shows that in the rat, like in the chicken (Toyota and Shimada, 1981; Cooper and Ordhal, 1985; Ogasawara *et al.* 1987), two TnT isoforms antigenically and electrophoretically similar to those expressed in the embryonic and adult heart are transiently expressed in fetal and neonatal skeletal muscle. This is a further example of developmentally regulated sarcomeric protein isoforms, like embryonic myosin light chain (Whalen *et al.* 1978), embryonic and neonatal MHCs (Whalen *et al.* 1981), and α-cardiac actin (Minty *et al.* 1982), which are transiently expressed in developing skeletal muscle. Embryonic and adult cardiac TnT are sequentially expressed during skeletal muscle development: the embryonic isoform is predominantly expressed in fetal stages while both isoforms are present in
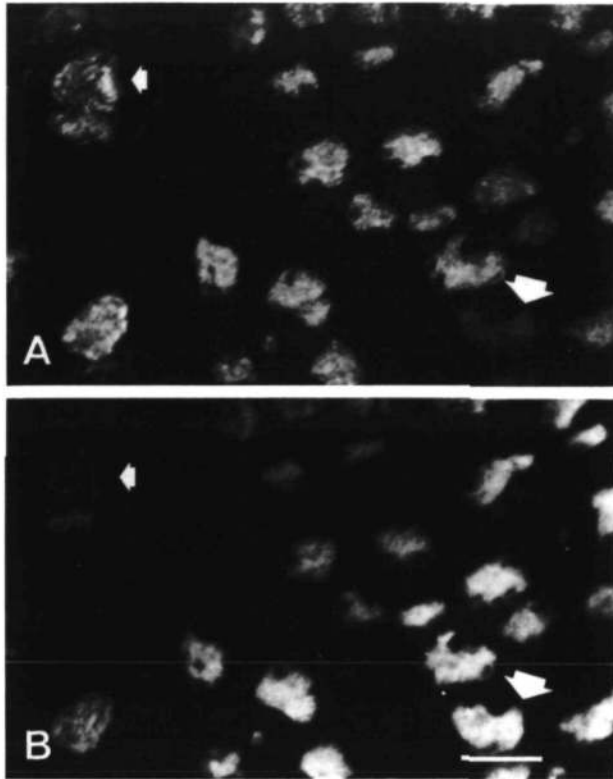
Fig. 8. Double immunofluorescence of regenerating tibialis anterior muscle 5 days after a cold injury, stained for cardiac TnT (A) and for embryonic MHC (B). Most of the regenerating fibers are labelled by both antibodies, but some stain only for cardiac TnT (small arrow) or embryonic MHC (large arrow). Bar: 28 μm.

Fig. 9. Serial cryosections of soleus muscle 14 days after denervation stained for cardiac TnT (A) and for embryonic MHC (B). Note the presence of fibers reactive for cardiac TnT and unreactive for embryonic MHC (square), fibers reactive for embryonic MHC and unreactive for cardiac TnT (circle), and fibers reactive for both markers (triangle). Immunoperoxidase staining. Bar: 36 μm.

1-day and 7-day newborns and the adult form is the only detectable TnT species by 15 days. These findings suggest that in the rat, like in the chicken (Cooper and Ordhal, 1985), a similar switch in splicing of cardiac TnT pre-mRNA takes place during development in cardiac and skeletal muscle. However, the level of expression of the cardiac TnT gene in developing skeletal muscle appears to vary according to species. In the chicken, the cardiac TnT gene is expressed at similar levels in embryonic skeletal and cardiac muscle (Cooper and Ordhal, 1985). In the rat, the pattern of accumulation of cardiac TnT closely parallels that of the corresponding mRNA, which is present in much lower amounts in fetal skeletal compared to fetal cardiac muscle (Ausoni et al. in preparation). These findings suggest that the level of cardiac TnT protein in developing skeletal muscle is regulated by changes in the level of the corresponding mRNA.

Immunoblotting analysis with a mAb that reacts with both cardiac and skeletal muscle TnT species reveals a distinct TnT profile in neonatal skeletal muscle. In addition to the cardiac TnT isoforms, hindlimb muscles from 1- and 7-day-old rats contain two other TnT bands that migrate ahead of cardiac TnTs but have lower mobility than the TnT species found in fast and slow skeletal muscles from adult animals. An additional lower mobility component is seen in muscles from 15-

day-old animals. The nature of these neonatal-specific TnT variants remains to be determined. Previous genomic and cDNA analyses have shown that alternative splicing of the fast skeletal muscle TnT gene generates multiple mRNAs, which are differentially expressed in developing and adult rat skeletal muscle (Medford et al. 1984; Breitbart et al. 1985). It is therefore likely that the neonatal-specific TnT forms are the product of mRNAs derived from alternative splicing of the fast skeletal muscle TnT gene. Multiple TnT variants, presumably derived by developmentally regulated pre-mRNA splicing of the fast skeletal TnT gene (Bucher et al. 1989), have been detected in neonatal chicken breast muscle (Abe et al. 1986).

Non-coordinated expression of cardiac TnT and embryonic MHC in skeletal muscle

Cardiac TnT is re-expressed during muscle regeneration, like embryonic and neonatal MHCs (Sartore et al. 1982) and embryonic myosin light chain (Carraro et al. 1983), and can thus represent a useful marker for regenerating fibers. Cardiac TnT is also re-expressed in a number of muscle fibers after denervation, as previously described for embryonic and neonatal

MHCs (Schiaffino *et al.* 1988). A reversion to an embryonic pattern of troponin T synthesis has also been described in regenerating and denervated chicken muscle, where the leg-type TnT typical of the embryonic stages is re-expressed in those muscles that contain the breast-type TnT as the major adult isoform (Obinata *et al.* 1984; Matsuda *et al.* 1984; Shimizu and Shimada, 1985).

In this study, we have used an immunocytochemical approach to investigate at the single fiber level the expression of two developmentally regulated sarcomeric proteins, cardiac TnT and embryonic MHC, during normal development, regeneration and denervation. Our findings show that there is no close correlation between the expression of the two proteins. Non-coordinated regulation for the synthesis of myofibrillar proteins has previously been observed in regenerating and denervated chicken skeletal muscle (Matsuda *et al.* 1984; Saad *et al.* 1988). The difference in the pattern of disappearance of cardiac TnT and embryonic MHC during postnatal development is particularly striking: whereas the level of cardiac TnT declines progressively and homogeneously in all fiber types, embryonic MHC rapidly disappears after birth in all fiber types except in type 2A fibers where it persists for several weeks (see also Schiaffino *et al.* 1988). In developing chicken skeletal muscle repression of the cardiac TnT gene is controlled at the level of transcriptional initiation (Long and Ordhal, 1988), and is probably nerve-dependent (Toyota and Shimada, 1983). It is not known whether transcriptional and/or post-transcriptional mechanisms operate to repress the embryonic MHC gene, but our results suggest that this process is modulated by fiber-type-specific factors that may also be implicated in the selective re-appearance of embryonic MHC immunoreactivity in type 2A fibers after denervation. In contrast, fiber-type-specific factors do not appear to be involved in the regulation of the cardiac TnT gene.

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