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Novel developmentally regulated exon identified in the rat fast skeletal muscle troponin T gene

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SUMMARY

In theory, the rat fast skeletal muscle troponin T gene can generate 64 different isoforms. Here we report the identification of a novel alternative exon (exon y) that increases the potential isoform variation to 128. The inclusion of exon y in fast skeletal muscle troponin T mRNA occurs in perinatal, but not adult, skeletal muscle. Exon y is located between exons 8 and 9. This is the first time that a developmentally regulated exon located amongst a set of alternatively spliced exons has been described. Exon y is included in two mRNA isoforms. The proteins that these mRNAs would encode have molecular masses greater than that of the largest fast skeletal muscle troponin T isoform lacking exon y. These two proteins correlate well in both size and pattern of expression with the two fast skeletal muscle troponin T isoforms expressed in perinatal skeletal muscle. These results indicate that there is coordinated regulation of the splicing of exon y with other alternative exons.

Key words: skeletal muscle, troponin T, alternative splicing

INTRODUCTION

Alternative splicing plays an important part in the generation of isoform variation of the contractile and regulatory muscle proteins (Nadal-Ginard et al., 1991). The ability of the rat fast skeletal muscle troponin T (fTnT) gene to generate theoretically 64 isoforms is well documented (Breitbart et al., 1985). Troponin T, together with troponin C and troponin I, form the troponin complex. The troponin complex interacts with tropomyosin and mediates a calcium ion sensitivity to the actomyosin ATPase. The rat fTnT transcript of 18 exons is modulated by alternative splicing at two distinct sites. Exons 4 to 8 inclusive, correspond to the N-terminal region of the protein. It has been proposed that the N-terminal region interacts with the head to tail overlap of tropomyosin on the actin filament (Tanokura et al., 1983). The implication in conferring calcium ion sensitivity cannot be excluded, but this property has been confirmed in the C-terminal region of the protein (Heeley et al., 1987). The C-terminal region of the protein contains sites of interaction with tropomyosin, troponin I and troponin C. Exons 16 and 17 have the constraint of mutual exclusion and reside near the C terminus. The remaining exons are constitutively spliced.

Alternative splicing regulation of the rat fTnT gene is both developmental stage- and tissue-specific. This has been demonstrated at the mRNA level (Breitbart et al., 1985) and also suggested at the protein level (Sabry and Dhoot, 1991a). The regulation of splicing is therefore not random and cannot rely exclusively on pre-mRNA secondary structure. It must include the interaction of *trans*-acting factors (Breitbart et al., 1987). Furthermore, the existence of specific isoforms at certain developmental stages implies a biochemical and a physiological rationale to their regulation.

The rat fTnT has two main distinct perinatal protein isoforms (Saggin et al., 1990; Sabry and Dhoot, 1991a). These two isoforms have high molecular masses compared to other fTnT isoforms and cannot be explained by the alternatively spliced mRNAs of the fTnT gene. S_1 nuclease analysis (Breitbart et al., 1985) demonstrated the combinatorial behaviour of the cassette exons 4 to 8 through skeletal muscle development but did not explain the existence of these perinatal isoforms. Many of the detected transcripts increased in amount from foetal to 28-day neonatal muscle. Furthermore, although exon 17 is used in preference to exon 16 in developing muscle, exon 17 is still present in adult muscle transcripts (Medford et al., 1984). Are the perinatal TnT isoforms therefore derived from the fTnT gene (Saggin et al., 1990; Sabry and Dhoot, 1991a)?

In the present study TnT clones isolated from neonatal rat limb muscle cDNA library have been characterised. The sequence of one of these clones matched other fTnT transcripts (Garfinkel et al., 1982; Breitbart et al., 1985). It also

contained an additional fragment, designated exon y, between exons 8 and 9, identical to a region within that intron on the genomic sequence (Breitbart and Nadal-Ginard, 1986). The existence of exon y, its ability to be alternatively spliced, and its developmental regulation were further confirmed by northern blotting and by polymerase chain reaction (PCR) analysis.

