Expression Pattern of Troponin I and Distinct Alternatively Spliced Developmental Isoforms of Fast Troponin T *In Vitro* and in Neonatally Denervated Rat Skeletal Muscles

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Abstract

To investigate the role of innervation in muscle cell differentiation, the expression pattern of troponin I and troponin T isoforms was investigated in aneural *in vivo* and *in vitro* myotubes using immunoblotting and Northern blot hybridisation procedures. The immunoblotting procedure showed that slow troponin I was expressed at high levels in cell cultures prepared from both fetal and neonatal rat skeletal muscles. With increased time in culture, the relative level of slow troponin I decreased while that of fast troponin I increased although this transition was not complete. The transformed rat myogenic cell line L6 also expressed a high level of slow troponin I but unlike the primary cell cultures, the changes in the relative proportions of fast and slow troponin I were not observed with increased time in culture:

The cell cultures prepared from 16 day fetal rat skeletal muscles expressed only low levels of fetal isoforms FF1 and FF2 of fast troponin T. The expression of FF1, FF2, neonatal NF1 and a small amount of an adult troponin T, AF1, was detected in cell cultures prepared from 18 day fetal, newborn and neonatal rat hindlimb muscles. The neonatal and adult isoforms of troponin T in cell cultures were detected in appreciable amounts after only 5-10 days in culture. The expression of both fetal, neonatal and three adult (AF1-AF3) isoforms of troponin T was detected in rodent transformed myogenic cell lines L6 and C2. Unlike the primary cell cultures, all isoforms in myogenic cell lines appeared within 2 days of *in vitro* growth.

The synthesis of fast troponin T mRNA investigated by Northern blot hybridisation procedure, continued in the neonatally denervated rat skeletal muscles. Post transcriptional changes included a delayed decline in the amount of fetal and neonatal fast troponin T mRNA containing exon y. The level of fast troponin T mRNA containing exon 17 in these muscles also gradually declined after denervation.

Key words: muscle, troponin I, troponin T, *in vitro* myotubes, denervation.

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Differentiation or maturation of skeletal muscle fibres is accompanied by changes in the expression pattern of different fibre type and developmental stage specific isoforms of a number of contractile and regulatory proteins of the myofibril during development. For example, in addition to the expression of some fibre type specific isoforms of myosin such as slow myosins, the early embryonic myotubes express fetal myosin which during later development is replaced by neonatal and finally adult myosins [25]. It is not clear, however, how much of this differentiation process is genetically determined and to what extent it is influenced by innervation, hormones or stretch. Thyroid hormones have been shown to play some role in the completion of myosin isoform transitions in rat developing muscles [4,8]. Hypothyroidism in developing rats also retards the isoform transitions of troponin T but does not inhibit this process completely [18]. Some myosin isoform transitions have been reported to continue when the innervation is interrupted in the neonatal rats [3]. The lack of innervation *in vitro*, however, has been reported to limit the normal myosin isoform transitions in muscle cell cultures [7]. For example, while fetal and neonatal myosins are detected in aneural muscle cell cultures [7, 21], the adult myosin is detected in myotubes co-cultured with spinal cord explants only [7].

The *in vitro* growth ofmyotubes provides a model system in which to investigate the growth and differentiation of cells in isolation from innervation. Most of the studies so far have been carried out using myosin as a muscle fibre type and developmental stage specific marker. The myosin in mammals is encoded by a multi gene family and diversity in this protein is generated at the gene level. It is not known whether similar changes are observed in other proteins such as troponin I or troponin T which are also encoded by multigene families. Calcium ions working through the troponin complex (troponin I, T and C) and tropomyosin, regulate the ATPase activity ofmyosin. Troponin T and troponin I in striated muscle are both encoded by three genes, each of which is predominantly expressed in either fast skeletal, slow skeletal or cardiac muscle [6, 11, 12]. Troponin T in addition generates further diversity by alternative RNA splicing which produces a large number of both cell type and developmental stage specific isoforms [12, 17]. Changes in the isoforms may influence the contractile properties of the muscle.

