Exon Skipping in Cardiac Troponin T of Turkeys with Inherited Dilated Cardiomyopathy*

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Troponin T is a central component of the thin filament-associated troponin-tropomyosin system and plays an essential role in the Ca\(^{2+}\) regulation of striated muscle contraction. The importance of the structure and function of troponin T is evident in the regulated isoform expression during development and the point mutations resulting in familial hypertrophic and dilated cardiomyopathies. We report here that turkeys with inherited dilated cardiomyopathy and heart failure express an unusual low molecular weight cardiac troponin T missing 11 amino acids due to the splice out of the normally conserved exon 8-encoded segment. The deletion of a 9-bp segment from intron 7 of the turkey cardiac troponin T gene may be responsible for the weakened splicing of the downstream exon 8 during mRNA processing. The exclusion of the exon 8-encoded segment results in conformational changes in cardiac troponin T, an altered binding affinity for troponin I and tropomyosin, and an increased calcium sensitivity of the actomyosin ATPase. Expression of the exon 8-deleted cardiac troponin T prior to the development of cardiomyopathy in turkeys indicates a novel RNA splicing disease and provides evidence for the role of troponin T structure-function variation in myocardial pathogenesis and heart failure.

In vertebrate striated muscle, actomyosin ATPase-based contraction is regulated by Ca\(^{2+}\) through the thin filament-associated troponin (Tn)-tropomyosin (Tm) system (1–3). The Tn\(^{1}\) complex is composed of three subunits as follows: troponin C (TnC), troponin I (TnI), and troponin T (TnT). Coupling Tn to the thin filament, TnT is proposed as a molecular organizer in the Ca\(^{2+}\) signaling system regulating muscle contraction (2, 4, 5). Three homologous TnT genes have evolved in vertebrates encoding the cardiac, fast, andslow skeletal muscle fiber typespecific TnTs (4). In fast skeletal muscle TnT, alternative RNA splicing of multiple exons encoding the NH\(_{2}\)-terminal variable region produces acidic and basic TnT isoforms exhibiting functional differences in Ca\(^{2+}\) activation of muscle contraction (6–8). In contrast, the NH\(_{2}\)-terminal region in cardiac TnT (cTnT) is less variable. Alternate RNA splicing in most avian and mammalian cTnT is limited to a single exon (exon 5) in the NH\(_{2}\) terminus. Inclusion or exclusion of the 10 amino acids encoded by exon 5 is developmentally regulated in all vertebrate cTnT and is responsible for the perinatal switch from the high molecular weight, more acidic embryonic isoform to the low molecular weight, less acidic adult isoform (9–13). In some mammals (e.g. human (11), bovine (14), rabbit (15), and mouse (16)), developmental independent alternate RNA splicing of exon 4 encoding four or five amino acids also occurs resulting in the expression of two cTnT isoforms in the heart. Whereas functional differences in Ca\(^{2+}\) activation have also been found between these cTnT isoforms (17), no other alternative splicing pathway has been observed in the NH\(_{2}\)-terminal coding exons of cTnT.

The NH\(_{2}\) terminus of TnT does not directly bind to the other regulatory proteins of the thin filament, and its role in TnT function is largely debated. The NH\(_{2}\) terminus of TnT can be removed (e.g. deletion of the first 45 amino acids from fast skeletal muscle TnT) without abolishing the core activity of TnT (18). However, deletion of the NH\(_{2}\)-terminal region of TnT has been shown to result in a significant reduction in the maximum activation of reconstituted myofibrils (19). Consistent with the effects on Ca\(^{2+}\) sensitivity and cooperativity of muscle contraction (20), hypertrophic and failing cardiac muscle with impaired Ca\(^{2+}\) activation also exhibits altered expression of cTnT isoforms with NH\(_{2}\)-terminal variations (21, 22). To explore the structure-function relationship of the NH\(_{2}\)-terminal domain of TnT, we have shown previously (23–25) that the structure of the alternatively spliced NH\(_{2}\)-terminal region may modulate the overall conformation of TnT, causing changes in the binding affinity for Tm, TnI, and TnC. This mechanism may form the foundation for the physiological and pathological significance of the various TnT isoform expressions in the heart.

The pathological effects of alterations in TnT structure and function are further demonstrated by the development of familial hypertrophic cardiomyopathy (26, 27) and dilated cardiomyopathy (DCM) (28) resulting from point mutations throughout the cTnT polypeptide chain. These dominant mutations alter the contractility of cardiac muscle leading to different disease states by relatively minor changes in TnT function (27, 29, 30). Turkey is one of the very few species that has a high incidence of spontaneous DCM (31). The turkey DCM exhibits many features similar to that in human DCM and has

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† The abbreviations used are: Tn, troponin; BSA, bovine serum albumin; cTnT, cardiac troponin T; DCM, dilated cardiomyopathy; AE8, exclusion of exon 8; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PVA, polyvinyl alcohol; S1, myosin subfragment 1; Tm, tropomyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T; WT, wild type.

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been used as a model system in heart failure studies (32–34). Although stress factors such as furazolidone treatment promote the rate of DCM development, turkey DCM clearly contains a genetic basis and exhibits a pattern of familial inheritance (34–37).