MATERIALS AND METHODS

Electrophoresis and immunostaining of western blots

Muscle samples were prepared as previously described (Sabry and Dhoot, 1991a). A 10 μl sample of a 10% (w/v) muscle in sample buffer was loaded onto a sodium dodecyl sulphate denaturing polyacrylamide gel. Western blots were prepared and stained with the monoclonal antibody F24 (Dhoot, 1988; Sabry and Dhoot, 1991a). Horseradish peroxidase-linked rabbit anti-mouse immunoglobulins (Dako Ltd.) were used to localize the primary antibody in the presence of diaminobenzidene and hydrogen peroxide. Densitometric scanning of the western blots was carried out using a Joyce Loebl laser densitometer.

RNA preparation

RNA was isolated from a selection of Wistar rat tissues using a guanidium isothiocyanate extraction method (Chomczynski and Sacchi, 1987). Quantification of RNA was by absorbance spectrophotometry at 260 nm wavelength.

Construction and screening of cDNA library

Generation of the cDNA library used materials and protocols provided by Stratagene's Lambda Zap system. The poly(A) RNA was isolated from 3- to 7-day neonatal Wistar rat hind-leg skeletal muscle RNA. The library, in phage lambda, was screened with the coding region *Pst*I fragment of the rat fTnT construct pTNT-15 (Garfinkel et al., 1982). Through recombination the phage from isolated positive plaques were maintained as pBluescript SK plasmids in *Escherichia coli* Xl1-Blue and screened for a second time with the same probe.

Subcloning and sequencing cDNA clones

Restriction fragments of clones were inserted into the replicative form of M13mp18 and M13mp19 strains (Sigma Chemicals), allowing the generation of single-stranded templates (Sambrook et al., 1989). The method for sequencing is based on the one by Sanger et al. (1977) using a Sequenase V2 kit. The materials and protocols necessary for sequencing were provided by U.S. Biochemicals. [α-^{35}S]ATP (Amersham Int. plc.) was incorporated to allow detection by autoradiography. The fragments generated through sequencing were size separated on 4% to 8% polyacrylamide urea denaturing gels.

Northern blot analysis

Total cellular RNA samples were separated by size on formaldehyde denaturing agarose gels. Removal of residual formaldehyde allowed efficient capillary transfer of the RNA onto nylon membranes (Hybond-N, Amersham Int. plc.). Hybridisation of ^{32}P-labelled probes to the membranes was carried out over one hour at 68°C using Quikhyb solution and the associated procedure (Stratagene). Subsequent removal of unbound probe off the membranes included three washes at room temperature with 2× SSC, 0.1% SDS over 30 minutes and a final wash at 60°C with 1× SSC, 0.1% SDS for 30 minutes. Autoradiographic exposure to Hyperfilm MP (Amersham Int. plc.) ranged from 16 hours to 4 days. The 180 bp *Pst*I fragment, from the fTnT construct pTNT-15, was used to detect fTnT mRNA. 50mer oligonucleotides for exon y and exon 17 were generated using the published genomic sequence. To remove hybridised probe, the nylon blots were washed for 15 minutes twice at room temperature in 250 ml of boiling 0.1% SDS.

PCR analysis

Single-stranded (ss) cDNA was prepared from 10 μg RNA using oligo(dT_{18}) and MMuLTV reverse transcriptase (Boehringer and Mannheim; Sambrook et al., 1989). The cDNA equivalent of 0.1 μg RNA was used for each PCR experiment. The reaction mixture for PCR in 100 μl included 1 unit *Taq* polymerase (Promega), 1× Reaction Buffer (Promega), 4 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP and dTTP, and 100 ng of each primer. The thermal cycling for the synthesis of fragments between exon 1 and exon y was 31 cycles including incubations: 30 seconds at 94°C, 30 seconds at 61°C and 30 seconds at 72°C. There was also an initial incubation for 30 seconds of 94°C and a final incubation of 270 seconds at 72°C.

