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Identification of a fetal exon in the human fast Troponin T gene

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Abstract

A developmentally regulated exon has been identified in the 5'-alternatively spliced region of the human fast Troponin T (TnT) gene. Expressed in fetal (but not adult) muscle, this exon is homologous with the fetal exons recently described in the rabbit and rat fast TnT genes. They all exhibit a split codon organization and encode a highly acidic peptide. To determine if the splicing pathways, including the human fetal exon, are also conserved, we defined the major TnT splicing patterns in fetal muscle. They generate fetal TnT 1, fetal TnT 3, and TnT1f, and TnT3f, species previously described in rabbit and rat skeletal muscles.

Key words: Troponin T; Alternative splicing; Gene expression; Human muscle; Fetal exon

1. Introduction

The heterogeneity of fast Troponin T's (TnT) in vertebrate skeletal muscle is due largely to variation in their N-terminal amino acid sequences [1–3]. In adult fast skeletal muscle, the different N-terminal sequences arise from alternative splicing involving five 5'-exons which create a hypervariable region in the molecule [4]. A sixth exon that contributes to the N-terminal heterogeneity of TnT's in fetal and early neonatal muscle was described recently in rabbit and rat skeletal muscles [5–7]. This exon, designated the fetal exon F, shares several properties with the other 5'-miniexons. They all exhibit the same split codon organization and similar purine-rich sequences. These sequences encode acidic peptides that account for the more acidic pIs of fetal TnT's [8]. The developmentally regulated alternative splicing of the fetal exons, however, distinguishes them from the other 5'-miniexons, which are expressed in both developing and adult muscles. In contrast, the fetal exon is included in the majority of late fetal TnT mRNAs, but its ability to be recognized as an exon is lost during neonatal development. The aims of this study were to determine whether a fetal exon is also present in human fetal muscle TnT mRNAs and to compare the alternative splicing of TnT mRNAs in human fetal muscle with those observed in rabbit and rat.

2. Materials and methods

2.1. Amplification and sequencing of the 5'-alternatively spliced regions of human fetal TnT

Total RNA was prepared [9] from 12–13 week (X87-2) and 18 week (X87-1) fetal muscle samples kindly provided by John Gilbert of the Joseph and Kathleen Bryan Alzheimers Research Center at Duke University Medical Center. Adult muscle RNA was provided by William Kraus at Duke University Medical Center.

Reverse transcription-PCR with the thermostable enzyme r*Tth* (Perkin Elmer-Cetus) was performed as described [6]. Primers that flanked the alternatively spliced 5'-region were selected from a full-length human fast TnT coding sequence [10], using the MacVector primer pair subroutine. Their sequences are CCCACCTTCAC-CATGTCT and CGAAGTCCACTTTCTCCC. After 5 min reverse transcription at 70°C, the buffer conditions were adjusted, and the PCR reactions denatured at 94°C for 2 min, and amplified using 25–35 cycles of 1 min at 94°C, 30 s at 53°, and 1 min at 72°C. Products were cloned in the pCR II TA vector (Invitrogen), and 16 clones were sequenced using the Circumvent DNA sequencing kit (New England Biolabs).

2.2. Analysis of TnT cDNAs by gel-electrophoresis

r*Tth* reactions were performed with a ^{32}P-endlabeled upstream primer, and the products were resolved with a 4% polyacrylamide/7 M urea gel. Their sizes were determined by comparison with sequencing reactions run in adjacent lanes. The radiolabeled products were visualized with a Fujix phosphoimager, and quantified with the McBas program.

Radiolabeled samples were prepared for restriction enzyme analysis by phenol/chloroform extraction and precipitation. Following digestion with *Tha*1, which recognizes a site present only in the fetal exon, the samples were analyzed on a 4% gel as described above or by autoradiography.

3. Results

3.1. rTth amplification of the 5'-alternatively spliced region of human TnT mRNAs yields four major products

Based on the sequence of a human fetal TnT cDNA [10], primers spanning the 5'-alternatively spliced exons of fast TnT were used to generate cDNAs by reverse transcription-PCR with the thermostable enzyme r*Tth*. Amplification of total RNA isolated from two fetal skeletal muscle samples, X87-1 and X87-2, generated cDNA products of 214, 190, 181, and 157 nucleotides (nt) (Fig. 1). Previous studies indicated that this approach provides a representative sample of the TnT RNA population [6], and is preferable to screening cDNA libraries for analyzing the 5' alternative splicing patterns because

