Adult cardiac muscle normally expresses a single cardiac troponin T (cTnT). As a potential pathogenic mechanism for turkey dilated cardiomyopathy, the splice-out of a normally constitutive exon generates an additional low molecular weight cTnT with altered conformation and function. We further found that aberrant splicing of cTnT also occurs in several mammals correlating to dilated cardiomyopathy. Skipping of the same exon as that in the turkey was found in the canine cTnT. Splice-out of the adjacent exon 6 occurred in the guinea pig cTnT. Retention of the embryonic exon 5 was found in the cTnT of cat, dog, and guinea pig. These aberrant splicing variants significantly altered the structure of cTnT to sustain functional effects that as in the myopathic turkey cTnT. The genomic sequence of canine cTnT gene shows no specific alterations. However, the alternative splicing patterns of canine cTnT are different in developing cardiac and skeletal muscles, suggesting abnormality of trans-regulatory factors. Transgenic expression of the aberrant cTnT variants resulted in contractile changes in mouse cardiomyocytes. The findings support the hypothesis that thin filament heterogeneity due to the co-expression of alternatively spliced cTnT variants may desynchronize myocardial contraction and contribute to the pathogenesis and pathophysiology of cardiomyopathy and heart failure.

Troponin T (TnT) is the anchoring subunit of the troponin complex in the thin filament regulatory system of vertebrate striated muscle. Three muscle type-specific TnT isoform genes have evolved in higher vertebrates, and alternative RNA splicing further produces more protein isoforms. Three exons in the cardiac TnT (cTnT) gene may be alternatively spliced. Exon 5 encode 10 amino acids in the NH$_2$-terminal region and is included in the embryonic cTnT but excluded from the adult cTnT (3, 4). The tightly regulated alternative splicing of exon 5 is responsible for the developmental switch from the high molecular weight, more acidic embryonic cTnT to the low molecular weight, less acidic adult cTnT. In addition to exon 5, a few mammals such as bovine (5), rabbit (6), rat (7), and mouse (7) exhibit developmentally independent alternative splicing of the 4 or 5 amino acids encoded by exon 4 in the cTnT gene (7). Similarly, exon 12 encoding for the 3 amino acids located between the T1 and T2 functional fragments of cTnT (8) is also alternatively spliced by a developmentally independent mechanism (9). Although the functional significance of exon 12 region is unclear, the alternative splicing of exons 4 and 5 alters the modulatory NH$_2$-terminal variable region of TnT (10). Inclusion or exclusion of the 10 amino acids encoded by the embryonic exon 5 in cTnT results in embryonic and adult cTnT isoforms with significant changes in activation of the actomyosin ATPase (11). In comparison, alternate splicing of exon 4 results in much smaller structural and functional differences (12). Nevertheless, these observations demonstrate that structural variations in the NH$_2$-terminal region of cTnT can affect the function of cardiac muscle.

In contrast to the fact that most adult avian and mammalian hearts express only a single cTnT isoform, we have found the expression of an additional cTnT splicing variant resulting from the exclusion of 12 amino acids encoded by the exon 8 in the heart of the dilated cardiomyopathy (DCM) turkey (13). This abnormal splicing pathway resulted in a low molecular weight cTnT exhibiting altered molecular conformation and binding affinity for troponin I (TnI) and tropomyosin (Tm). These structure-function changes in turkey cTnT altered the calcium activation of reconstituted thin filaments (13). The pathological role of cTnT structure-function abnormalities in cardiomyopathy and heart failure has been demonstrated by multiple point mutations in cTnT as well as the altered expression of splicing variants (14–19). It is worth noting that all turkeys, including domestic, wild, and the DCM models, constitutively express the abnormal low molecular weight cTnT lacking the exon 8 segment (13). The presence of two classes of cTnT that are different in function will generate heterogeneity in the thin filament regulatory system. Because the myocardium normally contracts as a syncytium, the heterogeneity will desynchronize the myocardial contraction and may be a key factor responsible for the high incidence of DCM in turkeys. This example indicates that abnormalities in cTnT mRNA splicing may play a role in the pathogenesis of DCM.