In the present study we found that turkeys with inherited DCM and heart failure express an unusually low molecular weight cTnT due to an exclusion of the normally conserved exon 8-encoded segment. A deletion of 9 bp in intron 7 of the turkey cTnT gene may be responsible for the weakened splicing of the downstream exon 8. The exon 8-deleted cTnT showed changes in overall conformation and binding affinity for TnI and Tm and resulted in an increase in the calcium sensitivity of myosin ATPase. This finding demonstrates a novel RNA splicing disease and provides evidence for the role of TnT structure-function relationship in the pathogenesis of DCM and heart failure.

EXPERIMENTAL PROCEDURES

SDS-PAGE and Western Blotting—SDS gel samples of ventricular muscle from furazolidone-induced DCM turkeys were provided by Dr. Henderson State University, Statesboro, GA. The samples were subjected to furazolidone treatment at 7 days of age for 2–3 weeks. Fresh muscle tissues were homogenized in SDS-PAGE sample buffer containing 1% SDS. The samples were stored below −70 °C until used. After heating at 80 °C for 5 min, the total protein extracts were resolved by 14% Laemmli gel with an acrylamide-to-bisacrylamide ratio of 180:1. Resulting gels were stained with Coomassie Blue R-250 to reveal the resolved protein bands. Duplicated gels were electronically transferred to nitrocellulose membranes as described previously (23). The nitrocellulose membranes were incubated with an anti-cTnT monoclonal antibody (mAb) (CT3) (25) or a rabbit anti-TnT polyclonal antibody RαTnT (23). The membranes were then washed with high stringency using Tris-buffered saline containing 0.5% Triton X-100, 0.1 mM EDTA, 0.1 M β-mercaptoethanol, and IgG second antibody (Sigma Chemical Co.), washed again, and developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution as described previously (23).

Two-dimensional Gel Electrophoresis—As described previously (10), total turkey cardiac muscle protein extracts were analyzed by two-dimensional gel. The first dimension was isoelectric focusing in Bio-Rad mini tube gels containing pH 4–6 and pH 3.5–10 Ampholine (Amersham Biosciences) in a 4:1 ratio. After electrophoresis at 400 V for 6 h and 650 V for 1.5 h, the isoelectric focusing gel was equilibrated in SDS-PAGE sample buffer for 10 min and loaded on a 14% Laemmli slab mini gel with an acrylamide-to-bisacrylamide ratio of 180:1 for the second dimension SDS-PAGE. Five min after the bromophenol blue dye front ran off the bottom edge, the gel was stained with Coomassie Blue R-250 to reveal the resolved protein spots or transferred onto nitrocellulose membrane for Western blotting as above.

cDNA Cloning and Sequencing—As described previously (38) total turkey ventricular muscle RNA was isolated by the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two µg of the cardiac RNA was used to synthesize cDNA encoding cTnT by reverse transcription using an oligonucleotide primer (5′-GAGAGACCAGAAAAGACCGAGGAG-3′) complementary to the exon 18 sequence of the chicken cTnT gene (9) flanking the translation stop codon plus an EcoRI restriction enzyme site (underlined). The turkey cTnT cDNA was then amplified by PCR using the exon 18 reverse primer and a forward primer (5′-CACATTGCTCGAGACTGAGGAGTCC-3′) synthesized corresponding to the exon 2 sequence of the chicken cTnC gene plus an Ndel restriction site (underlined) at the translation initiation codon. Resultant PCR products were cut at the Ndel and EcoRI sites built in at the ends of the cDNA and cloned into the pET17b expression plasmid (Novagen, Madison, WI). Recombinant plasmid DNA was purified, and the cDNA insert was sequenced by the dyeodeoxy chain termination method.

Genomic Cloning and Sequencing—Genomic DNA was prepared from the liver of domestic turkey by proteinase K digestion and phenol/CHCl₃ extraction (39). Two oligonucleotide primers were synthesized for PCR amplification of the segment containing the exon 7 to exon 10 region of the turkey cTnT gene. Sequence of the forward primer (5′-GGAGAGGCCACCGAGGGGCGATGAT-3′) was derived from the antisense sequence of the exon 10 of turkey cTnT gene. By PCR using Pfu DNA polymerase with proofreading activity (Stratagene), a DNA fragment of ~1.1 kb was amplified from the turkey genomic DNA (Fig. 4B). This PCR product was purified by agarose gel electrophoresis, cloned into the pBluescript plasmid vector, and then amplified as described (23).

Restriction Endonuclease Mapping of Turkey and Chicken cTnT Genomic Structure—As described previously (39), the exon 7 to exon 10 segment of turkey cTnT gene was amplified by PCR from the cloned plasmid DNA. The corresponding region of the chicken cTnT gene was amplified by PCR directly from domestic chicken genomic DNA as described above for the cloning of the turkey cTnT genomic segment. The specific PCR product of chicken genomic DNA was purified by agarose gel electrophoresis and recovered by the Prep-A-Gene glass bead method (Bio-Rad). Following PCR verification using an exon 8-specific internal primer versus the flanking exon 7 primer, the chicken cTnT genomic DNA fragment was re-amplified by PCR. After extraction with phenol/CHCl₃ and precipitation in ethanol, the turkey and chicken genomic DNA fragments were digested with a battery of restriction endonucleases under standard conditions. Agarose gel electrophoresis was carried out to identify the restriction fragments.

Expression and Purification of Tn Subunits—The wild type turkey cTnT, low molecular weight turkey cTnT, and mouse cardiac TnC (25) were expressed from cloned cDNA and purified with slight modification of protocols (23).