Oligonucleotides

All oligonucleotides were purchased from Oswell DNA Service. The relevant sequences were obtained from the published genomic sequence. Oligonucleotide sequences for the PCR primers are altered to include restriction endonuclease cut sites. The sequences are as follows: exon 1 sense primer, 5′-tgctg caggt cctgt ccaca aggag-3′; exon y anti-sense primer, 5′-tcgag ctcct cctcg gcgac agcat-3′; exon y northern blot probe (including 11 nucleotides corresponding to the constitutively spliced exon 9), 5′-tggtt tctcc tcttc ctcgt cctcc tcccg ctcct cctcg gcgac agcat-3′; exon 17 northern blot probe, 5′-ctgtg cttct gggct tggtc aatgc ggctc ctgag ggtgg taata tcgta-3′.

Preparation and sequencing of PCR fragments

The separation of radiolabeled PCR preparations on a polyacrylamide sequencing gel permitted selection of fragments, which were detected by autoradiographic film. Removal of gel slices and subsequent 'freeze and squeeze' isolation (Sambrook et al., 1989) allowed asymmetric PCR to be used to generate ssDNA fragments for sequencing. The reaction mixture for PCR was the same as above except that 4 ng:400 ng was the ratio of primers to exon 1 and to exon y, respectively. The thermal cycling was the same as described above for both of these primers. The resultant fragments were separated using agarose gel electrophoresis and purified according to the procedures outlined using the MERmaid kit (Bio 101, Inc., USA.). PCR fragments were then sequenced using a Sequenase V2 kit (U.S. Biochemicals), with the inclusion of dimethyl sulphoxide according to an improved PCR sequencing protocol (Winship, 1989).

RESULTS

Isolation and characterisation of a TnT cDNA clone containing exon y

A large size range of fTnT proteins is expressed in the neonatal skeletal muscle. In addition to adult and neonatal isoforms, this includes two large perinatal isoforms. The molecular mass of fTnT protein, deduced from the known 18 exons, ranges from 27.8 kDa to 30.9 kDa. This range probably cannot account for the size of all the fTnT isoforms expressed in the neonate. Fig. 1 shows the relative

mobilities of fTnT isoforms from a variety of muscles. The estimated sizes are indicated on Fig. 1 where it is assumed that the isoform with the highest mobility corresponds to 27.8 kDa and the vertical black line corresponds to the range described above. The two perinatal isoforms have a low mobility. To investigate whether the fTnT gene could generate these isoforms, a cDNA library was constructed using poly(A) RNA isolated from 3- and 7-day neonatal rat skeletal muscle. The library was screened with the coding region fragment of the fTnT construct pTNT-15.

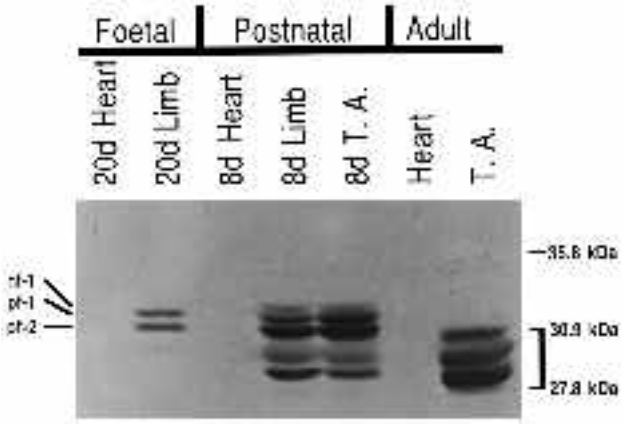

Fig. 1. The expression of fTnT. Western blot of the following rat muscle samples stained with the monoclonal antibody F24; 20-day foetal cardiac, 20-day foetal hind limb, 8-day neonatal cardiac, 8-day neonatal hind limb, 8-day neonatal *tibialis anterior* skeletal muscle, adult cardiac, adult *tibialis anterior*. Approximate size markers are shown. The total possible size range of fTnT isoforms that could be generated from the fTnT gene is indicated by the bracket. The perinatal isoforms pf-1 and pf-2, and the neonatal isoform nf-1, are indicated.

