Developmental and Muscle-specific Regulation of Avian Fast Skeletal Troponin T Isoform Expression by mRNA Splicing*  

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We have investigated the developmental regulation of the avian fast skeletal muscle troponin T (TnTf) gene of the Japanese quail. Sequence analysis of troponin T mRNA, cDNA clones, and a genomic DNA segment demonstrate that the avian, fast skeletal TnT, protein isoforms are produced from a single gene. This TnTf gene is expressed in skeletal muscle, but not in adult cardiac muscles or in non-muscle tissues. In addition to known TnT isoforms, three new isoforms of TnT are described. These isoforms arise by regulated alternative RNA splicing of exons in the 5' and 3' regions of TnTf transcripts. Alternative splicing of the 5' TnTf exons involves splicing of multiple exons in different combinations (i.e. not mutually exclusive), whereas 3' alternative splicing involves mutually exclusive splice choices between two exons (α or β exons). S1 nuclease protection and primer extension analyses show that alternative splicing of both 5' and 3' exons is precisely regulated and coordinated in physiologically different striated muscles, which express distinct, restricted combinations of 5' and 3' alternatively spliced exons in mRNA transcripts. In contrast, different embryonic muscles and clonal embryonic myoblast cultures coexpress the 3' alternative splice choices. This indicates that alternative splicing of TnTf mRNAs is controlled in different adult muscles by specific trans factors, and not by the restricted expression of different spliced forms in different embryonic myoblast lineages. Comparison of TnTf isoform expression in quail and chicken flight muscle (Wilkinson, J. M., Moir, A. J., and Waterfield, M. D. (1984) Eur. J. Biochem. 143, 47-56) to TnTf isoforms of the rat (Breitbart, R. E., and Nadal-Ginard, B. (1986) J. Mol. Biol. 188, 313-324), and rabbit (Pearlstone, J. R., Carpenter, M. R., and Smillie, M. B. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1902-1906) indicates that the avian gene contains an additional exon(s) not present in mammalian genes. The alternative exon sequences TnTf, mRNAs expressed in anatomically distinct quail muscles can be correlated with sequences in TnTf protein isoforms in these chicken muscles. Thus, the regulated splicing of alternative exons in TnTf transcripts, and not selective translation of stochastically spliced TnTf mRNAs, regulates TnTf isoform expression in specific muscles.  

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05006.

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Troponin T is a 30-35-kDa protein that is the largest of three subunits of the troponin complex. Troponin T (TnTf), troponin C (TnC), and troponin I (TnI) mediate the calcium-regulated contraction of striated muscle. Protein binding and crystallographic studies (1; for reviews, see Refs. 2 and 3) show that the N-terminal region of troponin T binds stably to tropomyosin (thereby attaching the troponin complex to the thin filament), and the C-terminal region binds in a Ca2+-sensitive interaction to TnI, TnC, and tropomyosin. Polymorphic forms exist for each of the troponin genes and are the result of gene duplication events and differential expression of multigene family members (4-8). TnI and TnTf isoforms are encoded by distinct fast skeletal, slow skeletal, and cardiac genes. TnC isoforms are encoded by a slow/cardiac gene and a fast skeletal muscle gene. The expression of specific troponin isoforms in physiologically and biochemically different adult striated muscles suggests that specific combinations of troponin isoforms directly influence Ca2+-sensitive muscle contraction. This has led investigators to propose coordinated control of troponin protein expression (7-9). However, several lines of evidence suggest that the genetic control of TnTf isoform expression is more complex than can be accounted for by expression of multiple gene family members. Protein sequencing and two-dimensional gel analysis of chicken fast skeletal muscles show that multiple TnTf isoforms are differentially expressed in embryonic and specialized adult muscle (10-15) and that these different isoforms result from cassette-like insertions or deletions of amino acid sequences internal to the N terminus of the protein (aa9-38) (10). These protein sequence data led Wilkinson to hypothesize that TnTf isoforms arise from a single gene by differential splicing events. Subsequent studies involving genomic and cDNA analyses of rat TnTf isoforms have shown that multiple isoforms are expressed by differential mRNA splicing of a single gene in the N- and C-terminal regions (15-17). This is consistent with the chicken protein amino acid sequence predictions, but specific avian amino acid sequences are different, and some are absent from the rat protein. Also, variant amino acid sequences at aa228-242 near the C terminus have been shown to arise by alternative splicing of α and β exons in both the rat and quail genes (15, 18). cDNA analyses of human slow skeletal TnTf (TnTf) (19) isoforms and genomic and cDNA analyses of chicken cardiac TnTf (TnTf) (20) isoforms have revealed unique, gene-specific differential splicing of these gene transcripts to generate additional isoforms. Therefore, TnTf polymorphic forms are in part the result of gene duplication and tissue-specific expression of the TnTf isoform gene family members, and in