A small amount of fast skeletal muscle troponin T mRNA is detected in rat fetal skeletal muscles and its level increases during postnatal development [13]. The multiple isoforms produced by this gene are subject to tight regulation in a developmental and tissue specific manner [10]. Recent work has shown that the inclusion of a novel exon, termed exon y [2, 13] into three different final transcripts correlates well with the size and expression pattern of developmentally regulated protein isoforms of fast troponin T, FF1, FF2 and NFL Exon y is found in perinatal transcripts up to postnatal day 28. The mutually exclusive splicing regulation of exon 16 and 17 at protein level is undetectable. However, analysis of troponin T mRNA suggests that there is a preference for exon 16 over exon 17 in adult fast skeletal muscles [12, 13].

In the present study, the role of innervation in the completion of differentiation process was investigated in aneural muscle cell cultures and also in the neonatally denervated rat skeletal muscles.

Methods

Preparation ofmuscle cell cultures

Fetal hindlimbs or specific neonatal muscles were excised aseptically and washed twice with sterile PBS. The tissue was added to 1 ml of enzyme mixture containing 1 mg/ml collagenase and 10 mg/ml DNase in PBS and incubated at 37°C with gentle stirring. After centrifugation, the supernatant was pipetted off into 2 ml 10% fetal calf serum (FCS) in DMEM (Life Technologies) supplemented with 1% L-Glutamine and 1% Pencillin/Streptomycin solution (Life Technologies). Cycles of enzyme disaggregation and supernatant collection were repeated until no intact material remained. The 2 ml aliquots of disaggregated material were pooled, centrifuged and the pellets resuspended in 8 ml of 10% FCS in DMEM. The resulting suspension was filtered through a double layer of 22.4 μm nylon mesh. The nonmyogenic cells were depleted by preplating the cells into uncoated 60 mm tissue culture dishes (Nunc). Unattached cells were collected after 30 minutes incubation at 37°C and 5%CO2. Cells isolated from fresh tissue or recovered from frozen cell stocks (L6 and C2) were plated at $2x10^4$ cells/cm^2 in 10% FCS DMEM supplemented with glutamine and antibiotics in 35 mm or 50 mm gelatin-coated tissue culture dishes. The medium was changed after 24 hours and every three days thereafter.

Electrophoresis and immunostaining of Western blots

After different time intervals, the cells were harvested (3-5 petri dishes/time point) by washing and resuspending the cells in cold PBS. The resultant suspension was centrifuged and the pellet resuspended and boiled for 5 minutes in sample buffer [17]. Weighed cell or muscle samples removed from normal developing rats (3-5 animals) used as controls were homogenised in 10 volumes of 0.0625 M Tris (pH 6.8)/2%SDS/5% 2-mercaptoethanol and 0.001% bromophenol blue and boiled for 5 minutes. The samples were stored at -70°c until required. 10 μl of each tissue sample was loaded onto sodium dodecyl sulphate denaturing polyacrylamide gel (SDS PAGE). Western blots were prepared and stained with the troponin I (42/25) or troponin T (F24 or JLT12) antibodies. Troponin I antibody stained blots were scanned by laser densitometer. Antibody 42/25 recognises all three isoforms of troponin I [16]. Antibodies F24 and JLT12 recognise all the fast isoforms of troponin T but JLT12 also reacts with cardiac isoforms in addition [18]. Horse radish peroxidase-linked rabbit anti mouse immunoglobulins (Dako Ltd.) were used to localize the primary antibody in the presence of diaminobenzidine and hydrogen peroxide.