For cTnT and mouse cardiac TnC expression, BL21(DE3)pLysS Escherichia coli cells were transformed with the recombinant pET17b plasmid. Freshly transformed E. coli was cultured in rich liquid media containing ampicillin and chloramphenicol and induced at mid-log phase with isopropyl-1-thio-β-D-galactoside. After three additional hours of culture the bacterial cells were harvested for purification.

The turkey cTnTs were purified by the following method. The induced bacterial cells were washed with cold ethanol and acetonefollowed by protein extraction with 1 M KCl, 0.1 mM EDTA, 15 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0. Extracted proteins were fractionated by ammonium sulfate precipitation, and the fraction between 20 and 50% saturation was dialyzed against 10 mM Tris-HCl, pH 8.0, containing 6 mM β-mercaptoethanol. Following dialysis the fraction was brought to 6 M urea, 0.1 mM EDTA, 15 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, clarified by centrifugation, and chromatographed on a DE52 anion-exchange column equilibrated in the same buffer. The column was eluted with a linear KCl gradient (0–300 mM), and the protein peaks were analyzed by SDS-PAGE. The fractions containing turkey cTnT were diazylzed against water and concentrated by lyophilization. The cTnT was further purified to homogeneity by G-75 gel filtration chromatography in 6 M urea at pH 7.0 as described previously (38).

Mouse cardiac TnC was purified as follows. The induced bacterial cells were lysed by three passes through a French press. Cellular proteins were extracted in 6 M urea, 0.1 mM EDTA, 15 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, and fractionated on a DE52 anion-exchange column equilibrated in the same buffer. The column was eluted with a linear KCl gradient (0–300 mM), and the protein peaks were analyzed by SDS-PAGE. The fractions containing mouse cardiac TnC were diazylzed against water and concentrated by lyophilization. The cardiac TnC was further purified to homogeneity by G-75 gel filtration chromatography in 6 M urea at pH 7.0 as described previously (23).

Chicken and bovine cardiac TnI were purified from adult ventricular muscle. The tissue was homogenized in 30 mM Tris-HCl, pH 8.0, containing 0.5% (w/v) Triton X-100, 0.05% SDS, incubated with alkaline phosphatase-labeled anti-mouse or anti-rabbit IgG second antibody (Sigma Chemical Co.), washed again, and developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution as described previously (23).

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peak was identified by SDS-PAGE, dialyzed against 0.5% formic acid, and lyophilized.

**Purification of Other Myofilament Proteins**—Rabbit cardiac TnI, skeletal muscle actin, and myosin were all purified from muscle tissue. Chicken and rabbit cardiac α-TnI were isolated as described previously (41). Chicken cardiac actin was isolated from skeletal acetone powder and prepared as described previously (10). Following polymerization F-actin was stored in 50% glycerol at −20 °C until used. Rabbit skeletal myosin was isolated from back muscle, and myosin sub-fragment 1 (S1) was obtained by limited α-chymotrypsin digestion (42) and frozen in small aliquots at −80 °C until used.

**Antibody Conformational Analysis**—Myofilament protein antibodies (RAcTnT, generated against fast skeletal muscle TnT that cross-reacts to multiple epitopes on the conserved central and COOH-terminal domains of cTnT (23)) were used to monitor the conformational changes that alter the antibody binding affinity. Similar to that described previously (23), purified turkey cTnT was dissolved in Buffer A (0.1 M KCl, 3 mM MgCl2, 10 mM PIPES, pH 7.0) and coated on microtiter plates by incubation at 4 °C overnight. The unbound cTnT was washed away with Buffer A containing 0.05% Tween 20 (Buffer T), and the remaining plastic surface was blocked with 1% bovine serum albumin (BSA) in Buffer T. The immobilized cTnT was incubated with serial dilutions of CT3 or RAcTnT antibody at room temperature for 2 h. Following washes with Buffer T to remove the unbound first antibody, the plates were further incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin second antibody (Sigma) at room temperature for 1 h. The unbound second antibody was washed away with Buffer T and H2O2/2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) was added for substrate reaction. A0.05 nm of each assay well was recorded at a series of time points by an automated microtiter plate reader (Bio-Rad Benchmark). The A0.05 nm values in the linear course of the color development were used to plot the antibody titration curves for the quantification of binding affinity for the epitopes on turkey cTnT. All experiments were done in triplicate.

**Protein Binding Assay**—A solid phase protein binding assay (23) was used to investigate the interactions of turkey cTnT with TnI and TnM. Purified wild type and exon 8-deleted turkey cTnT or BSA control was dissolved at 5 μg/ml in Buffer A and coated onto triplicate wells of micrometer plates by incubation at 4 °C overnight. After washes with Buffer T to remove the unbound protein, the plates were blocked with BSA containing 1% BSA. The plates were then incubated with serial dilutions of chicken cardiac TnI or α-TnI in Buffer T containing 0.5% BSA at room temperature for 2 h. After washes with Buffer T, the bound TnI or TnM was quantified via an anti-TnI mAb TnI-1 (40) or an anti-Tm mAb CH1 (43), respectively, using the standard ELISA procedure described above.