One cDNA clone pTnT-38 was isolated and sequenced over approximately 800 nucleotides through to the untranslated region and polyadenylation sequence at the 3′ end (Fig. 2E). Apart from a 39-nucleotide segment, the sequence matched previously reported cDNA sequences and the exons described for the genomic sequence of the rat fTnT gene. The 5′ end of the clone begins at the fourth codon of exon 5. Exon 17 is included but not exon 16. Exon 6 and exon 7 are both absent. The additional 39 nucleotides map to a sequence in the intron between exons 8 and 9 (Fig. 2A). A hexanucleotide sequence that fits the consensus donor site for splicing exists at the 3′ end of this 39-nucleotide region on the genomic sequence. The flanking 5′ region also has a consensus acceptor sequence (Mount, 1982). A sequence that could promote lariat formation on to an upstream adenosine residue has not yet been identified. Lariat branch sites have not been located for exons 3, 15 and 17 (Breitbart et al., 1985). However, if the sequence underlined in Fig. 2A (5′-TTCTTGC-3′) is altered, where guanidine is replaced with an adenosine residue, then it matches the consensus sequence (5′-YNYTRAY-3′; Hartmuth and Barta, 1988).

The fact that the 39-nucleotide sequence is flanked by introns in the genomic sequence demonstrates that it is inconceivable for the clone to be derived from an incompletely processed pre-mRNA. This sequence also maintains the reading frame of the processed message. We have designated this sequence as exon y. Furthermore, exon y must be alternatively spliced because other reported fTnT cDNA sequences, isolated from adult rat skeletal muscle, do not contain exon y. Fig. 2B schematically represents the updated complement of exons in the rat fTnT gene, where the amino acid sequences for the alternative exons, including exon y, are also shown. Expression of the clone, as a fusion protein with the α-peptide of β-galactosidase, in *E. coli* is still detected by the fTnT-specific monoclonal anti-