Denervation procedure

New-born Wistar rat pups were used for control and surgical denervation. Using Halothane anaesthesia, denervation was performed in the right hindlimb by removal of a 5-6 mm segment of sciatic nerve from the upper thigh region. This led to paralysis of the hindlimb muscles. The muscles denervated at birth were removed from groups of 3-5 rats killed at days 3, 5, 7, 10, 14, 21, 28 and 35.

RNA preparation and Northern blot analysis

Total cellular RNA was isolated from rat muscles pooled together in each group (3-5 animals) using a guanidinium isothiocyanate extraction method [5]. To obtain sufficient material, the whole lower hindlimb muscles were used for earlier stages up to week 2 but only gastrocnemius muscle was used at day 28 and 35. 10 μg of each RNA sample was size separated on formaldehyde denaturing agarose gels. Removal of residual formaldehyde allowed efficient capillary transfer of the RNA on to nylon membranes (Hybond-N, Amersham Int.). Hybridisation of ^{32}P-labelled probes to the membranes was carried out over one hour at 68°C using Quikhyb solution and the associate procedure (Stratagene). Subsequent removal of unbound probe off the membranes included three

washes at room temperature with 2x SSC [20], 0.1% SDS over 30 minutes and a final wash at 60°C with 1xSSC, 0.1% SDS for 30 minutes. Autoradiographic exposure to Hyperfilm MP (Amersham Int.) ranged from 16 hours to 4 days. To remove hybridised probe for rehybridisation with another probe, the nylon blots were washed for 15 minutes twice at room temperature in 250 ml of boiling 0.1% SDS.

Oligonucleotides and DNA probes

The 180 bp PstI fragment from the fast troponin T construct (pTNT-15) corresponding to exon 18 was used to detect all of the fast troponin T mRNA isoforms [9]. 50 mer oligonucleotides for exon y of fast troponin T also including 11 nucleotides corresponding to exon 9 (5'-tggtt tctcc tcttc ctcgt cctcc tcccg ctcct cctcg gcgac agcat-3'), exon 17 (5'-ctgtg cttct gggct tggtc aatgc ggctc ctgag ggtgg taata tcgta-3') and for neonatal myosin heavy chain (5'-cgcat cttga ggagg ccgcc aagtg gctga aggaa aggca cagaa tgtgc-3') [14] synthesiscd by Oswell DNA Service were used to analyse the expression pattern of these specific mRNAs. These probes were used for Northern blot hybridisations as described in the previous section.

Results

Expression pattern of fast and slow troponin I isoforms in vitro and in vivo developing muscles

Hindlimb rat muscles at 16 days *in utero* expressed over 95% slow troponin I, the level of which dropped to 74% two days later at day 18 (Figure 1). The proportion of slow troponin I in the newborn rat hindlimb muscles decreased to about 1% by day 3 after birth. While the level of slow troponin I decreased, the level of fast troponin I increased with both fetal and neonatal development. Hindlimb rat muscles therefore expressed a high level of slow troponin I during early fetal development leading to high levels of fast troponin I during late fetal and neonatal stages (Figure 1).

Hindlimb cell cultures prepared from 18 day fetal, newborn and 3 day postnatal skeletal muscles showed a high level expression of slow troponin I when examined after 3 days *of in vitro* growth (Figure 1). The relative level of slow troponin I in such cultures decreased with increased length of time *in vitro*. For example, at 3 days all muscle cell cultures expressed over 70% (73-77%) slow troponin I which decreased to 56-64% at day 5 and 43-60% at day 10. There was only a small difference in the level of slow troponin I expressed in cultures prepared from different fetal and neonatal age groups tested (Figure 1).

The relative proportions of slow and fast troponin I were also investigated during *in vitro* growth of rat myogenic cell line L6. The L6 cell line expressed a high level (80%) of slow troponin I. Unlike the primary cell cultures, the level of slow troponin 1 in this myogenic cell line did not change with the length of time in culture when tested up to 2 weeks of growth (Figure 1).