In the present study, we found that the aberrant splicing of
cTnT also occurs in mammalian hearts that exhibit a high correlation to the spontaneous development of DCM. The canine cTnT demonstrates the skipping of the exon 7 segment, the mammalian equivalent to the avian exon 8 previously found to be excluded from the DCM turkey cTnT. An exclusion of the adjacent exon 6-encoded segment occurs in the guinea pig cTnT. We also found abnormal retention of the embryonic exon 5 in the human, cat, dog, and guinea pig cTnT. The aberrant splicing patterns of canine cTnT and cardiac cTnT are consistent with both the mRNA degradation and misregulated splicing of pre-mRNA. The differences in cTnT expression between the embryonic and the adult muscle indicate the normal developmental regulation of cTnT splicing in the mammalian heart muscle.

Trans-regulatory factors. Transgenic (TG) expression of the aberrantly spliced cTnT variants resulted in functional changes in adult TG mouse cardiomyocytes. The findings support the hypothesis that thin filament heterogeneity due to the co-expression of alternatively spliced cTnT variants may desynchronize myocardial contraction and contribute to the pathogenesis and pathophysiology of cardiomyopathy and heart failure.

MATERIALS AND METHODS

Anti-cTnT Monoclonal Antibody—A mouse monoclonal antibody (mAb) previously developed by immunization with purified bovine cTnT (CT3) (2) and subsequently used in the present study cross-reacts with slow skeletal muscle TnT but not fast skeletal muscle TnT. The distinct mobility of cTnT and slow TnT in SDS-gel electrophoresis allows an easy identification of cTnT in Western blots. The CT3 epitope has been mapped in the COOH-terminal domain of TnT (20).

SDS-PAGE and Western Blotting—Muscle tissues were collected from experimental animals immediately post mortem and stored at −80°C. The frozen or fresh tissues were directly homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 1% SDS. Caution was applied to minimize the time of sample processing to avoid protein degradation. After being heated at 80°C for 5 min, the samples were stored at −80°C until use. The muscle protein extracts were resolved by 14% Laemmli gel with an acrylamide-to-bisacrylamide ratio of 180:1. The gels were stained with Coomassie Blue R-250 to reveal the resolved protein bands. Duplicated gels were transferred onto nitrocellulose membrane for Western blotting using the CT3 mAb as described above.

RESULTS

Aberrant Splicing of Cardiac Troponin T in Dilated Cardiomyopathy

cDNA Cloning and Sequencing—As described previously (13) total RNA from domestic cat, Hartley guinea pig, and Doberman pincher dog ventricular muscle was isolated by TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Two micrograms of the cardiac RNA was used to synthesize cDNA by reverse transcription (RT) using an oligonucleotide primer (5'-TnYV-3') complementary to the beginning of the poly-A tail of mRNAs. Cardiac TnT cDNA was amplified from the total cardiac cDNA by PCR using a degenerative forward primer (5'-CACTATGTCGACCTGCGGCTGGTGGCCT-3') corresponding to the exon 2 sequence in mammalian cTnT covering the translation initiation codon and a degenerative reverse primer (5'-GAATTCATTCTGATGGCTGGTGG-3') synthesized corresponding to the complementary sequence of exon 17 in mammalian cTnT covering the translation stop codon. Restriction endonuclease NdeI and EcoRI cleavage sites were engineered in the underlined sequence for later cloning into a prokaryotic expression vector. Resultant PCR products were purified by agarose gel electrophoresis as described previously (13) and ligated into the pCR4 vector using the TOPO system (Invitrogen). The recombinant plasmid DNA was purified, and the cDNA insert was sequenced by the dyeodeoxy chain termination method.

Expression of Cloned cDNA in E. coli—cDNAs encoding the cTnT splicing variants were isolated by NdeI and EcoRI digestion, subcloned into the pET17b expression vector, and used to transform BL21(DE3)pLysS Escherichia coli cells. 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 (13). After three additional hours of culture the bacterial cells were harvested, lysed in SDS-PAGE sample buffer, and subjected to Western blot as described above.