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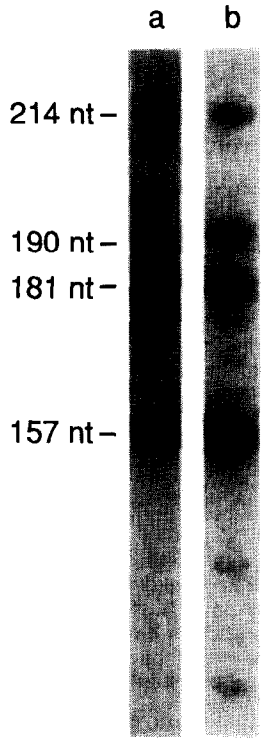

Fig. 1. Analysis of the RT-PCR products from human fetal muscle RNA. RNA prepared from two human fetal muscle samples, (a) X-87-1 and (b) X-87-2, was amplified with r*Tth* and TnT primers spanning the 5′-alternatively spliced region. A ^{32}P-endlabeled upstream primer was included in the PCR, and the resulting radiolabeled products were resolved by denaturing polyacrylamide gel electrophoresis and phosphoimaging.

most TnT clones isolated from cDNA libraries are not full-length ([4,11]; Briggs and Schachat, unpublished observations). The mixture of cDNAs was cloned and their sequences were determined from at least two independent r*Tth* reactions.

3.2. *Identification of a 24 nt fetal exon in the human fast TnT gene*

Nucleotide sequences corresponding to the four major cDNA products were obtained (Fig. 2). Overall these sequences are quite similar to those of rat and rabbit cDNAs, and the translated amino acid sequences are nearly identical over much of the region (Fig. 3). Combining this sequence information with the exon organization of the rat fast TnT gene [12] allows several splicing patterns of the human cDNAs to be defined. The smallest major product at 157 nt contains exons 4, 5, and 8 and thus corresponds to TnT3f, as previously defined in rabbit and rat TnT's. The additional sequence in the 190 product lies between exons 5 and 8. This sequence is homologous to that of exons 6 and 7; thus the 190 nt product most likely corresponds to TnT1f. The 214 and 181 nt products exhibit the same exon combinations as TnT1f and TnT3f, but both contain an additional 24 nt sequence. This insert is located between exons 8 and 9, in the same position as the fetal sequences from rabbit [5,6] and rat [5–7]. Thus, by analogy with the rabbit and rat fetal TnT's, the 24 nt insert results from incorporation of the fetal exon, and the 214 nt product encodes the

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HUMAN                (M) S  D  E  E  V  E  Q  V  E  E  Q  Y  E  E  E  E  E  A  Q  E  E

 all          CCTTCACCATGTCTGACGAGGAAGT|TGAACAGGTGGAGG|AGCAGTACGAAGAAGAAG|AGGAAGCCCAGGAGGAAG|

RAT TnT1f     CAGCCACTATGTCTGACGAGGAAAC|TGAACAAGTTGAGG|AACAGTACGAAGAGGAAG|AGGAGGCCCAGGAGGAAG|
             exon                    2 | 3          3 | 4              4 | 5              5 |


HUMAN              A  A  E  V  H  E  E  V  H  E  P  E  E  V  Q  E  D  T  A  E  E  D  A  E

fetal TnT1  (214)  CTGCAGAAGTCCATGAGGAAG|TTCATGAACCAG|AGGAAGTTCAAGAAG|ACACCGCAGAGGAGGACGCGGAAG|
     TnT1f  (190)  ++++++++++++++++++++|++++++++++++|++++++++++++++|------------------------|
fetal TnT3  (181)  --------------------|------------|++++++++++++++|++++++++++++++++++++++++|
     TnT3f  (157)  --------------------|------------|++++++++++++++|------------------------|
    TnT3f*  (142)  --------------------|------------|---------------|------------------------|

RAT TnT1f          ------AAGTCCAGGAGGAAG|CCCCGGAACCAG|AGGAAGTCCAAGAAG|------------------------|
                     6               6 | 7        7 | 8           8 |  F(FETAL)    (FETAL) F  |