1 The abbreviations used are: TnT, troponin T; TnC, troponin C; TnI, troponin I; TnTf, fast skeletal muscle TnT; aa, amino acid; bp, base pair(s).
part the result of differential mRNA splicing for each isoform gene.

The evident complexity of TnT isoform regulation makes the study of TnT mRNA expression in birds of particular interest. In contrast to mammals, birds have several specialized muscles, such as the pectoralis major, that are relatively homogeneous in fiber type, facilitating biochemical studies to correlate isoform mRNA and protein expression with fiber type. The complexity of the TnT gene family and splicing of the TnT transcripts makes a comparative analysis in birds and mammals important to understanding the regulation and function of TnT gene splicing.

We have investigated TnT isoform expression in the Japanese quail. TnT isoform expression has been examined by cDNA and genomic cloning, as well as by S1 nuclease protection and primer extension analyses of mRNAs from embryonic muscle, adult muscles, and cloning myoblast cultures. These studies provide evidence that the avian TnT gene is expressed abundantly in embryonic and adult skeletal muscles. Different TnT isoforms are produced by alternative, but not mutually exclusive, splicing of multiple exons near the 5' end, and by alternative, but mutually exclusive, splicing of two exons (α and β) near the 3' end of the TnT primary mRNA transcript. The expression of mRNAs with specific 5' and 3' splice choices is developmentally regulated and is correlated with expression of specific protein isoforms (10, 11). Embryonic muscles and clonal embryonic myoblast cultures coexpress the two alternative exons, but adult skeletal muscles express restricted combinations of splice choices, indicating that TnT isoform expression is regulated at the level of mRNA splicing mediated by developmental influences. The TnT splicing process is generally similar in birds and mammals, although birds have additional exons that encode flight muscle pectoralis isoforms. The amino acid sequences encoded by regulated, alternatively spliced exons in both bird and mammal genes are divergent, suggesting that these domains are important for specialized muscle function.

**MATERIALS AND METHODS**

**cDNA Cloning and Screening**—We generated cDNA libraries from 5 μg of poly(A)^+^ mRNA isolated from cultured pectoralis myofibers (21) and adult leg muscle. RNA isolation and cDNA cloning procedures have been described (22-24). Each amplified library, containing approximately 1 × 10^6^ unique recombinants, was screened with triplicate BA85 (Schleicher and Schuell) nitrocellulose filter and probed independently with nick-translated (25) TnT cDNA clone (cC122) (19), and T4-polyadenylate kinase-labeled oligonucleotides specific to α or β exons in TnT mRNA. The oligonucleotides were synthesized in the University of Virginia sequencing facility and T4-polyadenylate kinase labeled according to manufacturer's specification (Pharmacia LKB Biotechnology Inc.). Filters were prehybridized, hybridized (26) with nick-translated cC122, and stringently washed in 0.1× SSC, and 0.1% sodium dodecyl sulfate at 65 °C. Probe-labeled oligonucleotide probes were used as described by Wood et al. (27) using tetramethylammonium chloride to stabilize A-T base pairs.

cDNA Subcloning and Sequencing—Purified TnT positive λgt10 clones were subcloned into the EcoRI site of the pEMBL 18® vector (28). The DNA sequences were determined by the dideoxy chain termination method (29) using α-^32^P-dATP (500 Ci/mmol, Du Pont-New England Nuclear) using Sequenase (United States Biochemical Corp., Cleveland, OH). Reaction products were electrophoresed on buffer gradient gels (30). The gels were transferred onto Whatman No. 3MM, dried under heat and vacuum, and exposed to Kodak XAR film. Sequencing of the TnT primary cDNA was verified with oligonucleotide probes synthesized for priming single-stranded clones for dideoxy nucleotide sequencing reactions.