Two-dimensional Gel Electrophoresis—The total ventricular muscle protein extracts were analyzed by two-dimensional gel electrophoresis as described previously (21). The first dimension was isoelectric focusing (IEF) in Bio-Rad mini tube gels containing pH 3.5–10 Ampholine (Amersham Biosciences). After electrophoresis at 350 V for 16 h and 700 V for 1 h, the IEF gel was equilibrated in SDS-PAGE sample buffer for 10 min and loaded onto a 14% Laemmli slab mini gel with an acrylamide-to-bisacrylamide ratio of 180:1 for the second dimension SDS-PAGE. Five minutes 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 using the CT3 mAb as described above.

Genomic Cloning and Sequencing of the Exon 6 to Exon 8 Region of Canine cTnT Gene—Genomic DNA was isolated from a lymphonodus of a male Doberman pincher dog by proteinase K digestion and phenol/CHCl3 extraction as previously described (13). Two oligonucleotide primers were synthesized for PCR amplification of the segment containing the exon 6 to exon 8 region of the canine cTnT gene. Sequence of the forward primer (5'-GGCTGCGACGACGGACACAGCGG-3') was chosen within the exon 6. Sequence of the reverse primer (5'-GGTTCGACTCTGCCGCCGCGCCCTG-3') was complementary to the exon 8 sequence. By PCR using Pfu DNA polymerase with proofreading activity (Stratagene), a DNA fragment of ~1.2 kb was specifically amplified from the canine genomic DNA. This PCR product was purified by agarose gel electrophoresis, cloned into the pCR4-TOPO plasmid vector, and sequenced as described above.

Sequence Analysis—The cDNA and genomic DNA sequences as well as the deduced protein primary structures were analyzed using the DNASTAR computer program.

Contractility Analysis of Cardiomyocytes Isolated from Transgenic Mouse Hearts Expressing the Aberrant cTnT Variants—As described previously (22), transgenic mice were constructed on a C57BL/6 background using cloned mouse cardiac c-mysinn heavy chain gene promoter (23, generously provided by Dr. Jeffrey Robbins, University of Cincinnati) to direct a heart-specific, post-natal expression of mouse cTnT DNA encoding the high molecular weight embryonic isoform or the exon 7-deleted variant. For reliable functional characterization, segregation of the transgene allele was confirmed for both transgenic constructs during breeding of the founder lines. The expression of the exogenous cTnT in the transgenic mouse hearts was verified by Western blots using the CT3 mAb as above. The relative amounts of exogenous and endogenous cTnT in the transgenic mouse cardiac muscle were determined by densitometric quantification of the Western blots as described above. The incorporation of exogenous cTnT into the cardiac muscle was confirmed by an increase in the resolved protein spots or transferred onto nitrocellulose membrane for Western blotting using the CT3 mAb and TnI and Tm in the transgenic mouse cardiac muscle. Mice of both sexes, 4–5 months old, were used for functional characterization. Wild type C57BL/6 mice of similar age (Charles River) were used as controls.

Cardiomyocytes were enzymatically isolated from the transgenic and wild type mice by perfusion with Ca2+ free Joklik solution containing collagenase and returned to 1.25 mM Ca2+ as described previously (24). Rod-shaped single cardiomyocytes with a sharp outline and clearly visible striations were selected for contractile analysis. The cardiomyocytes were loaded into a close-coupled lucifer yellow dye solution using a Zeiss Axiovert 100 inverted microscope through a heating adapter and continuously superfused with oxygenated physiological buffer containing 132 mM NaCl, 4.8 mM KCl, 12 mM MgCl2, 10 mM HEPES, 5 mM pyruvic acid, 1.8 mM CaCl2, pH 7.2, at 37°C. The cardiomyocytes were paced using a Myopac field stimulator (IonOptix, Milton, MA) to produce trains of 10 Hz at 1, 2, 5, and 10 mmol/L catecholamines (the underlined sequence was the sequence of the cTnT genetic variant of the aberrancy splicing on the cDNA and the cell length by two-edge detection. Data were acquired at a sampling rate of 240 Hz and analyzed by the SoftEdge computer program from IonOptix. The data were exported to the program Excel, and statistical analysis was carried out using Student t test.

RESULTS

Several Mammalian Species Express Abnormally Spliced cTnT Variants—It was documented that a switch from the
cTnT have been submitted to the GenBank/EBI data bank (accession numbers AF519619, AF519620, AF519741, AF519742, AF519743, AF519744, AF519745, AF519746, AY120356, AY120357, AY005140, AY005141, AY005142, and AY005143). 