HUMAN         E  E  K  P  R  P  K  L  T  A  P  K  I  P  E  G  E  K  V  D  F

 all          AGGAGAAACCGAGACCCAA|ACTCACTGCTCCTAAGATCCCAGAAGGGGAGAAAGTGGACTTCG

RAT TnT1f     AGGAGAAACCAAGACCCAA|ACTTACTGCTCCTAAGATCCCGGAAGGAGAGAAAGTAGACTTCG
               9              9 | 10
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Fig. 2. Nucleic acid sequences of five human TnT cDNAs. The first four sequences correspond to the major cDNA products observed in Fig. 1. The locations of the primers used in Fig. 1 are underlined. Identical sequences are indicated by (+), and the nucleotides absent from some sequences are indicated by (–). The rat TnT sequence and boundaries of exons 4, 5, 6, 7, 8, and 9 are taken from Breitbart [4,12], and that of fetal exon from Briggs and Schachat [6]. The presence or absence of homologous blocks of sequence was used to define the exon boundaries in the human sequences. Due to greater sequence divergence and the absence of different splicing patterns involving exons 6 and 7, their boundary was estimated by homology with the rat exons.

Exon	2	3	4	5	6	7	8	fetal	9
Hum	MSDEEV	EQVE	EQYEEE	EEAQEE	---AAEVHEE	---VHEP	EEVQE	DTA-----EEDAE	EEKPRPK
Rat	MSDEET	EQVE	EQYEEE	EEAQEE	-----EVQEE	APE---P	EEVQE	DAVAEEEREEDEE	EEKPRPK
Rab	MSDEEV	EHVE	EQYEEE	EEAQEE	APSPAEVHEP	APEVHVP	EEVHE	DAL-EDMREEEEE	EEKPRPK

Fig. 3. Alignment of the predicted amino acid sequence of human fetal TnT1 with rat and rabbit TnT's. The translated N-terminal sequences of rat and rabbit TnT's [4,11,25] are augmented with the fetal exons of rat [5–7] and rabbit [5,6] TnT.

N-terminal region of fetal TnT 1. Similarly, the 181 nt product arises from mRNAs specifying fetal TnT 3. Although the human fetal exon is considerably shorter than its rat and rabbit counterparts (24 vs. 39 and 36 nts, respectively), like them it encodes an acidic peptide (DTAEEDAE), exhibits a 1:2 split exon organization, and is the most distal of the 5′-miniexons in the fast troponin T gene.

The four major cDNAs all represent exon combinations that are expressed in rabbit and rat muscles. A fifth cDNA, which has not been reported previously in fetal muscle, was also observed. It is labeled TnT3f* because it, like TnT3f, does not contain exons 6 and 7, but it also lacks exon 8. Based on densitometric analysis, this cDNA represents less than 1% of the total PCR products.

3.3. *Evidence for the developmental specificity of the fetal exon*

To determine whether expression of the human fetal exon is specific for developing muscle, the 5′-alternatively spliced regions of TnT mRNAs were amplified from both fetal and adult skeletal muscle RNA (Fig. 4). Densitometric analysis showed that TnT's containing the fetal exon (fetal TnT1 and fetal TnT3) are 24 and 53% of the total in the fetal muscle samples X-87-1 and X87-2, respectively (Figs. 1 and 4), but they comprise less than 1% of the TnT in adult muscle RNA. To determine whether the fetal exon is present in the other TnT's in adult muscle, the amplified products were digested with a restriction enzyme, *Tha*I, that recognizes a unique site in these cDNA products, in the fetal exon (Fig. 4,a′,b′). Fetal TnT1 and fetal TnT3 are cleaved in the fetal sample, but none of the other bands in the adult sample is susceptible to *Tha*I cleavage. Thus, like its rabbit and rat counterparts, the human fetal exon is not expressed at significant levels in adult skeletal muscles.

4. Discussion

The identification of a new, developmentally regulated exon in the rabbit and rat fast TnT genes [5–7] led us to investigate whether a similar exon is incorporated in human fetal skeletal muscle TnT mRNAs. Wu et al. [10] isolated the first full-length human fetal cDNA clone. While it had many of the properties expected of fetal TnT's, notably the expression of the 3′-exchangeon exon 17, it appeared to lack a fetal exon. Given the difficulty in aligning a single N-terminal TnT cDNA due to repeating sequence motifs, sequence divergence, and the multiplicity of potential alternative splicing pathways, we took a direct approach to determining whether a fetal exon is expressed. In previous studies [5,6] we found that reverse transcription-PCR with r*Tth* yields a representative sample of the 5′-alternatively spliced sequences in rabbit muscle RNA. Sequence comparison showed that the resulting cDNAs were derived from several different alternative splicing patterns, which allowed the miniexon boundaries of the rabbit fast TnT gene to be defined.