**Actomyosin ATPase Assay**—The tropomyosin complex was reconstituted by mixing wild type or exon 8-deleted turkey cTnT, bovine cardiac TnI, mouse cardiac TnM, and rabbit skeletal actin at molar ratio of 1:1:1:4.2 in 4.6 M urea, 1 mM KCl, 5 mM MgCl2, 0.05 mM CaCl2, 0.5 mM dithiothreitol, 20 mM imidazole, pH 7.0. The mixture was dialyzed against the same buffer without urea, followed by a change of 100 mM KCl, 5 mM MgCl2, 0.05 mM CaCl2, 0.5 mM dithiothreitol, 20 mM imidazole, pH 7.0. Insoluble proteins were removed by centrifugation for 10 min at 120,000 × g in a microcentrifuge, and the supernatant was incubated on ice for 2 h. To the TnM-TnC complex, F-actin was added as a sub-saturated molar ratio and incubated at 4 °C for 1 h. After incubation at room temperature for 15 min, the reconstituted thin filaments were used for actomyosin S1 ATPase assay. The protein contents of the reconstituted thin filament were verified by 12% SDS-PAGE with an acrylamide/bisacrylamide ratio of 29:1 (40).

The Ca2+-regulated myosin S1 ATPase activity was analyzed using a modified malachite green method (44). Briefly, the reconstituted thin filaments were suspended in assay buffer (6.5 mM KCl, 3.5 mM MgCl2, 0.5 mM dithiothreitol, 2.5 mM EDTA, 20 mM imidazole, pH 7.0) containing various concentrations of free Ca2+ calculated as described previously (42) and added to the wells of a microtiter plate. After incubation at room temperature for 10 min, 2.5 μg of myosin S1 in assay buffer was added per well to the thin filaments, and the reaction was initiated by the addition of ATP to 0.5 mM of the 150-μl reaction. The reaction was mixed by shaking a microtiter plate reader and allowed to proceed to room temperature for 10 min before being stopped by the addition of ammonium heptamolybdate plus sulfuric acid. After the addition of malachite green and polyvinyl alcohol (PVA), the inorganic phosphate produced from ATP hydrolysis was determined by absorbance at 595 nm recorded by an automated microplate reader (Bio-Rad Benchmark). The A595 values were plotted versus the pCa values and fit to the Hill equation (Sigma Plot) to construct the Ca2+ activation curves for the myosin S1 ATPase. Thin filaments containing wild type or exon 8-deleted turkey cTnT were assayed in parallel, and all experiments were done in triplicate.

**Antibody Conformational Analysis**—Densityometry analysis of the Western blots was done using the NIH Image program version 1.61. The DNA and protein sequence analyses were done using computer programs from DNAstar. Statistical analysis of the ELISA antibody titration, protein binding assays, and ATPase assays was done by Student test.

**RESULTS**

**An Unusual Low Molecular Weight cTnT Missing the Exon 8-Encoded Segment in the DCM Turkey Heart**—Normally the adult avian heart expresses only a single cTnT (Fig. 1A). In contrast to several evolutionarily closely related birds, Western blots on total cardiac muscle homogenates from the DCM turkeys (31) detected an additional low molecular weight cTnT (Fig. 1A). The low molecular weight cTnT is also present in the hearts of non-DCM domestic turkey and wild turkeys and is proportionally incorporated into the myofibrils (Fig. 1B). Denstometry of the Western blots determined a 69:31 expression ratio of the wild type/low molecular weight turkey cTnTs. By utilizing reverse transcription-PCR, we cloned cDNAs encoding the wild type (WT) and low molecular weight turkey cTnTs. Sequencing analysis revealed that the primary structure of the low molecular weight turkey cTnT differs from the WT by an unusual exclusion of the segment encoded by exon 8 (ΔE8) (Fig. 2A). Expression of the cloned cDNAs in *E. coli* yielded proteins that are recognized by the anti-cTnT mAb CT3 with sizes identical to that of the cTnT variants found in turkey cardiac muscle (Fig. 2B). Physical properties of the wild type and ΔE8 turkey cTnT calculated from their primary structures are shown in Table I. The splicing out of this unusual acidic 12-amino acid segment results in changes of both size and isoelectric point (pl) of the cTnT (Table I) which were confirmed by two-dimensional gel electrophoresis (Fig. 2C). The cDNA sequence of wild type and exon 8-deleted turkey cTnTs have been submitted to GenBank with accession numbers AF274301 and AY005139, respectively.

Amino acid sequence alignment of cTnT from a number of mammalian and avian species demonstrates that the exon 8-encoded segment is conserved across the vertebrate phyla (Fig. 3). This conservation of primary structure indicates the importance of this region in the function of cTnT. Therefore, a
deletion of this segment due to the skipping of exon 8 during mRNA splicing results in structural and functional consequences.