Western blot analysis of total ventricular muscle homogenates from 11 mammalian species shows the protein integrity in the samples. A duplicate gel was transferred to nitrocellulose membrane for Western blot analysis using the anti-cTnT mAb CT3. The blot in the lower panel shows a single or two slightly different adult isoforms of cTnT in most of the species. Significant expression of high molecular weight cTnT bands was detected in adult monkey, pig, cat, dog, and guinea pig hearts. Multiple low molecular cTnT bands are found in the hearts of the dog and guinea pig. 

Fig. 1. Expression of cTnT variants in mammalian hearts. The upper SDS-PAGE gel of total ventricular muscle homogenates from 11 mammalian species shows the protein integrity in the samples. A duplicate gel was transferred to nitrocellulose membrane for Western blot analysis using the anti-cTnT mAb CT3. The blot in the lower panel shows a single or two slightly different adult isoforms of cTnT in most of the species. Significant expression of high molecular weight cTnT bands was detected in adult monkey, pig, cat, dog, and guinea pig hearts. Multiple low molecular cTnT bands are found in the hearts of the dog and guinea pig. MW, molecular weight.

Aberrant Splicing of Four Exons Generates Two Classes of Abnormal cTnT Variants—Utilizing RT-PCR we cloned cDNAs encoding the two cat, six guinea pig, and six canine cTnT variants detected by Western blots. Sequencing of the cDNAs revealed alternative RNA splicing of four NH2-terminal coding exons in the cTnT gene of the three species. The cDNA sequences of the splicing variants of cat, guinea pig, and dog cTnT have been submitted to the GenBank/EBI data bank with accession numbers AF519619, AF519620, AF519741, AF519742, AF519743, AF519744, AF519745, AF519746, AY120356, AY120357, AY005140, AY005141, AY005142, and AY005143. Protein primary structure maps of the mammalian cTnT variants were constructed from the deduced amino acid sequences and are shown in Fig. 3. Comparison of the cTnT variants demonstrates that the high molecular weight cTnT found in the adult cat heart is the result of the abnormal inclusion of embryonic exon 5 encoding 10 amino acids. The six cTnT variants found in the adult dog heart were due to alternative splicing of exons 4, 5, and 7. In addition to the normal adult isoforms, abnormal inclusion of the embryonic exon 5 was responsible for the two high molecular cTnT variants, whereas the skipping of exon 7 deleted 12 amino acids and produced the low molecular weight cTnT. The exon 7 of mammalian cTnT gene is equivalent to the exon 8 in avian cTnT gene. Therefore, the aberrant low molecular weight dog cTnTs are equivalent to the low molecular weight cTnT found in the DCM turkey heart (13). As seen in the bovine and mouse cTnT, alternative splicing of exon 4 encoding 5 amino acids produced two variants each of the adult, embryonic and exon 7-deleted cTnT. Similar to the dog cTnT variants, the two high molecular weight cTnTs found in the adult guinea pig heart were the result of the abnormal inclusion of the embryonic exon 5. However, unlike the splicing of the dog and turkey low molecular weight cTnT variants, the two low molecular weight cTnTs found in the guinea pig heart resulted from abnormal exclusion of the exon 6-encoded segment (Fig. 3). Inclusion or exclusion of exon 4 encoding 4 amino acids is responsible for the two variants of adult high and low molecular weight guinea pig cTnTs. Both exons 6 and 7 are normally constitutively expressed exons in all cTnTs previously characterized. Exon 6 in the mammalian cTnT genes encodes a large segment (9) corresponding to that encoded by two exons, exon 6 and 7, in the avian cTnT gene (3). Exon 6 of guinea pig cTnT gene encodes for 25 amino acids, and its abnormal exclusion results in a rather large structural change (Fig. 3 and Table I).