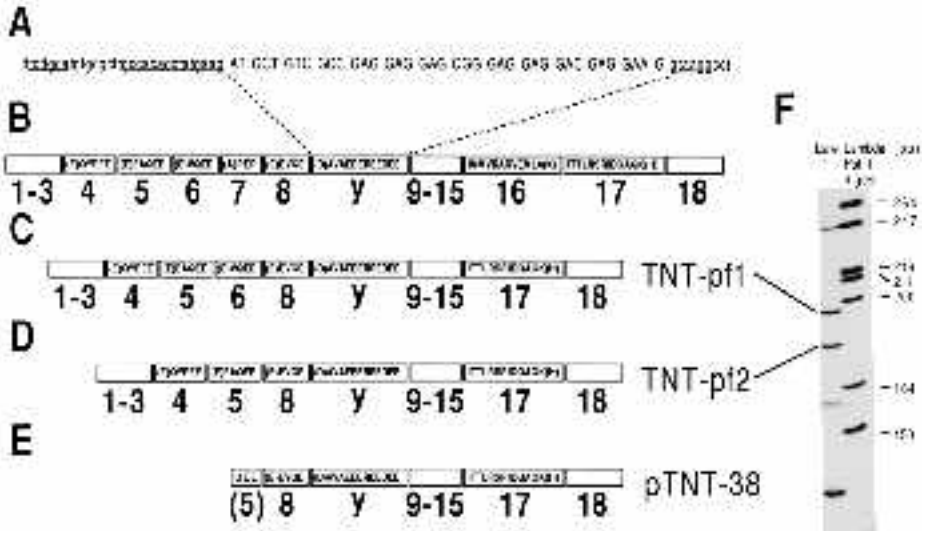

Fig. 2. Schematic representation of exon y and its association with the other fTnT exons. (A) The sequence of exon y and flanking intronic sequences. Exon y is represented in upper case with the bases grouped into appropriate codons. The intronic sequences are represented in lower case. The sequences that are underlined correspond to consensus sequences required for splicing. (B) All 19 exons of the rat fTnT gene. The predicted amino acid sequence is shown for all the alternative exons including exon y. Exon y is located between exons 8 and 9, and codes for 13 amino acids. Amino acids shown in parenthesis represent split codons where that exon contains two out of the three bases. (C) and (D) The predicted exon arrangement of the two mRNAs that contain exon y, designated TNT-pf1 and TNT-pf2. Exon 17 is included in the transcript but not exon 16. (E) The exon arrangement of our isolated clone pTNT-38. Note that the sequence is not full length and terminates in exon 5. (F) Lane 1 shows the separation on a polyacrylamide gel of PCR fragments generated from 3-day neonatal rat skeletal muscle cDNA using primers to exon 1 (sense) and exon y (anti-sense). Two of the fragments indicated correspond to TNT-pf1 and TNT-pf2. This was demonstrated by the size and subsequent sequencing of the fragments. The other fragments present were sequenced but did not correspond to the rat fTnT gene.

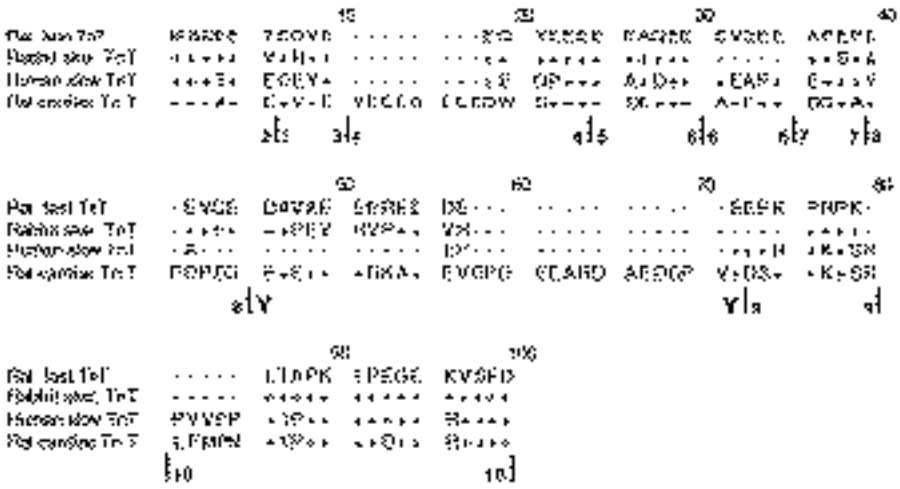

Fig. 3. Comparisons of TnT amino acid sequences of the N-terminal region. A dash (−) indicates the absence of an amino acid. A plus sign (+) indicates an identical residue. Vertical lines with bold face numbers indicate exon boundaries corresponding to the rat fast troponin T gene. Each protein sequence is a deduction made from its DNA sequence.

body F24 (data not shown). Exon y, therefore, does not disrupt the F24 antibody epitope.

Exon y is associated with two fTnT messages

Exon y is positionally connected with the cassette exons 4 to 8. The combination of exon y and exons 4 to 8 inclusive during splicing could be random or specific. PCR was used to investigate alternative splicing encompassing these exons. Two primers were generated matching exon 1 (sense) and exon y (anti-sense). cDNA from neonatal rat skeletal muscle was used as the template material for PCR. ^{32}P-end-labelling of the exon 1 primer permitted detection and isolation of the PCR fragments, once they were separated on an 8% denaturing polyacrylamide sequencing gel.

Fig. 2F indicates the presence of fragments that, due to their size, could be associated with specific exon combinations. To confirm the nature of these fragments, they were isolated, reamplified and sequenced.