The Expression of Turkey ΔE8 cTnT Is a Constitutive Event—To investigate whether the altered exon 8 splicing pathway is developmentally regulated in comparison with the embryonic specific alternative splicing of exon 5 (9, 45), we examined the expression of cTnT variants in turkey hearts at days 7, 14, and 21 in ovo, days 0, 7, and 15 days post-hatch, as well as in the adult. Western blots using the CT3 mAb against a constitutive epitope in the COOH-terminal region of cTnT demonstrated that in addition to the normally expressed single embryonic cTnT (46), a band with a lower molecular weight was detected in the day 7 embryonic heart at an abundance similar to the WT/ΔE8 cTnT ratio observed in the adult heart (Fig. 4). This band may represent the embryonic cTnT with exon 5 inclusion (+10 amino acids) and exon 8 exclusion (−12 amino acids) (Table I). During embryonic and post-hatch heart development, the expression level of embryonic cTnT decreases due to the increased splice out of the exon 5 segment (9, 45), resulting in a corresponding increase of the adult cTnT. The emergence of a low molecular weight adult cTnT during this developmental isoform switching indicates the continuous presence of the exon 8 deletion event (Fig. 4A). During this transition, both wild type adult cTnT and the embryonic cTnT with exon 8 deletion are expressed in the turkey cardiac muscle. By Western blot, the WT adult cTnT overlaps with the ΔE8 embryonic cTnT due to their similar sizes (Table I). The similar levels of exon 8 exclusion in embryonic and adult cTnT indicate that the abnormal skipping of exon 8 in the turkey cTnT-ΔE8 is not developmentally regulated but occurs as a constitutive splicing pathway of the cTnT mRNA. Taking advantage of the transient expression of cTnT gene in embryonic skeletal muscle (9, 46, 47), we further found that when cTnT is expressed in day 14 embryonic leg muscle the splice out of exon 8 occurs at a ratio to the WT similar to that in the day 14 embryonic heart (Fig. 4B). This result indicates that the splicing pathway resulting in the skipping of exon 8 is not due to a change restricted to the cardiac muscle cellular environment but may be the result of a weakened recognition for this exon by the RNA splicing mechanism.

**Genomic Structure of Turkey cTnT Gene in the Regions Flanking Exon 8**—To further explore the mechanism responsible for exon 8 skipping, a genomic DNA fragment corresponding to the region of exon 7 to exon 10 in the turkey cTnT gene was cloned, and the DNA sequences flanking exon 8 were determined. The DNA sequence has been submitted to GenBank™ with accession number AF374417. DNA sequence alignment demonstrated that the turkey and chicken cTnT genes are highly similar (Table II), which is consistent with their close evolutionary relationship (Fig. 1A) (48). The DNA sequence alignment of exon 8 and flanking regions of turkey and chicken cTnT cDNA demonstrate only one T to C transition at a wobble base in the exon 8 sequence (Fig. 5C). Although it has been demonstrated that purine-rich sequences in cTnT exon 5 may enhance splicing (49), the effect of this pyrimidine transition on exon 8 splicing is unclear. The intron sequences flanking exon 8 in the turkey cTnT gene have preserved consensusesplicing boundary elements (Fig. 5C). DNA sequence alignment revealed that the intron 7 in turkey cTnT gene has a 9-bp deletion compared with the chicken cTnT gene (9) (Fig. 5A). This structural difference between the turkey and chicken cTnT genes is confirmed by restriction enzyme mapping (Fig. 5B). In the alignment of turkey and chicken cTnT genomic sequences, very few other single base pair deletions or insertions are found in the intron 7 and intron 8 sequences (data not shown). In contrast, the deletion of the 9-bp segment from the turkey cTnT intron 7 is a significant change and may disrupt a potential cis-active regulatory sequence, responsible for the weakened splicing of exon 8 during cTnT gene expression in both cardiac and skeletal muscles.

**Polymorphism of Exon 7 Sequence in Turkey cTnT Gene—**
Two alleles differing in the presence or absence of a GAA codon at the 3' end of exon 7 encoding a Glu residue are found among the cloned turkey cTnT cDNAs (Fig. 6). As demonstrated by the genomic DNA structure (Fig. 5), the inclusion or exclusion of a Glu codon is due to a difference in the exon 7 sequence rather than resulting from mRNA splicing using alternative acceptor or donor sites. This polymorphism was found in both the wild type and ΔE8 turkey cTnT, indicating its independence of the exon 8 splicing pathways. Such single amino acid polymorphism was also found previously among the exon 4 sequences of rabbit cTnT cDNA clones (15), and the functional significance remains to be investigated.

**Exon 8 Deletion Caused Conformational Changes in cTnT**—By using wild type and exon 8-deleted turkey cTnT proteins purified from E. coli expression (Fig. 7), we compared the cTnT-ΔE8 to the WT for differences in molecular weight using gel electrophoresis analysis. We have shown previously that the NH₂-terminally originated conformational changes of TnT detected by ELISA epitope analysis agree well with that detected by fluorescence spectra (24). The results in Fig. 8A demonstrate that the exon 8 deletion resulted in an increased binding affinity of mAb CT3 to an epitope in the COOH-terminal region of cTnT (25), indicative of an NH₂-terminal structure alteration-induced conformational change in the COOH-terminal domain of cTnT. The exon 8 exclusion induced conformational change of cTnT was further shown by the increase in binding affinity of the anti-TnT polyclonal antibody RAtnT against multiple epitopes throughout the ΔE8 molecule (Fig. 8B), demonstrating the conformational change is not limited to the CT3 epitope. The increase in antibody accessibility to the epitopes on cTnT-ΔE8 may also reflect an NH₂-terminal structure-originated change in the molecular flexibility of TnT (24).