**Full-length TnT cDNA Clones**—We constructed three cDNA libraries in λgt10 of mRNAs isolated from myofiber cultures of day 10 embryonic pectoralis and adult muscle. We screened these libraries with a partial quail TnT cDNA, cC122, and with oligonucleotides complementary to alternative exons encoding amino acids 229-242, that were previously identified in the quail cDNA11 (α) and cC113 (β) cDNAs (18). This region is called α and β, according to the rat gene designations (15). A primary screen of 20,000 myofiber cDNA identified seven positive phage, two of which were α and five of which were β. One clone, cC501, is nearly full-length based on DNA sequence and Northern blot analyses (35). Another nearly full-length cDNA, cC605, was identified in TnT clones isolated from the adult leg cDNA library.

Fig. 1 compares the sequences of the myofiber cDNA clone, cC501 and the adult leg cDNA clone, cC605. These cDNAs encode the complete amino acid sequence for the fast skeletal isofrom of tropinin T (10, 36). The myofiber cDNA clone, cC501 is 1097 bp and spans from 6 nucleotides upstream of the ATG codon to the polyadenylation site. The adult leg cDNA clone, cC605 is 1107 bp and spans from 1 nucleotide upstream of the ATG codon to the polyadenylation site. The entire nucleotide sequence of cC501 was determined. cC605 was sequenced with the exception of a short region which encodes the common amino acid sequence from 78 to 125. The sequence of this region in five non-full-length adult leg cDNA clones was identical to the sequence of cC501. cC501 and cC605 both encode the α form of TnT and have identical nucleotide sequences with the exception of a region in cC605 encoding an additional four-amino acid peptide near the N terminus (exon 7). The cDNAs are identical to the previously published cDNA cC113, cC119, and cC122 except for the alternative β exon in cC13 (18). The nucleotide sequence identity of cC501 and cC605, including their 3' untranslated sequences and the cassette-like sequence difference near their 5' ends, indicates that these cDNAs are produced from a single gene by a differential mRNA splicing mechanism. These cDNAs encode two novel DNA sequence of this clone was determined as described above for the cDNAs and in certain regions by the method of Maxam and Gilbert (33). The genomic sequence encodes exon sequences identical to sequences found in the cDNAs (Fig. 1; 18). For specific regions, sequence-specific oligonucleotides were synthesized for priming single-stranded clones for dideoxy nucleotide sequencing reactions.

**METHODS**

**Nuclease Protection Assays**—To determine the regulation and function of TnT gene splicing, we performed S1 nuclease protection assays as described by Konieczny and Emerson (22). The cC113 TnT probe (18) is a 479-nucleotide fragment spanning from the HinfI site in the 3'-untranslated region of the mRNA probe (Fig. 1). This fragment was T4-polynucleotide kinase end-labeled with [γ-^32^P]ATP according to the manufacturer's specification. The optimum hybridization temperature was empirically determined to be 49 °C. S1 nuclease-resistant fragments were resolved on 6% denaturing polyacrylamide gels and exposed to Kodak XAR film. Quantitation of the relative amounts of α or β RNAs assayed by this probe was determined by cutting out protected fragments, and determining the ^32^P counts/min with an LS-230 scintillation counter (Beckman Instruments, Inc.). All samples were normalized to control for ^32^P background.

**Primer Extension and Dideoxynucleotide Sequencing Analysis**—We performed primer extension and dideoxynucleotide sequencing of 3', 5', and 3' ends of TnT mRNAs with oCO89 (34). The oligonucleotides were hybridized to mRNA samples at 30 °C and the β oligonucleotide was hybridized at 34 °C. Primer extension and mRNA sequencing of TnT mRNAs with oC089 and oC090 oligonucleotides (Fig. 1). were as described by Geliebter (34). To determine the full-length products, dideoxynucleotides were applied from one reaction aliquot and the specific dideoxynucleotides were added to the others for sequencing. The fragments were resolved on 8% denaturing acrylamide gels, dried under heat and vacuum onto Whatman No. 3MM paper and exposed for 8-30 h to Kodak XAR film.