Two fragments had sequences that could be mapped to the fTnT gene. Both fragments contained exons 1 to 3. Of the cassette exons both included exons 4, 5 and 8. The larger fragment also included exon 6. The remaining sequences could not be matched to the rat troponin T sequence (data not shown). The pattern of fragments on a gel did not alter when skeletal muscles of different ages and type were analysed (data not shown), suggesting that exon y is only associated with these two isoforms. The two isoforms are designated as TNT-pf1 (Fig. 2C) and TNT-pf2 (Fig. 2D), where TNT-pf2 is the smaller of the two fragments and corresponds to the clone TNT-38 described above. The additional inclusion of exon 7, that is, the full complement of exons including exon y, could explain the size of the nf1 protein isoform (Sabry and Dhoot, 1991a) but has not yet been detected at the mRNA level. With the inclusion of exon 17 in the fully processed 10 message, the predicted protein molecular masses are 32.0 kDa and 31.4 kDa. The largest predicted molecular mass of fTnT lacking exon y is 30.9 kDa. The inclusion of exon 16 instead of exon 17 makes little difference to the overall predicted molecular mass. Using PCR, exon y was only detected in mRNA containing exon 17 and not exon 16 (data not shown).

The corresponding protein sequence of exon y is acidic, containing seven glutamic acid and two aspartic acid residues out of a 13 amino residue peptide. Exon y can be compared with the foetal exon of rat cardiac TnT (Jin and Lin, 1989) (Fig. 4, below). Both exons are rich in glutamic acid residues and are of similar size. The cardiac developmentally regulated exon is 19 amino residues in from the N terminus, whereas the skeletal exon y starts at amino residue 37. The N-terminal region between exons 3 and 10 exhibits greater variation than the C-terminal region when comparisons are made across species. This variation is in addition to the effects of alternative splicing (Fig. 3). Yet a feature of the skeletal troponin T proteins is the similar length of the N-terminal divergent region upstream of exon 10. With rat skeletal troponin T this region can now extend to 56 amino residues as opposed to 43 amino residues (with the inclusion of exon 7); human slow troponin T (Gahlmann et al., 1987), 49 amino residues; and rabbit skeletal troponin T (Fujita et al., 1991), 47 amino residues. But with the rat cardiac troponin T this region extends for 85 amino residues.

Exon y is a developmentally regulated exon

To investigate the utilisation of exon y in certain RNA populations, in comparison with exon 17 and the total population of TnTf mRNAs, ^{32}P-labelled probes were hybridised to northern blots (Fig. 4A, B and C). Compatible western blots were stained with the monoclonal antibody F24 to assess the correlation with protein expression and RNA production (Fig. 4D). To detect fTnT mRNA, a 180 bp fragment at the 3′ end of pTNT-15 was used. This fragment corresponded to the untranslated region of exon 18. The initial production of the fTnT gene mRNA occurs at low levels in 20-day foetal hind-limb skeletal muscle, reaching maximum levels at day 3 in the neonate and is maintained at high levels in adult fast *vastus lateralis* and mixed *gastrocnemius* skeletal muscles but not in the slow *soleus* skeletal muscle.

Oligonucleotides, 50 bases in length, were used to detect both exon y and exon 17. The mobility of the RNA detected by both the exon y and exon 17 probes was the same as that of the fTnT probe. The presence of mRNA containing exon y was limited to neonatal skeletal muscle and, to a lesser extent, to 20-day foetal skeletal muscle. There was