**Exon 8 Deletion Altered the Binding Affinity of cTnT to TnI and Tm**—The effective incorporation of the exon 8-deleted turkey cTnT into cardiac myofibrils (Fig. 1B) indicates that it may impose a functional significance on the Ca²⁺-regulation of contraction. To examine the effect of the exon 8 deletion on interaction of cTnT with other thin filament regulatory proteins, we compared the binding of cTnT-WT and cTnT-ΔE8 to TnI and Tm. The results of ELISA solid phase protein binding experiments demonstrate that cTnT-ΔE8 has an increased binding affinity for TnI compared with that of cTnT-WT (Fig. 9A). This is observable in the significantly lower concentration of TnI required to reach 50% of maximum binding (p < 0.01), reflecting a higher Kₐ value during the initial phase of equilibrium binding. However, under saturated coating of cTnT on the microtiter plates, a significantly lower maximum binding of TnI was observed for cTnT-ΔE8 compared with that of cTnT-WT (Fig. 9B, p < 0.02). This may reflect a weakened coupling between cTnT-ΔE8 and TnI and thus rendering a lower resistance to the postincubation washes under non-equilibrium conditions in the ELISA procedure (50). The higher Kₐ value in cTnT-ΔE8-TnI binding may facilitate incorporation of the mutant cTnT into the Tn complex and the thin filament, whereas the less stable coupling between cTnT-ΔE8 and TnI may result in changes to the allosteric feature of the Ca²⁺-regulatory system.

The binding of cTnT-ΔE8 to Tm exhibits no significant difference in affinity from that observed in the cTnT-WT (Fig. 9C). However, the level of its maximum binding to Tm is significantly increased in cTnT-ΔE8 compared with that of the cTnT-WT (Fig. 9D, p < 0.001). In contrast to the weakened coupling of cTnT-ΔE8 to TnI, the results may reflect a strengthened coupling between cTnT-ΔE8 and Tm, which may also affect the allosteric feature of the thin filament regulatory system. This hypothesis is in agreement with the observation that a COOH-terminal truncation of cTnT found in human familial hypertrophic cardiomyopathy was shown to alter the activation of thin filament through destabilization of the thin filament inhibition state, which is responsible for the pathological phenotypes (51).

**Incorporation of Turkey cTnTΔE8 into Reconstituted Thin Filaments Alters the Ca²⁺-regulated Actomyosin S1 ATPase Activity**—In light of the central role TnT plays in the regulation of muscle contraction, it is likely that the conformational change of cTnT-ΔE8 and its altered interactions with TnI and Tm may affect Ca²⁺-activation of the cardiac muscle. To evaluate whether the deletion of exon 8 from the turkey cTnT does alter muscle contractility, we measured the Ca²⁺-regulated myosin S1 ATPase activity using reconstituted thin filaments composed of either turkey cTnT-WT or cTnT-ΔE8 (Fig. 10). Measurement of the actomyosin ATPase activity demonstrated that the cTnT-ΔE8 thin filaments were more sensitive to Ca²⁺ than those containing cTnT-WT (Fig. 11). This is evident by the leftward shift of the ATPase-pCa curve and the significantly higher pCa₅₀ value (p < 0.01) exhibited by the cTnT-ΔE8 thin filament (pCa₅₀ = 5.83) compared with that of the cTnT-WT (pCa₅₀ = 5.60). The results suggest that the deletion of the exon 8 segment from turkey cTnT alters the interactions within the
thin filament regulatory system, leading to an alteration of the thin filament-based Ca\textsuperscript{2+}/H11001 signaling and affecting the activation of cardiac muscle contraction.

**DISCUSSION**

Abnormal Exclusion of the Exon 8 Segment from Turkey cTnT—Although the exon 8-encoded region in turkey cTnT gene (E7-F and E10-R, respectively) was used in PCR to amplify and clone a genomic DNA segment of the turkey cTnT gene. DNA sequencing detected a 9-bp deletion in the intron 7 sequence of the turkey cTnT gene as compared with the corresponding region in the chicken cTnT gene. B, corresponding DNA segments were amplified from the cloned turkey genomic DNA or directly from total chicken genomic DNA using the E7-F and E10-R primer pair. Restriction endonuclease mapping showed comparable cleavage sites in the turkey and chicken cTnT genomic DNA segments by multiple enzymes (data not shown). Analyzed by 1.2% agarose gel electrophoresis, the PstI and BstXI maps show restriction fragments confirming the 9-bp difference between the intron 7 region of turkey (T) and chicken (C) cTnT genes. C, the nucleotide sequences of the exon 8 and flanking regions of turkey and chicken cTnT genes are aligned to show a few differences (in boldface letters), which may also contribute to the weakened exon 8 splicing.

**FIG. 5.** Genomic structure of the turkey and chicken cTnT genes in the exon 7 to exon 10 region. A, oligonucleotide primers derived from the exon 7 and exon 10 sequences of turkey cTnT gene (E7-F and E10-R, respectively) were used in PCR to amplify and clone a genomic DNA segment of the turkey cTnT gene. DNA sequencing detected a 9-bp deletion in the intron 7 sequence of the turkey cTnT gene as compared with the corresponding region in the chicken cTnT gene. B, corresponding DNA segments were amplified from the cloned turkey genomic DNA or directly from total chicken genomic DNA using the E7-F and E10-R primer pair. Restriction endonuclease mapping showed comparable cleavage sites in the turkey and chicken cTnT genomic DNA segments by multiple enzymes (data not shown). Analyzed by 1.2% agarose gel electrophoresis, the PstI and BstXI maps show restriction fragments confirming the 9-bp difference between the intron 7 region of turkey (T) and chicken (C) cTnT genes.