Using this approach with human fetal muscle, we found four major r*Tth* products, which can be divided into two pairs; the 214 and 190 cDNAs differ only by a

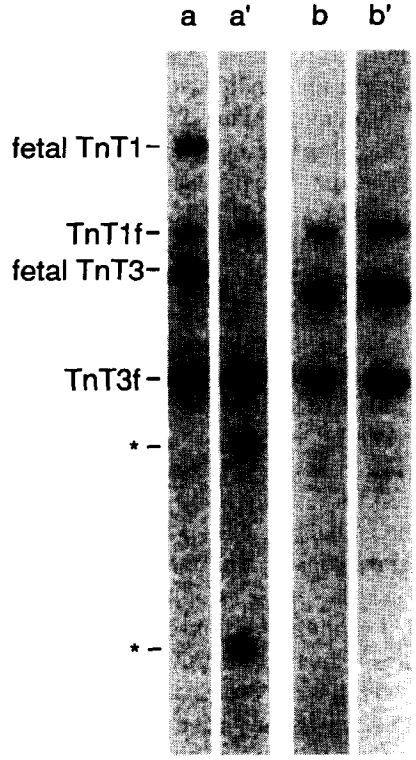

Fig. 4. Comparison of fetal exon expression in fetal and adult muscle. The 5′-alternatively spliced TnT sequences present in fetal (X87-1) and adult muscle RNA (lanes a, b) were amplified by the r*Tth* reaction using the same primers as in Fig. 1. These cDNAs were digested by *Tha*I (lanes a′, b′) and the cleavage products marked with an asterisk (*). The lack of change in the adult sample (b,b′) indicates that none of the additional bands in that sample contain the fetal exon.

24 nt insert, as do the 181 and 157 nt cDNAs. Since the 214 and 181 nt cDNAs exhibit the same splicing patterns as fetal TnT1 and fetal TnT3 in rabbit and rat skeletal muscle [1,6], the 24 nt insert is identified as the human fetal exon by its homology with the rabbit and rat fetal exons. First, it is expressed in fetal but not adult muscle. Second, although the human fetal exon is smaller than its homolog in rat and rabbit, it has a similar acidic character, split exon organization, and genomic location between exons 8 and 9.

Because many 5′-alternatively spliced variants of mammalian fast TnT mRNAs have been postulated [4], but far fewer protein isoforms [1] or full-length cDNAs have been found [4,11], we analyzed the splicing patterns of the other human fetal TnT's to determine whether new splicing patterns are utilized. Like the fetal TnT's, the two other products observed, TnT1f and TnT3f, also correspond to isoforms identified in rat or rabbit fetal muscle. The remaining sequence (TnT3f*) is a new splicing pathway; although it is not easily detected among the fetal muscle PCR products, a band of the appropriate size (142 nt) makes up approximately 2% of the TnT cDNAs in the adult muscle sample.

The acidic character of the peptide specified by the human fetal exon may be essential to both the function of the fetal TnT proteins and indirectly to recognition of the exon during the splicing process in fetal muscle. In rat and rabbit, expression of the fetal exon results in the insertion of nine and eight acidic residues, respectively, in the TnT protein. Similarly, expression of the human fetal exon results in the insertion of eight residues, five of which are acidic. Because variation in N-terminal sequences appears to alter the calcium-sensitivity and responsiveness of thin filaments [13], the insertion of a string of acidic residues may be related to the reduced calcium sensitivity of fetal muscle fibers [14,15]. The expression of TnT3f at this stage of development is also consistent with the physiology of fetal muscle, as it is the least Ca^{+2}-sensitive of the fast TnT's, as measured in adult muscles [13].

The indirect role of these acidic residues in enabling the fetal exon to be recognized during RNA processing relates to the sequences of their codons. Short exons, like the 24 nt fetal exon, generally are not efficiently spliced [16–18]. In the case of the rat [6] and the rabbit (Maready and Schachat, unpublished observations), the splicing of the fetal exon is further negatively affected by the presence of a suboptimal 3′ splice acceptor site in intron 8. In several genes, these negative factors are compensated for by the presence of a polypurine sequence in an exon or its 3′ intron that allows it to be recognized and incorporated into the spliced RNA [19–24]. In the case of the human fetal exon, the codons for the glutamic and aspartic acid residues (GAA, GAG, GAC) provide a polypurine tract that may be critical to the definition of the fetal exon and, in the presence of developmentally regulated *trans*-acting factors, enable it to be recognized as an exon in fetal muscle.

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