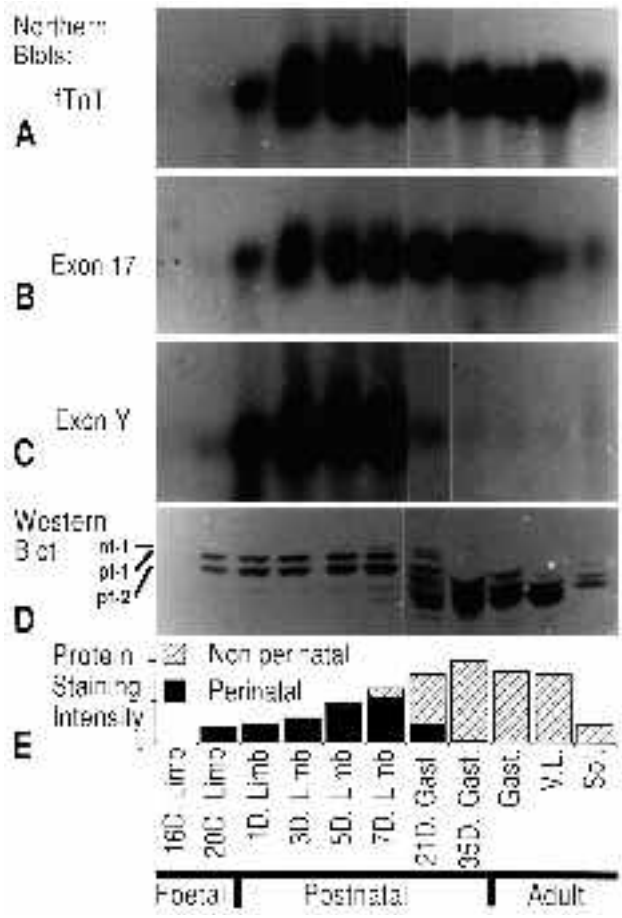

Fig. 4. (A-C) The sequential hybridization of three ^{32}P-labeled probes to the same northern blot of 10 μg total cellular RNA samples. The probes inluded a 180 bp cDNA fragment to the 3′ untranslated region of rat fTnT mRNA (A); a 50mer oligonucleotide to exon 17 (B); and a 50mer oligonucleotide to exon y (C). (D) A comparison of the same muscle samples with a western blot stained with the fTnT monoclonal antibody F24. The perinatal isoforms pf-1 and pf-2, and the neonatal isoform nf-1 are indicated. (E) A corresponding stack graph representation of a laser densitometric scan of the western blot. The values represented correspond to the integrations of the signals reflected off the blots and are standardised such that the maximal value represented equals unity. The filled bars represent the signal generated by to the two perinatal bands, pf-1 and pf-2, whereas the hatched bars represent the signal from the remainder of the bands. The combination of both bars therefore represents the total signal detected. The samples were isolated from the rat and included 16-day foetal hind-limb muscles; 20-day foetal hind-limb muscles; 1-day postnatal hind-limb muscles; 3-day postnatal hind-limb muscles; 5-day neonatal hind-limb muscles; 7-day postnatal hind-limb muscles; 21-day postnatal *gastrocnemius*; 35-day postnatal *gastrocnemius*; adult skeletal muscles: *gastrocnemius*, *vastus lateralis* and *soleus*.

very little detection of exon y in the adult skeletal muscle samples. Exon 17 was detected wherever the fTnT mRNA was present. The preferential inclusion of exon 17 in the *gastrocnemius* and *soleus* adult muscles fTnT mRNA compared to the *vastus lateralis* followed a previously described pattern of tissue-specific regulation (Medford et al., 1984).

The total fTnT protein concentration for each sample was estimated from the western blots (Fig. 4D) by integrating the laser densitometric signals for all the bands located by the antibody F24 (Fig. 4E). The expression of fTnT followed a similar pattern to that of the mRNA levels. Significant detection occurred in the day 20 fetal sample and maximal expression occuring at 35 days in the *gastrocnemius*. The expression of the two perinatal proteins shows a similar pattern to the inclusion of exon y in fTnT mRNA. Both the detection of these perinatal bands and of exon y occur initially at day 20 in the foetus, reaching maximum levels at 7 days in the neonatal limb muscle and are not detected in the adult muscle samples. Furthermore, all the fTnT protein detected up to postnatal day 3 is constituted solely of the two perinatal proteins.