Expression of the cloned cDNA encoding the four abnormal dog cTnT variants in E. coli yielded proteins recognized by the anti-cTnT mAb CT3 with sizes identical to those found in the dog cardiac muscle (Fig. 4). The results confirm the authenticity of the cDNA cloning. The physical properties of the alternatively spliced cat, dog, and guinea pig cTnT variants calculated from the deduced primary structures are summarized in Table I. The data demonstrate that the abnormal inclusion and/or exclusion of exons 5, 6, or 7 in the cat, guinea pig, and dog cTnTs resulted in substantial changes in the molecular weight and isoelectric point (pI). Western blot analysis by the mAb CT3 of two-dimensional gel electrophoresis on left ventricular homogenates from the adult cat, dog, and guinea pig hearts further confirmed the changes in both molecular weight and pI of the cTnT splicing variants (Fig. 5) as calculated from the amino acid sequences (Table I). 

Fig. 2. The nature of the abnormal high and low molecular weight cTnT variants. Western blot analysis of total ventricular muscle homogenate using the anti-cTnT mAb CT3 compared the multiple cTnT variants in the adult heart of the cat, pig, dog, and guinea pig with the neonatal mouse and DCM turkey cardiac muscle samples. The alignment shows that the cat, pig, dog, and guinea pig high molecular weight cTnT variants are of similar size to that of the mouse embryonic cTnT. The low molecular weight cTnT variants found in the dog are similar to that in the turkey heart. The guinea pig low molecular weight cTnT variants were the smallest size observed.
the size of the alternatively spliced cTnT variants and their overall charge, confirming that the NH2-terminal structure determines the charge features of TnT (25). The alternative splicing of exon 4 encoding 5 amino acids in dog cTnT and 4 amino acids in guinea pig cTnT further added differences to the structural variations. Fig. 6.

Comparison of the combined effects of exons 4, 5, 6, and 7 on the physical property of canine and guinea pig cTnT. The results demonstrate that the inclusion or exclusion of the 4 or 5 amino acids encoded by exon 4 only results in relatively small changes of pI when exon 7 in dog or exon 6 in guinea pig cTnT is included. However, it produces significantly larger effects when both exons 5 and 7 in dog cTnT or exons 5 and 6 in guinea pig cTnT are excluded. The progressively additive effects of the alternatively spliced NH2-terminal exons on the structure of cTnT suggest that the abnormal exclusion of exon 7 or 6 from adult cTnT results in a critical decrease in the buffering capacity of the protein structure, which significantly reduces the tolerance of the NH2 terminus to structural variation such as the non-developmentally regulated alternative splicing of exon 4.

The relative amounts of the cTnT variants expressed in the adult cat, dog, and guinea pig hearts were determined by densitometric analysis of the Western blots and are summarized in Table II. Alignment of the amino acid sequences encoded by the alternatively spliced NH2-terminal coding exons 4, 5, 6, and 7 in a number of mammalian species demonstrates a high degree of similarity (Fig. 7). The sequence conservation

<table>
<thead>
<tr>
<th>Physical properties of the guinea pig, cat, and dog cTnT variants</th>
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</thead>
<tbody>
<tr>
<td>cTnT Variant*</td>
</tr>
<tr>
<td>Turkey embryonic WTab</td>
</tr>
<tr>
<td>Turkey adult WT</td>
</tr>
<tr>
<td>Turkey adult ΔE6</td>
</tr>
<tr>
<td>Cat embryonic</td>
</tr>
<tr>
<td>Cat adult</td>
</tr>
<tr>
<td>Guinea pig embryonic #1</td>
</tr>
<tr>
<td>Guinea pig embryonic #2</td>
</tr>
<tr>
<td>Guinea pig adult #1</td>
</tr>
<tr>
<td>Guinea pig adult #2</td>
</tr>
<tr>
<td>Guinea pig ΔE6 #1</td>
</tr>
<tr>
<td>Guinea pig ΔE6 #2</td>
</tr>
<tr>
<td>Dog embryonic #1</td>
</tr>
<tr>
<td>Dog embryonic #2</td>
</tr>
<tr>
<td>Dog adult #1</td>
</tr>
<tr>
<td>Dog adult #2</td>
</tr>
<tr>
<td>Dog ΔE7 #1</td>
</tr>
<tr>
<td>Dog ΔE7 #2</td>
</tr>
</tbody>
</table>

*WT, wild type; ΔE, exon deletion; aa, number of amino acids; MW, molecular weight; pI, isoelectric point.