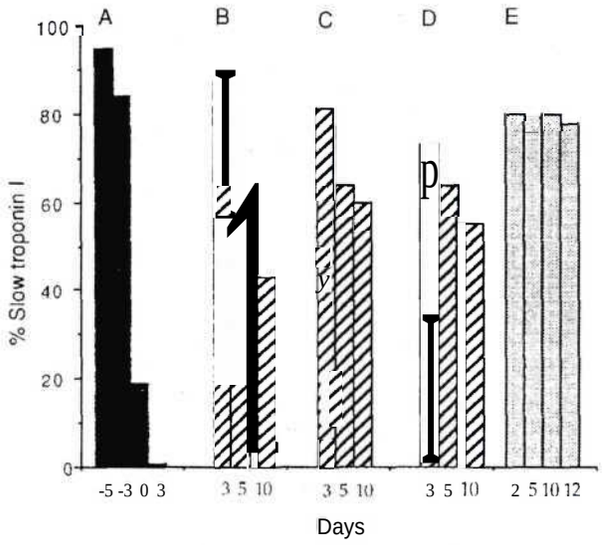

Figure 1. The proportion of slow troponin I expressed in vivo hindlimb (block A), in primary cell cultures (blocks B, C, D) and in rat myogenic cell line L6 (block E) studied from immunoblots. The remaining proportion of troponin I represents the fast isoform of troponin I. In block A, the 1-4 columns represent fetal stages at day 16 (-5), day 18 (-3), newborn (0) and neonatal day 3. Blocks B-D represent in vitro growth of primary cell cultures prepared from 18 day fetal (block B), newborn (block C) and neonatal day 3 (block D) hindlimb after 3, 5 and 10 days of growth. Block E represents myogenic cell line L6 after 2, 5, 10 and 12 days of growth in vitro.

Expression pattern of cardiac and developmental isoforms of fast troponin T in vitro and in vivo developing muscles

Fetal isoforms, FF1 and FF2, were expressed at low levels in 16 and 18 day fetal skeletal muscles (Figure 2A). The neonatal isoform NF1 was absent at day 16 and expressed at very low level in 18 day fetal skeletal muscles but its expression increased during the neonatal period. Adult isoforms of fast tropnin T, AF1-AF5 are present in the adult muscles which appear during neonatal period [16].

The cell cultures prepared from 16 day fetal rat hindlimb muscles showed a low level expression of FF1 and FF2 after 3 as well as 5 days of *in vitro* growth (Figure 2A). The other fast isoforms were usually not detected. The cell cultures prepared from 18 day fetal hindlimb muscles also showed the expression of FF1 and FF2 with only a trace amount of NF1, the neonatal isoform, at 3 days of culture (Figure 2A). By 5 days, both NF1 and an adult isoform AF1 were easily detectable in these cultures with NF1 being expressed at higher level than AF1. By 10 days, NF1 appeared to be expressed at the same level as FF1 and FF2 whereas AF1 continued to be expressed at a low level.

Cell cultures prepared from the newborn hindlimb muscles expressed FF1 and FF2 but NF1 and AF1 isoforms also became easily detectable after only 3 days in culture