**FIG. 6.** Polymorphism of exon 7 sequence in turkey cTnT gene. Two alleles differing in the exon 7 sequence are found among the turkey cTnT cDNA clones. Genomic structure revealed that the presence or absence of a 3-bp (GAA) segment in the 3' region of exon 7 corresponds to the inclusion (E+) or exclusion (E−) of a Glu residue in the cTnT polypeptide chain.

thin filament regulatory system, leading to an alteration of the thin filament-based Ca\textsuperscript{2+}/H11001 signaling and affecting the activation of cardiac muscle contraction.

**Exon 7**

\textbf{Glu +:} AACACGAAGATGAAACAAAAGCACCAGAAGAGGAA\textit{G}tt
\textbf{Glu −:} AACACGAAGATGAAACAAAAGCACCAGAAGAGGAA\textit{G}tt

GluHisGluAspGluThrLysAlaProGluGluGlu

**FIG. 6.** Polymorphism of exon 7 sequence in turkey cTnT gene. Two alleles differing in the exon 7 sequence are found among the turkey cTnT cDNA clones. Genomic structure revealed that the presence or absence of a 3-bp (GAA) segment in the 3' region of exon 7 corresponds to the inclusion (E+) or exclusion (E−) of a Glu residue in the cTnT polypeptide chain.

thin filament regulatory system, leading to an alteration of the thin filament-based Ca\textsuperscript{2+}/H11001 signaling and affecting the activation of cardiac muscle contraction.
from turkey cTnT may constitute a significant structural and functional change.

Because this abnormal splicing pathway is also present in the wild turkey it is therefore not an isolated instance in a particular domestic stock. As indicated above, avian protein structure generally has a lower tolerance to changes compared with that of other species (52). Therefore, the exon 8 skipping event in turkey cTnT is considered an abnormal trait for the vertebrate cTnT although it occurs in all individuals tested in the species (Fig. 1B). This potential genetic abnormality carried by an entire species is supported by its direct linkage with the spontaneous development and susceptibility to stress-induced changes in molecular conformation and flexibility.

**Potential Role of Intron Mutation as a Cause of mRNA Splicing Diseases**—Turkey and chicken both belong to the Phasianidae family of birds. The striking protein and mRNA sequence similarities shown in Table II confirm the close evolutionary relationship between the turkey and chicken cTnT genes. Although the avian constraint hypothesis discussed above suggests that increased functional constraints on avian proteins reduces genetic divergence (52), the almost same percent sequence similarities at the protein and mRNA levels indicate little divergence at the codon wobble bases, further supporting the close evolutionary relationship between the turkey and chicken cTnT genes. Sequence alignment of the exon 7 to exon 10 region of the turkey and chicken cTnT genes (data not shown) demonstrates that the 9-bp deletion in the intron 7 of turkey cTnT gene (Fig. 5) is a significant difference in contrast to the scattered single base variations seen in the other regions.

**Effects of the Erroneous Skipping of Exon 8 Segment in Turkey cTnT on Muscle Thin Filament Function**—The incorporated cTnT expressed in E. coli.
ration of cTnT lacking the exon 8 segment into the myofibrils at over 30% of the total cTnT in the turkey cardiac myofibril (Fig. 1) may have a significant effect on the cardiac muscle contractility. The abnormal exon 8 splicing variant involves the NH₂-terminal region that is a proposed modulator domain (Fig. 2A). The conformation and protein binding changes in cTnT-E8 (Fig. 8 and Fig. 9) suggest that the deletion of exon 8 segment may have a profound effect on the function of cTnT based on the modulatory role of the NH₂-terminal structure of TnT, which affects the conformation and function of other domains of the molecule (23, 24, 54). The proximal position of the exon 8-encoded segment to the Tm-binding site in the central region of TnT (42) (Fig. 2A) and the altered binding of the cTnT-E8 to Tm (Fig. 9) suggest that the exon 8 deletion may impart an effect on the interaction of TnT with Tm in the thin filament.

As many cTnT point mutations found in human familial hypertrophic cardiomyopathy (55), the structural change due to the deletion of the exon 8 segment may lead to an alteration in the calcium activation and/or relaxation of the turkey cardiac muscle. Nevertheless, the functional effects of exon 8 deletion observed in the protein binding assay are similar to those we demonstrated previously (38) to occur in TnT as the result of NH₂-terminal structural variation or modification (23–25), which themselves may alter the contractility of the muscle (7). The clear but minor alteration in Ca²⁺ activation of the cTnT-E8 thin filaments as well as the distinct changes in the

Fig. 9. The deletion of the exon 8 segment from the turkey cTnT alters interactions with Tnl and Tm. Solid phase protein binding assays show that the interactions of cTnT-ΔE8 with Tnl (A and B) and Tm (C and D) are significantly altered as compared with that of the cTnT-WT. Although the binding affinity of cTnT-ΔE8 for Tnl was higher than that of cTnT-WT, as defined by the cTnT concentration required for 50% of maximum binding during equilibrium incubation (A), the maximum level of their binding after repeated washes was significantly lower than that of cTnT-WT, reflecting a weakened coupling (B). In contrast, the relative binding affinities of turkey cTnT-ΔE8 and cTnT-WT for Tm were not significantly different (C). However, the maximum level of their binding after non-equilibrium washes was significantly higher than that for cTnT-WT, indicating a stronger coupling in a myofilament assembly (D).