**RESULTS**

**Isolation of Full-length Quail TnT cDNA Clones**—We constructed three cDNA libraries in λgt10 of mRNAs isolated from myofiber cultures of day 10 embryonic pectoralis and adult muscle. We screened these libraries with a partial quail TnT cDNA, cC122, and with oligonucleotides complementary to alternative exons encoding amino acids 229-224, that were previously identified in the quail cDNA11 (α) and cC113 (β) cDNAs (18). This region is called α and β, according to the rat gene designations (15). A primary screen of 20,000 myofiber cDNA identified seven positive phage, two of which were α and five of which were β. One clone, cC501, is nearly full-length based on DNA sequence and Northern blot analyses (35). Another nearly full-length cDNA, cC605, was identified in TnT clones isolated from the adult leg cDNA library.

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avian TnT isoforms that include additional protein sequences (see Fig. 8) not predicted by the avian TnT protein sequence data (10).

Comparison of 5'-Untranslated Sequences of Troponin T isoforms—We next compared by oligonucleotide-primed sequencing the 5'-untranslated sequences and 5' termini of TnT mRNAs expressed in different specialized muscles. Poly(A') mRNA isolated from问我。
Developmental Regulation of Troponin T Isoforms

We first assayed accumulation of α and β mRNAs in cultures of embryonic pectoral muscle prepared from day 10 quail myoblasts. Fig. 4 shows that α and β mRNAs are coexpressed in myogenic cultures, although β predominates. We then compared α and β TnT expression in embryonic leg and pectoralis dissected from day 8 embryos when skeletal muscle is still physiologically distinct from adult muscle, having "slow" contractile properties (38-40). Fig. 4 shows that α and β mRNAs are coexpressed in embryonic leg and pectoralis muscles in proportions similar to those observed in cultured myofibers. The embryonic leg β form is visible after longer exposures. The α and β protected fragments were excised from gels and radioactivity was quantitated by scintillation counting. The results show that the relative levels of α/β expression are 15:85%.

We compared troponin mRNA isoform expression in five anatomically and physiologically distinct adult muscles to determine whether alternatively spliced α and β mRNAs are expressed differentially in specialized muscles. Fig. 4 shows that the TnTf mRNA is not expressed detectably in adult striated heart muscle and liver tissue. TnTf mRNA is abundantly expressed in all of the adult skeletal muscles tested, but specific muscles express very different proportions of α or β mRNA. In leg muscle, which is a mixture of fast and slow twitch muscle fibers, α and β mRNAs are coexpressed. In

E. A. Bucher and C. P. Emerson, Jr., work in progress.

FIG. 1. RNA sequence analysis of the 5′ ends of TnT mRNAs. 10 μg of poly(A+) mRNA from liver, cultured embryonic myofibers, leg, adductor, and pectoralis were hybridized to 5′-end-labeled oC089 (see Fig. 1) and sequenced with reverse transcriptase, deoxynucleotides, and the dideoxynucleotides indicated on the lanes ACGT to generate the sequences shown. For each RNA sample a control with no dideoxynucleotides was added (designated −) to show the full-length extension product. Leg RNA was not sequenced, but the full-length extension product was determined. The size of the extension product including the molecular weight contribution of the oligonucleotide is shown at the right.
Fig. 3. Structure of the fast skeletal TnT genomic clone gC106. The entire sequence of the 17,115-nucleotide gC106 λ clone has been determined, and the deduced intron/exon organization is shown. The exon numbers are based on the rat exon sequence designations (16).

adductor and sartorius leg muscles, which contain predominantly slow twitch fibers (11, 39), β is predominantly expressed and represents greater than 95% of TnTβ mRNA. In gracilis, α and β mRNAs are coexpressed. In pectoralis, which is a fast muscle (39), α mRNA is greater than 99% of the total TnT message. However, during early development, the pectoralis muscle expresses 85% β splice form, but by 3 weeks post-hatching the α splice form expression increases to greater than 99% of TnTα mRNA. Precise quantitation of these hybrids is limited by possible differential stabilities of shorter length hybrids, although quantitation with cC119 probes gave similar results (data not shown). The data presented establish that the relative levels of expression of α and β differ greatly in different tissues and at different developmental stages. Furthermore, mRNAs contained either α or β; mRNAs containing both or neither exons were never detected and thus are mutually exclusive (also see Refs. 15 and 18).