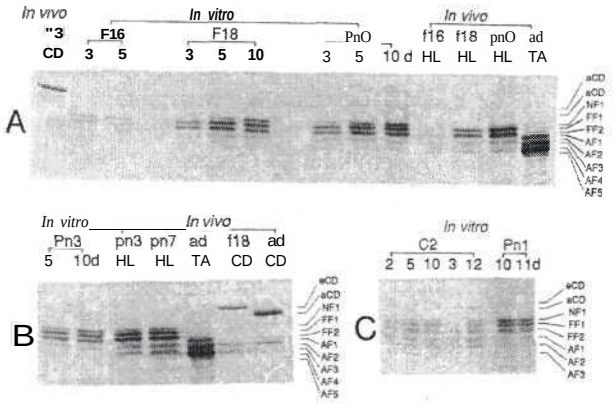

Figure 2. The expression of different troponin T isoforms in cultured myotubes and in some in vivo rat muscles studied by immunoblotting procedure using antibody JLT12 which recognises all fast and cardiac isoforms of troponin T. Part A: The tissue extracts for controls were prepared from Tibia Us anterior (TA), cardiac (CD) or hindlimb (HL) muscles from fetal days 16 (f16), 18 (f18) and postnatal day 0 (pnO) or adult (ad). The primary myotube cultures grown for 3, 5 or 10 days were prepared from hindlimb (HL) or Tibia/is anterior (TA) muscle from 16 day fetal (F16), 18 day fetal (F18), or postnatal day 0 (PnO) rat pups. Part B: The myotube cultures grown for 5 or 10 days were prepared from 3 day old rats (Pn3). The in vivo control hindlimb (HL), Tibialis anterior (TA) or cardiac (CD) muscles were removed from 18 day fetal (f18), postnatal day 3 (pn3), 7 (pn7) or adult (ad) rats. Part C: The expression of troponin T in mouse myogenic cell line C2 was investigated after 2, 5, 10, 3 and 12 days in vitro. The myotubes prepared from hindlimb of 1 day old rat (pn1) grown for 10 or 11 days were used as controls. eCD = embryonic cardiac troponin T, aCD = adult cardiac troponin T: FF1 and FF2 - fetal isoforms of fast troponin T; NF1 = neonatal isoform of fast troponin T; AF1-AF5 = adult isoforms of fast troponin T.

(Figure 2). The level of NF1 was higher in the cultured cells compared with the newborn *in vivo* muscles. The level of both NF1 and AF1 increased faster in newborn hindlimb cultures compared with the cultures from 18 day fetal skeletal muscles. When cell cultures were prepared from 3 or 7 day neonatal limbs, FF1 and FF2 were still the major isoforms expressed (Figure 2B). Neonatal and adult isoforms NF1 and AF1 became detectable after 3 days but the level of adult isoform in cultures prepared from these stages of development did not appear any higher than in the cell cultures prepared from newborn muscles. Cell cultures prepared from adult rat Soleus and Extensor digitorum longus muscles also showed the expression of mainly FF1, FF2 and NF1 isoforms (not shown). Cardiac troponin T was expressed at a low level at all stages of *in vitro* growth.

The rat transformed myogenic cell line L6 expressed a number of troponin T isoforms during *in vitro* growth. The developmental isoforms FF1, FF2 and NF1 were expressed throughout the growth period of two weeks in culture. These myogenic cells also expressed three adult isoforms AF1, AF2 and AF3 which appeared within a few days of culture with their levels maintained throughout the growth period (not shown). Similarly, C2 mouse muscle cell line also expressed these six fast troponin T isoforms throughout the 2-12 day period of culture. There were no apparent changes during this time period (Figure 2C).

Expression pattern of fast troponin T and neonatal myosin heavy chain mRNA in normal and neonatally denervated hindlimb muscles

Fast troponin T mRNA transcripts in normal and denervated muscles were identified using a Northern hybridisation procedure (Figures 3 and 4). Radiolabelled cDNA corresponding to exon 18 detected all fast troponin T mRNA whereas radiolabelled oligonucleotide probes specifically detected fast troponin T mRNA containing either exon y or exon 17. The fast troponin T mRNA isoforms detected had a mobility corresponding to approximately 1.0 kb. An additional transcript, 3.2 kb in length was detected up to day 10 in controls but up to day 35 in denervated hindlimb muscles.

The level of fast troponin T mRNA in denervated muscles was noticeably greater from 7 to 28 days. The pattern of alternative splicing of the fast troponin T mRNA also changed with denervation. The level of fast troponin T mRNA containing exon y was greater in the denervated muscles compared with the controls. This was particularly apparent during later stages of development when this mRNA isoform was only detectable, albeit in small amounts, in the denervated muscles (Figure 4). The delayed decline in the production of fast troponin T mRNA containing exon y followed a trend similar to that of neonatal myosin heavy chain mRNA (Figure 4). The most noticeable difference in the usage of exon 17 was observed at day 35 where there was less fast troponin T with this exon in the denervated muscle.