DISCUSSION

The present study demonstrates the presence of a novel exon in the rat fTnT gene. The fact that this exon is included in the first transcripts after the switch-on of the rat fTnT gene implies that it has an important role in the development of muscle. Similarly, in the cardiac TnT genes of both rat and chicken (Cooper and Ordahl, 1986) there is the inclusion of an exon encoding an acidic peptide. This is the first time that the use of an exon encoding an acidic peptide under developmental regulation has been described for a skeletal muscle TnT gene. Protein sequence comparisons of the N-terminal region demonstrate considerable variation between species in terms of amino acid sequence and its acidic nature compared to the corresponding comparisons of the C-terminal region. However, the length of the divergent region is similar when comparing isoform types across species, although it is greater in cardiac isoforms than in the skeletal isoforms. Therefore, the inclusion of a developmentally regulated exon suggests that the increased acidity and length of the divergent region may be more functionally important than the specific amino acid sequence of the additional developmentally regulated exon. Postulated structures of the troponin T protein contain a filamentous N-terminal domain interacting with two overlapping tropomyosins and a globular carboxyl domain closely associated with troponin C and troponin I (Heeley et al., 1987). Yet it is still unclear what effect the developmentally regulated addition of an acidic peptide may have. This is also the case with the acidic peptide encoded by the alternatively spliced cardiac TnT exon (Jin and Lin, 1989). The changes may affect the strength of attachment and the position of the troponin complex in relation to the tropomyosin overlap or the sensitivity of the troponin complex to the level of intracellular calcium.

Although the proportion of fTnT mRNA containing exon y has not been determined there is a good correlation between the detection of exon y and the two fTnT perinatal proteins. If the correlation is true the data suggest that a significant proportion of the initial perinatal fTnT mRNA includes exon y. Furthermore, the mRNA transcripts TNT-pf1 and TNT-pf2 code for proteins larger than proteins that could be derived from the previously reported fTnT gene. This calculation fits well with our observation that the fTnTproteins pf1 and pf2 probably have molecular masses

that are too high to be accounted for by the previously reported fTnT gene, further suggesting that exon y is involved in the generation of the these perinatal protein isoforms.

It has been suggested that in the chicken embryonic cardiac muscle the TnT isoform is involved in myofibrillar construction (Cooper and Ordahl, 1986). The rat fTnT gene is switched on in the late foetus when hyperplasic muscle development is coming to an end (Goldspink, 1972). The utilisation of the TNT-pf1 and TNT-pf2 isoforms in the myofibril may involve the replacement of existing embryonic/foetal skeletal TnT proteins, such as the slow muscle types Es1 to Es5 (Sabry and Dhoot, 1991b) that have not been characterised at the mRNA level. Alternatively, the utilisation of the perinatal isoforms could occur in the new myofibrils created as a consequence of hypertrophy.

This is the first time that a developmentally regulated exon that is located in a cassette of alternatively spliced exons has been described. Yet exon y is included in only two mRNA isoforms. The implication is that there is a coordinated regulation of the alternative splicing of these exons. But the regulatory mechanism remains unclear. The inclusion of exon y may only allow a specific pre-mRNA secondary structure to affect the selection of the other exons, or the inclusion of each of the alternative exons may be regulated individually. It is difficult to detect specific *cis*-elements on the pre-mRNA that would distinguish exon y from other exons. This problem is also encountered with the mutually exclusive exons 16 and 17 versus the constitutive exons (Medford et al., 1984). Therefore, the putative regulatory *trans*-acting factors may not bind to specific RNA sequences or to RNA at all.

The fTnT gene can now theoretically generate 128 different isoforms, although the permutation of the alternative exons in vivo is almost certainly limited. The identification of exon y and its probable association with the two perinatal protein isoforms of fTnT provides a biochemical rationale for the specificity and coordinate regulation of alternative splicing. These results should allow the identification of developmentally regulated RNA *trans*-acting factors in skeletal muscle and perhaps a clearer understanding of coordinate alternative splicing regulation.

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