Clonal Analysis of α and β Expression in Myogenic Cultures—We then examined whether the coexpression of α and β in embryonic muscle reflects a heterogeneity of myogenic lineages, each of which express only one splice form, α or β. Primary cultures of day 10 pectoralis muscle were replated at clonal densities of approximately 50 myoblasts/plate. Eight colonies were picked and plated onto separate culture dishes. The clones were allowed to grow until myofibers had formed, and RNA was isolated for analysis of α and β expression.

Fig. 5 shows that all of the clones that express TnTα mRNA coexpress α and β. Furthermore, the relative abundance of α and β coexpression in the clonal cells is identical to pooled populations of myofibers. These studies do not distinguish whether coincident splicing of α and β can occur within a single nucleus; however, they do demonstrate that embryonic myogenic lineages are not programmed to specifically accumulate only one splice form. The regulation of α and β splicing observed in embryonic and adult muscles, therefore, is likely controlled by environmental influences and physiological properties of different muscles, and not the selection of distinct myoblast lineages.

Analysis of the 5′ Splice Combinations of TnT mRNAs in Specialized Adult Muscles and Myogenic Cultures—We examined the combinations of 5′ splice choices in TnT transcripts expressed in different muscles by comparing primer extension products to determine if 5′ alternative splices also show muscle specific patterns. Since certain combinations of 5′ alternative exons are predicted to give different length hybrids, although quantitation with cC119 probes gave similar results (data not shown). The data presented establish that the relative levels of expression of α and β differ greatly in different tissues and at different developmental stages. Furthermore, mRNAs contained either α or β; mRNAs containing both or neither exons were never detected and thus are mutually exclusive (also see Refs. 15 and 18).

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mRNA isolated from pooled cultures of myofibers gel and exposed to Kodak XAR film for cultures. The expected protected fragment sizes are to the sequences are identical to TnT cDNA lane. The liver and adult heart controls performed in this experiment were combinations of similar-sized resistant products were resolved on a 6% denaturing polyacrylamide gel and exposed to Kodak XAR film for 24 h. The S1 probe is schematically shown below the autoradiogram. The probe size and expected protected fragment sizes are to the right. demonstrates that the sequences are identical to TnT cDNA sequences to the 5' border of exon 9 (data not shown). 5' to exon 9, divergent sequences are detected in primed products from myofiber and pectoralis mRNA samples, indicating that the differentially spliced region begins at exon 8. Nine size classes represent a minimum estimate since different combinations of similar-sized 5' exons would result in unique mRNAs having the same length.

oc090 primer extension products are tissue-specific. Primer extensions of myofiber mRNA results in eight specific extension products. In contrast, the mRNA accumulated in leg consists of one major and two minor mRNA extension products similar to the shorter length forms detected in myofibers. Adductor mRNA has one unique major product that is not found in the major leg and embryonic myofiber mRNAs. This unique adductor extension product is not detected in mixed leg muscle, which does not have adductor tissue. The adductor product was sequenced and represents a new TnTf isoform, whereas adult specialized muscles express a more restricted subset of 5' splice combinations, indicating that 5' alternative splicing is precisely regulated during the development of adult muscles.

Analysis of Combinations of 5' and 3' Splice Choices in TnT, Transcripts Expressed in Specialized Adult Muscles and Embryonic Myofibers—Primer extension analysis was also used to examine whether different muscles produce TnTf transcripts with restricted combinations of 5' and 3' alternative