Discussion

Slow troponin I is expressed at high levels in early fetal skeletal muscles, the level of which decreases during subsequent fetal and neonatal *in vivo* development. High levels of slow troponin I were also expressed in cultures from both fetal and neonatal skeletal muscles. The primary myotube cultures showed some reduction in slow troponin I with time but this isoform was still expressed at quite high levels after 10 days *in vitro*. Sunderland et al [23] have reported 100% slow troponin I mRNA in primary cell cultures prepared from 16 day fetal limbs and 62% in cell cultures from 19 day fetal limbs. While we observed abundance of slow troponin 1 in such fetal cell cultures, we never observed levels approaching 100%. This may reflect the different sensitivities of the two techniques or the differences in protein and mRNA levels. Unlike slow

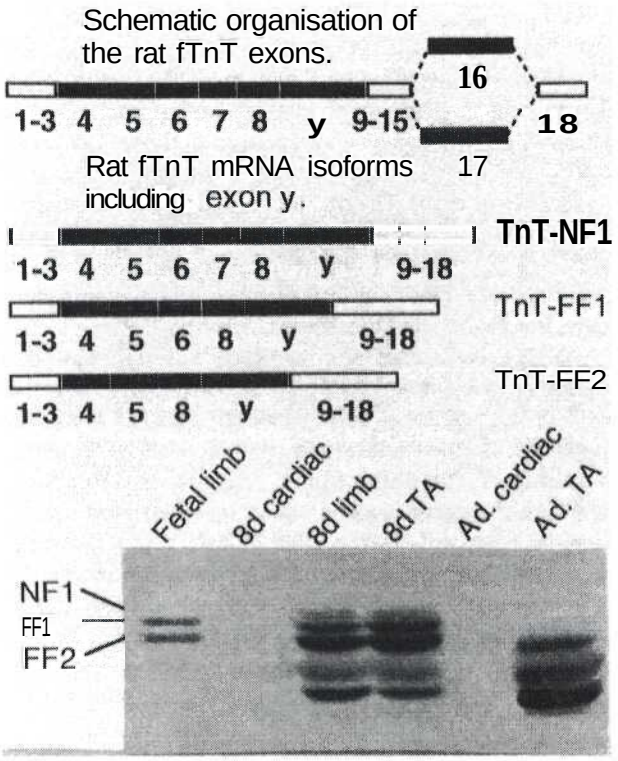

Figure 3. Schematic representation of the splicing variants of the rat fast troponin T gene. Constitutive exons and alternative exons are represented by open and filled rectangles and annotated accordingly. Exons 16 and 17 are subject to mutually exclusive incorporation into the processed mRNA. The three transcripts that encode exon y are NF1, FF1 and FF2. These transcripts correspond to the protein isoforms detected by the fast troponin T monoclonal antibody F24 on SDS PA GE Western blot. Antibody F24 does not react with cardiac isoforms of troponin T. The rat muscle samples represented are from fetal limb, postnatal 8 day cardiac muscle, limb muscle and Tibialis anterior (TA), adult cardiac and adult TA.