bCalculated using the adult sequences plus the chicken embryonic exon 5 segment, assuming the turkey exon 5 segment is identical to the chicken counterpart.
Aberrant Splicing of Cardiac Troponin T in Dilated Cardiomyopathy

50279

Relative amounts of the alternatively spliced cTnT variants in the adult hearts of cat, dog, and guinea pig

<table>
<thead>
<tr>
<th>cTnT splicing variants</th>
<th>Cat (%)</th>
<th>Dog (%)</th>
<th>Guinea pig (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic #1</td>
<td>26 ± 4.0</td>
<td>38 ± 3.0</td>
<td>13 ± 2.6</td>
</tr>
<tr>
<td>Embryonic #2</td>
<td>N/A</td>
<td>14 ± 3.8</td>
<td>1 ± 1.8</td>
</tr>
<tr>
<td>Adult #1</td>
<td>74 ± 4.0</td>
<td>22 ± 3.8</td>
<td>27 ± 3.0</td>
</tr>
<tr>
<td>Adult #2</td>
<td>N/A</td>
<td>13 ± 1.9</td>
<td>23 ± 1.9</td>
</tr>
<tr>
<td>Abnormal low MW #1</td>
<td>N/A</td>
<td>6 ± 3.8</td>
<td>20 ± 5.8</td>
</tr>
<tr>
<td>Abnormal low MW #2</td>
<td>N/A</td>
<td>7 ± 3.8</td>
<td>7 ± 1.8</td>
</tr>
</tbody>
</table>

* MW, molecular weight; N/A, not applicable. The quantification was done by densitometry analysis of the CT3 mAb Western blots. Each set of the data was from four to five blots. The relative amount (mean ± SD) of each splicing variant is given as the percentage of total cTnT in the cardiac muscle.

indicating the importance of this region in the function of cTnT, suggesting potential effects of the abnormal splicing of these exons on cardiac muscle contraction. Furthermore, a high degree of conservation at the level of nucleotide sequences was found among the abnormally spliced exons in the cat, guinea pig, and dog as compared with the counterparts normally spliced in human, rat, and mouse (data not shown). The conservation of the exon sequences suggests that their aberrant splicing was not due to mutations within the exon sequences.

Genomic Structure of the Dog cTnT Gene in the Regions Flanking Exon 7—a 9-bp deletion was found in intron 7 of the turkey cTnT gene and may contribute to the abnormal skipping of the downstream exon 8 (13). Therefore, we investigated whether the skipping of exon 7 in the dog cTnT gene is due to mutations within the flanking intron sequences. We have cloned and sequenced the dog cTnT genomic DNA fragment from exon 6 to exon 8. The genomic DNA sequence has been submitted to the GenBank™/EBI data bank with the accession number AF119684. There is no closely related genomic sequence available to verify the intron sequence of canine cTnT gene like that available for the comparison between turkey and chicken cTnT genes (13). However, the comparison between canine, human, and rat cTnT genes (Fig. 8) showed no apparent deletion or insertion in the canine gene structure. The consensus splicing boundary sequences in introns 6 and 7 of canine cTnT gene are preserved (Fig. 8). The data suggest that the aberrant splicing of exon 7 in the canine cTnT gene may be different from the splicing of turkey cTnT exon 8 (13) and is not due to the disruption of a cis-regulatory element. However, this hypothesis needs to be further investigated.