FIG. 6. Primer extension analysis of the 5’ alternatively spliced region of TnT mRNAs in specific muscle types. 10 μg of poly(A+) mRNA from liver, cultured myofibers, leg, adductor, and pectoralis were hybridized to end-labeled oc090 (Fig. 1) and extended with reverse transcriptase and deoxynucleotides. A dideoxy sequence was also determined (data not shown). The products were resolved on an 8% denaturing polyacrylamide gel. The extremes of the size distribution are represented to the right. The myofiber sample shows eight “bands” each of which is broad because of the multiple start sites over 10 bp (Fig. 2). These eight bands consist of three abundant size classes of higher molecular weight, the fourth and fifth size classes are less abundant and the low molecular weight size classes 6–8 are abundant. The molecular weights of the leg forms are similar to the myofiber low molecular weight forms. The major adductor form has a slightly higher mobility than the leg or myofiber forms and thus is the ninth size class. The pectoralis forms are consistent with the high molecular weight mRNA sizes expressed in myofiber mRNA. The sizes represent the predicted full-length TnT mRNA, but the sizes on a denaturing polyacrylamide gel were in the range of 150 nucleotides.
splice choices. We hybridized poly(A) mRNAs with \(^{32}\)P-end-labeled oligonucleotides complementary to either \(\alpha\) (oC003) or \(\beta\) (oC002) exon sequences (see Fig. 1), and extended 5' with reverse transcriptase and deoxy nucleotides. Fig. 7 shows that myofiber RNAs primed with either \(\alpha\) or \(\beta\) oligonucleotides produce two major size classes, each of which is composed of three products. A minor band is seen between the two triplets in oC090 primed myofibers (Fig. 6). Leg RNA primed with \(\alpha\) and \(\beta\) oligos has fewer products. One major, and two closely migrating bands, have the same size as several of the myofiber extension products. The major high molecular weight band seen in the \(\beta\) primed leg lane is likely not muscle-specific since it co-migrates with a \(\beta\) primed liver product and a similar size is not seen in the oC090 primed leg (Fig. 6). Adductor mRNA gives a single product when primed with \(\beta\) No extension product is produced when adductor is primed with \(\alpha\), consistent with the observation that adductor expresses greater than 95% \(\beta\) (Fig. 4). We consistently obtain high molecular weight background products in \(\beta\) primed adductor reactions. The adductor product size is slightly larger than leg products, consistent with adductor products in Fig. 6. In contrast, pectoralis mRNA gives a higher molecular weight band when primed with \(\alpha\), and no extension product is primed with \(\beta\), consistent with the observed predominance of \(\alpha\) expression in the pectoralis (Fig. 4). Thus, TnT mRNA in different tissues shows different extended products which also vary with \(\alpha\) or \(\beta\) inclusion near the 5' end, showing that combinations of 5' and 3' splice choices are developmentally restricted in different adult fast skeletal muscles (see "Discussion").

Amino Acid Sequence Comparison of Fast Skeletal Troponin T Isoforms—We compared avian and mammalian TnT amino acid sequence isoforms to determine the relationship of different fast skeletal isoforms as well as the contribution of differential splicing to TnT isoform heterogeneity. The amino acid comparisons shown in Fig. 8 are with respect to the rabbit fast skeletal protein sequence (36) and numbered accordingly. In the 5' alternative splice region from aa1 to aa37, the rabbit/rat sequences are 52% identical and the rabbit/quail sequences are only 43% identical. In contrast, the TnT proteins are highly conserved C-terminal to aa37, being 97% similar between different mammals and 92% similar between mammals and quail. The quail protein sequence becomes more divergent between aa207 and aa257, the region of alternative \(\alpha\) and \(\beta\) splicing. These data show that the regulated differential splicing of the alternative 5' and 3' splice regions generate variability in TnT protein sequences in regions that also are divergent between quail and mammals. These regions of sequence divergence generated by muscle-specific splicing regulation, therefore, may be functionally significant for TnT isoforms expressed in different muscles.

**DISCUSSION**

The quail fast skeletal TnT gene generates a diversity of isoforms by complex, developmentally regulated mRNA splicing of a single mRNA transcript. Our findings provide new information about the mechanisms of TnT splicing regulation. Primer extension studies show that embryonic muscles express the same complexity of 5' splice forms with either \(\alpha\) or \(\beta\) 3' alternative exon splice forms (Fig. 7). Therefore, the mechanism that controls \(\alpha\) and \(\beta\) splice choice is permissive for all possible choices of alternative 5' exons, and the splicing of \(\alpha\) or \(\beta\) exons into TnT transcripts does not restrict, intrinsically, the choices of 5' exons, or vice versa. However, during the development of adult leg muscles, mRNAs with \(\alpha\) and \(\beta\) 3' exons have different 5' alternative exons (Fig. 7). This shows that the splicing of the 5' and 3' alternative exons can be subjected to muscle-specific mechanisms that coregulate splicing \(\alpha\) and \(\beta\) isoforms with different 5' exon combinations. The muscle-specific mechanisms by which different 5' exons are selected for inclusion into mRNAs with \(\alpha\) or \(\beta\) exons is unknown. Selection of 5' and 3' exon splicing processes could be coordinated by a common mechanism or by mechanisms that independently select 5' and 3' alternative splices.