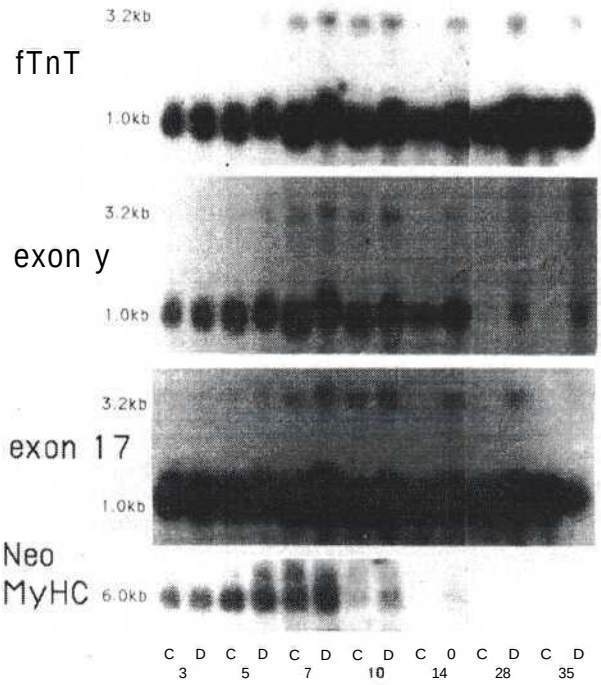

Figure 4. Northern hybridisation of four ^{32}P-labelle DNA probes: the 180 bp fast troponin T corresponding to exon 18 (all fast troponin T mRNA) and three 50 mer oligonucleotides specific for exon v (troponin T), exon 17 (troponin T) and neonatal myosin heavy chain. The 10 μg total RNA samples represented are from control (C) versus denervated (D) lower hindlimb muscles at 2, 5. 7, 10 and 14 day postnatal and gastrocnemius muscles at 28 and 35 day postnatal with denervation carried out at day 0. The processed fast troponin T transcripts are approximately 1.0 kb in size although an additional transcript was detected with an approximate size of 3.2 kb. The myosin heavy chain transcripts are approximately 6.0 kb in length.

troponin I, the expression of slow myosin *in vitro* is greatly influenced by the age of the developing muscle from which the cell cultures are prepared. For example, in rat, mouse and chicken, very little or no slow myosin is detected in cultures prepared from late fetal or neonatal limb skeletal muscles [22, 24]. The much higher levels of slow troponin I *in vitro* may mimic the early *in vivo* embryonic myotubes which also express high levels of slow troponin I. Unlike the *in vivo* muscles in which a rapid transition of slow troponin 1 to fast troponin I is observed during late fetal development, this transition was initiated but not completed *in vitro* over the 10 day time period investigated in this study. This may reflect the much lower level of stretch or tension induced in cultured myotubes which is necessary to induce changes similar to *in vivo* development.

Unlike myosin and troponin T, which express both cell type and developmental stage specific isoforms [17, 25], only three isoform types, fast, slow and cardiac, characteristic of fast, slow and cardiac muscle types, have been described for troponin I [6]. The slow troponin I is also expressed at high levels during early development of cardiac [16, 19] and skeletal muscles and therefore is believed to act as an early developmental isoform in both these tissues. It is not known if the nature of slow troponin 1 observed during early development is different from adult slow troponin I. Since slow troponin I in normal adult skeletal muscles is usually restricted to type I fibres, the changes observed in slow troponin 1 may reflect both the muscle cell type and developmental stage specific expression of troponin I in cultured muscle cells.

All fetal and neonatal rat skeletal muscle cell cultures initially expressed only the fetal isoforms of troponin T. The expression of neonatal and a trace amount of an adult isoform was observed with only increased time in culture. Compared with early fetal muscles, the induction of neona-

tal and a small amount of an adult troponin T isoform was faster in cultures prepared from late fetal or neonatal muscles. While the level of neonatal troponin T increased considerably with both time in culture and to a certain extent with the increased age of the muscle used to prepare the cultures, the expression of small amount of only one adult isoform remained very limited. The other adult isoforms were barely detectable in any of the primary cell cultures examined in this study. It therefore appears that some troponin T transitions can occur in the absence of nerve but this transition is incomplete since a number of adult isoforms are not observed and the fetal isoforms continue to be expressed at high levels. The low level expression of an adult troponin T isoform in aneural muscle cell cultures differs from myosin, the adult isozymes of which are not detected in such cultures [7, 21]. The presence of adult myosin heavy chains in primary cultures, however, has been reported by some other groups [10, 15]. While the changes in myosin occur by activating new genes, the changes in fast developmental troponin T isoforms are brought about by alternative RNA splicing of the same fast troponin T gene. The changes at the gene and RNA alternative splicing level respond to different signals and may explain the differences observed in myosin and troponin T.