Fig. 10. Reconstitution of cardiac muscle thin filament. SDS-PAGE gels demonstrate the initial protein material and thin filament reconstitution. Lane 1, rabbit actin and cardiac α-Tm; lane 2, turkey cTnT-WT reconstituted Tn complex; lane 3, turkey cTnT-ΔE8 reconstituted Tn complex; lane 4, turkey cTnT-WT reconstituted thin filament; lane 5, turkey cTnT-ΔE8 reconstituted thin filament. Note in lane 4 that cTnT-WT and actin co-migrate in this gel system producing a band larger than the actin alone band in lane 5.
interactions of cTnT-ΔE8 to TnI and Tm suggest that contrary to a simple disruption of the global structure, conformational alteration due to the NH₂-terminal exon 8 deletion produces a change in the functional state of TnT. These altered interactions between cTnT and the other regulatory proteins may alter the function of the assembled myofilament in the turkey cardiac muscle and contribute to the pathogenesis of DCM and heart failure. Therefore, conformational modulation may play a major role in the functional effect of cTnT-ΔE8, predisposing these animals to the development of DCM and heart failure.

The correlation between the cTnT NH₂-terminal splicing abberance and the DCM phenotype adds evidence for the functional significance of the NH₂-terminal region of TnT. These findings lend support to the role of the NH₂-terminal domain of TnT as a regulatory structure modulating the conserved central and COOH-terminal regions (Fig. 2A) (23, 24). In the rod-shaped TnT molecule (24), the deletion of the segment encoded by exon 8 (Fig. 2A) may enhance the effect of the distal NH₂-terminal negative charge on the central and COOH-terminal regions. We have shown previously (7) that TnT isoforms with more acidic NH₂ terminus confer a higher Ca²⁺ sensitivity for the activation of muscle contraction. This is consistent with the higher Ca²⁺ sensitivity of the cTnT-Δ8 thin filament as compared with the cTnT-WT control in myosin S1 ATPase assay (Fig. 11).

Heterogeneity of Myocardial Contractility in the Pathogenesis of Cardiomyopathy and Heart Failure—The incorporation into the turkey cardiac myofibrils renders a functional effect of the cTnT-ΔE8 on the thin filament regulation of contraction. Like that observed in the cTnT point mutations in human familial hypertrophic cardiomyopathy, the mechanism for the mainly quantitative functional changes that result in the pathogenesis of DCM and heart failure needs to be discussed.

A potential mechanism for a quantitative shift in the function of cTnT, including that resulting from the exon 8 erroneous splicing, to cause cardiomyopathy is the sustaining heterogeneity among the thin filament regulatory units resulting from the presence of two or more classes of functionally different vTnT in the adult myocardium. When cTnT undergoes the developmental isoform switching, there is only a transient presence of both embryonic and adult isoforms in the heart (10, 46). In contrast, the sustaining heterogeneity among myofilament contractile units is obviously harmful to the myocardium that needs to contract as a syncytium to maximize cardiac efficiency. Consistently, a single isoform of TnI, TnC, and Tm is present in the cardiac muscle (7). In comparison to the exon 8 splice variant, the two cTnT isoforms in adult human, bovine, rabbit, and mouse hearts resulting from the alternative splicing of exon 4 in the distal NH₂-terminal region would produce significantly less functional heterogeneity in the thin filament, although a potential negative effect cannot be excluded. To support this hypothesis, we have found that the overexpression of a wild type fast skeletal muscle TnT in transgenic mouse cardiac muscle (8) produced myopathic phenotypes (56). Mimicking cTnT mutants with quantitative functional changes, the fast skeletal muscle TnT shows functional differences from the endogenous cTnT in the transgenic mouse cardiac muscle (8). Although both are wild type TnT proteins that function normally in their specified type of muscle environment, the two classes of TnT (cardiac and fast skeletal) co-incorporated into the cardiac thin filaments lead to two classes of troponins slightly differing in their responses to Ca²⁺ signaling. This in turn will desynchronize the activation and inhibition of the contractile units to reduce overall working efficiency of the cardiac muscle. Therefore, the heterogeneity among the myocardial contractile units due to the constant co-expression of wild type and exon 8-deleted cTnT in the turkey heart may be responsible for the pathogenesis of DCM.

This hypothesis is supported by the observations that the cTnT mutants identified in cardiomyopathies, including single amino acid substitutions and sequence truncations, produced various alterations in the thin filament Ca²⁺-regulatory function but rather similar clinical phenotypes in terms of the structure and function of the cardiac muscle (30, 55). A previous study (57) showed that less than 5% incorporation of a COOH-terminal truncated cTnT mutant in the cardiac muscle of transgenic mice produced neonatal lethality. The fact that a mutant TnT can produce a significant pathologic phenotype at these low expression levels supports the hypothesis that it is the heterogeneity of the thin filament regulatory system that results in cardiomyopathy, rather than the quantity of contractile units containing the abnormal TnT. This hypothesis may reflect a common mechanism for the molecular pathology of cTnT and other myofilament protein mutation-induced cardiomyopathies and the development of chronic heart failure during various myocardial diseases and aging where the primary lesions in contractile and signaling proteins result in myocardial heterogeneity.

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