Alternative splicing of the \(\alpha\) and \(\beta\) exons does not require external factors provided in the embryo, such as innervation or specific hormones, since \(\alpha\) and \(\beta\) alternative exons are coexpressed in myogenic cultures at the onset of myoblast differentiation. This coexpression is also observed in embryonic muscles, and \(\beta\) exon expression predominates in both cultured and embryonic muscles. We show by clonal cell analysis of cultured embryonic pectoral muscle that both \(\alpha\) and \(\beta\) splicing occur in clonal lineages of normal embryonic myoblasts. Thus, selection of \(\alpha\) and \(\beta\) exons is not lineage
Fig. 8. Amino acid comparison of fast skeletal troponin T isoforms. Sequence comparisons are against the rabbit TnT protein sequence (16) and are numbered accordingly. The chicken and rat amino acid sequences are taken from Refs. 10 and 17, respectively. Amino acid sequence differences are marked with an $X$ for a nonconservative replacement and with an $a$ for a conservative replacement. Residues identical with the corresponding residue in the rabbit sequence have no marking. The sequences were compared using a modified version of the Lipman and Pearson FASTP computer program (43).

restricted. Our results do not resolve whether $\alpha$ and $\beta$ exons are coexpressed within a single nucleus. In contrast to the quail, rat embryonic muscles, and the rat embryonic cell line, L6E9, express only the $\beta$ exon (15), suggesting either a bird/mammal divergence in embryonic splicing regulation or in the development of cell lineages.

The expression of the $\alpha$ and $\beta$ exons is highly regulated during the maturation of specialized adult muscles. In embryonic pectoralis muscle, $\beta$ exon expression predominates and there is a transition to expression of greater than 99% $\alpha$ exon. This transition from $\beta$ to $\alpha$ exon expression is coincident with a fiber type transition in chicken pectoralis from embryonic fibers, which are predominantly type IIA fibers (red fast twitch) and have slow contractile rates, to greater than 99% type IIB fibers (white fast twitch) 4 weeks after hatching (39). This change to a predominance of the $\alpha$ splice form in pectoral muscle is temporally coincident with changes in TnT, tropomyosin, and C-protein isoforms (42, 44-48).

Analysis of TnT expression in the adult leg muscles provides further evidence for a relationship between the differential expression of $\alpha$ and $\beta$ exon forms and specialization of fiber type. Adductor and sartorius muscles are unusual muscles in birds because they contain slow twitch muscle fibers as well as fast twitch fibers (11, 46). Greater than 96% of the fast twitch fibers in adductor muscle are the red fast twitch type IIA (39). We find that $\beta$ exon expression in adductor represents greater than 95% of TnT mRNA. In the sartorius, $\beta$ also predominates. This establishes a correlation between expression of the $\beta$ exon form in embryonic, adductor, and sartorius muscles and the presence of type IIA fast fibers and slow contractile rate (38-40). The expression of $\alpha$ exon in pectoralis is correlated with the presence of type IIB fast fibers and contractile rate. Consistent with these findings, protein analysis of chicken adductor and sartorius revealed expression of a unique leg TnT protein isoform, fLT3 (11), that is correlated with their slow contractile speed.

When myoblasts differentiate, specific splicing factors are produced that allow the muscle-specific expression of embryonic splice forms of TnT (49). Our clonal cell lineage studies establish that developmentally controlled transcription factors must also be produced to regulate the splicing of TnT alternative exons during the maturation of specific muscle types.