Since the cell cultures prepared from 16 day fetal skeletal muscles express only FF1 and FF2, it is possible that these cells represent a different myogenic cell lineage as has been suggested for myosin [21]. The levels of FF1 and FF2 in these cultures were low suggesting that there is a compensatory higher level of slow or embryonic troponin T at this stage which may be expressed at lower levels in cultures from later stages of development which express higher levels of fast troponin T instead. The expression of an adult isoform in cell cultures prepared from later stages of development further indicates the possible existence of different myogenic cell lineages but the changes observed were very gradual and not a sudden change typifying the distinct myogenic cell lineages emerging at different stages of development.

The transformed myogenic cell lines in contrast expressed a higher and larger number of adult isoforms of fast troponin T. Unlike the gradual appearance of later developmental isoforms of troponin T in primary cell cultures, the sudden expression of six isoforms of fast troponin T was observed in transformed myogenic cell lines which indicates differences in normal and transformed cells and some programming of such an expression pattern in cell lines. The distinction between primary and transformed myogenic cell lines was also indicated by the expression of troponin I isoforms. For example, the reduction in slow troponin I with time was observed in only primary cell cultures and not in transformed myogenic cell line L6 which continued to express slow troponin I at high levels.

The synthesis of fast troponin T mRNA continued in neonatal denervated rat skeletal muscles at a slightly higher level than in the normally developing muscles. The enhanced production of fast troponin T mRNA with denervation may reflect the slow to fast fibre type change. The lower levels of fast troponin T with exon 17 at day 35 may also be indicative of slow to fast transition occurring with denervation. The changes observed in perinatal isoforms of troponin T were similar to those observed for neonatal myosin heavy chain. The regulation of alternative splicing of fast troponin T mRNA, as with the transcriptional regulation of fast troponin T and neonatal myosin heavy chain gene indicates tight control influenced by developmental stage, innervation and mechanical activity.

Although the level of exon y mRNA was higher in denervated muscles, it nevertheless was considerably reduced after 4 weeks of denervation indicating that this transition despite being slowed down can continue when innervation is interrupted. This differs from the situation in cell cultures in which the suppression of developmental isoforms of troponin T was not observed. This may be due to the much shorter time period investigated *in vitro* and the absence of other associated factors in addition such as tension and stretch. Unlike the newly formed myotubes in culture, the myotubes in denervated muscles probably already had sufficient length of neuronal contact to specify the transitions and expression of some adult troponin T isoforms.

The detection of a 3.2 kb RNA by the fast troponin T probes suggests the existence of a fast troponin T pre-mRNA. This mRNA species was detected up to day 10 in the controls but was still present through to day 35 in the denervated muscle samples. The size of this mRNA, and that each of the fast troponin T DNA probes detected it, suggests that in each case the same intron or introns were retained. The intron between constitutive exons 9 and 10, is 2258 bases in length [1] and could be such a candidate. If this were true, the accumulation of this pre-mRNA indicates that there is a rate limiting step in the production of the mature message of fast troponin T and hence a further level of post-transcriptional regulation.

In conclusion, both troponin I and troponin T showed a limited degree of progression of isoform transitions with increased time in culture but these transitions were not complete. The complement of developmental isoforms of troponin T appeared to be related to both the length of time *in vitro* and the developmental stage of the muscle from which the cell cultures were prepared. A comparison of the expression of troponin T *in vitro* and *in vivo* suggests that factors in addition to innervation also influence the expression of adult and developmental isoforms.

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