The regulation of TnT isoform expression is controlled by the abundance of specific TnT mRNA splice forms, not selective translation of a stochastically produced collection of TnT mRNAs. This conclusion is supported by our observation of a correlation between the expression of TnT proteins and mRNAs in embryonic and specific adult muscles. Embryonic muscles express multiple TnT protein isoforms, but specialized adult muscles express a more restricted array of isoforms (9, 10, 42, 47). Similarly, our primer extension studies show that embryonic muscles express multiple TnT mRNAs, whereas different adult muscles express a more restricted number of TnT mRNAs. There also is a specific correlation between the TnT proteins expressed and mRNA splice forms. Pectoralis expresses large TnT mRNAs, which likely encode the high molecular weight TnT protein isoforms found in...
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chicken pectoralis (10-12, 42). The presence of high molecular weight mRNAs in myogenic cultures is unexpected since large TnT proteins were not detected by two-dimensional gel analysis of chicken embryonic muscles (10, 11), although monoclonal antibody staining detects the large pectoralis-type TnT protein in day 17 chicken embryos (48). Additional analyses of expression in staged quail embryonic muscles are necessary to clarify this point. In addition to the correlation between TnT protein isoforms and mRNA forms in pectoral muscle, the adductor leg muscle expresses a unique-sized TnT mRNA that likely encodes the unique fLF3 protein found in chicken adductor (11).

The avian TnT gene must have additional 5' alternative exons to encode the pectoralis forms of TnT. The three quail TnT isoform sequences described in this study include sequences that correspond to all of the exons described in the rat cDNA and genomic sequences (Figs. 1 and 8; 16, 17). In addition, our primer extension results and the chicken protein data (10) reveal the existence of larger TnT mRNA and proteins than predicted from the quail cDNAs and the rat data. A recent report provides cDNA sequence information for a pectoralis form whose length is consistent with our primer extension predictions (50). These combined data increase the isoform coding potential from 64 (17) to at least 128. The prior suggestion that exons 9 of the TnTf gene is also alternatively spliced (17) is unlikely since all quail mRNA sequences encode this exon (see "Results"). Structural studies of the 5' end of the quail TnT gene will reveal the organization of these unique avian exons.

The abundance of specific splice forms of troponin T is highly regulated in any one muscle. It is theoretically possible that all 128 potential splice forms are made and that the differential abundances of TnT mRNAs in different muscles result from regulated mRNA stability. We think that this possibility is unlikely since previous studies show that TnTf and TnTl transcription rates and mRNA accumulation are very similar (51). If all 128 potential splice were made, then this would require the unlikely possibility that any one form of TnTf mRNA have a 100-fold greater stability than TnTl mRNA. Therefore, we favor the conclusion that there is direct regulation of TnTf mRNA splicing in different muscle types. Experimental studies to investigate the cis and trans components of this splicing regulation are now feasible using biochemical and transfection approaches (49).

Regulated alternative splicing of TnTf transcripts provides a genetic mechanism to produce isoforms that likely determine physiologically distinct Ca2+ sensitivities of the different fast skeletal muscle types (1-3, 52-60). In this regard, the expression of the α and the 5' exon forms of the quail TnTl gene is particularly interesting. The α exon is expressed uniquely in quail flight pectoralis muscle and is highly diverged from the mammalian forms (18), and the pectoralis specific 5' exon(s) are missing from the mammalian gene. These findings suggest a specialized avian flight muscle function for these exons.

Consideration of TnT protein function provides a possible rationale for the evolution of alternative splicing as the mechanism for generating TnT isoform diversity. Isoform diversity might have arisen by the evolution of large gene families for the fast, slow, and cardiac TnT isoforms. However, alternative splicing is the mechanism for generating isoform diversity of TnT as well as the evolution of cardiac TnT isoforms. One possible selective advantage of alternative splicing is evolution of isoform diversity without altering the stoichiometry of TnT expression, such as would result from TnTf gene duplications and the evolution of a larger, more diverse gene family.

Consistent with this idea, troponin T, I, and C isoform mRNAs and proteins are expressed coordinately in striated muscle types (13, 51, 63). Furthermore, genetic evidence in Drosophila shows that muscle gene dosage is essential for development of functional flight muscle (62). DNA transfection and transgenic approaches are now possible to examine the significance of TnT gene dosage and the functions of specific TnT exon protein sequences in physiologically specialized muscle types.

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