**The Expression of Canine cTnT Variants Are Regulated in a Developmental and Tissue-specific Manner**—To investigate the developmental and muscle type-specific regulation of the alternative splicing of canine cTnT gene, we compared the expression of cTnT in adult and neonatal dog cardiac and skeletal muscles. The Western blot in Fig. 9A demonstrates that the alternative splicing patterns of exons 4, 5, and 7 are similar in the four chambers of the adult dog heart, suggesting that the pressure load did not have a major effect on the regulation of the cTnT splicing variants. The splicing patterns in the embryonic dog heart are different from that in the adult. The neonatal dog atria expressed four cTnT variants identical in size to the two embryonic and two adult dog cTnT isoforms and similar to the four cTnT isoforms in the neonatal mouse heart (Fig. 9B). These isoforms are produced by combinations of the non-developmentally regulated splicing of exon 4 and the developmentally regulated splicing of exon 5 observed in the human cTnT (11). However, the neonatal dog ventricle expressed only two cTnT variants (Fig. 9B) with sizes corresponding to those of the embryonic and adult cTnT isoforms with the inclusion of exon 4 (Fig. 3 and Table I). The two low molecular weight cTnTs found in the adult canine heart resulting from the exclusion of exon 7 were not found in the neonatal dog cardiac muscle. Taking advantage of the low level expression of cTnT gene in embryonic skeletal muscle (26), we examined the regulation of cTnT splicing in the neonatal dog gastrocnemius muscle (Fig. 9B). The results demonstrate that, when the cTnT gene is expressed in the neonatal skeletal muscle, exon 7 is excluded, different from its inclusion in the neonatal cardiac muscle. The splicing pathway for exon 4 in the neonatal skeletal muscle was similar to that in the neonatal heart. However, the inclusion of exon 5 was at a much lower level in the neonatal skeletal muscle than that in the neonatal cardiac muscle (Fig. 9B).

Functional Effects of the Aberrantly Spliced cTnT Variants—We have successfully developed transgenic mouse lines...
overexpressing the exon 7-deleted (ΔE7) or high molecular weight embryonic (Emb) cTnT (Fig. 2). In Fig. 10A, SDS-PAGE and Western blots using anti-cTnT mAb CT3 on cardiac muscle from the transgenic mice confirmed the expression of ΔE7 cTnT or embryonic cTnT in the adult heart. Determined by densitometric measurement of the Western blots, the exon 7-deleted and embryonic cTnT were expressed as 84.1 ± 3.3% and 68.8 ± 10.1%, respectively, of the total cTnT in the adult transgenic mouse cardiac muscle. A similar ratio of the exogenous and endogenous cTnT was found in the transgenic cardiac myofibrils, demonstrating an effective incorporation of the aberrantly spliced cTnT into the muscle thin filament (Fig. 10A).

Western blots using mAbs TnI-1 and CH1 demonstrated that cardiac muscle of wild type (WT), Emb TG, and ΔE7 TG mice all exhibit similar expression of cardiac TnI and Tm (Fig. 10B). The transgenic mice expressing embryonic or exon 7-deleted cTnT as the predominant TnT in the adult cardiac muscle provide integrated physiological systems for the functional characterization of the aberrantly spliced cTnT variants. Fig. 10C shows cardiomyocytes isolated from the ventricular muscle of wild type and the transgenic mice overexpressing embryonic or exon 7-deleted cTnT. The cells of transgenic mice are of similar size, striation pattern, and sarcomere length as that observed in wild type cardiomyocytes.

**FIG. 7.** The amino acid sequences encoded by the alternatively spliced exons are conserved. Amino acid sequence alignment of the regions encoded by the alternatively spliced exons 4, 5, 6, and 7 of cTnT genes from multiple mammalian species demonstrates an evolutionary conservation. The exon boundaries are conserved in all known mammalian (human, rat, and mouse) cTnT genes. Residues identical to that in the human cTnT are illustrated as a “*”, and the gaps introduced to maximize the alignment are represented by an asterisk. The human, mouse, rat, rabbit, and bovine cTnT sequences cited were published in Refs. 17, 7, 9, 6, and 5, respectively.

**FIG. 8.** Genomic structure of the exon 6 to exon 8 region of the canine cTnT gene. Oligonucleotide primers derived from the exon 6 and exon 8 sequences of dog cTnT (E6-F and E8-R, respectively) were used for PCR cloning of the flanking genomic DNA segment of the canine cTnT gene. The DNA sequence was used to construct the genomic map. Common restriction enzyme sites are displayed to scale in the map. Uppercase letters denote exon sequences; lowercase letters denote intron sequences. No apparent disruption was found in the splicing boundaries. Compared with the corresponding region of the human (17) and rat (9) cTnT genes, no significant deletion or insertion was found in the canine genomic DNA segment.

**FIG. 9.** Alternative splicing of exons 5 and 7 in canine cTnT is regulated differentially in cardiac and skeletal muscles. A, Western blot examination of cTnT expression using mAb CT3 demonstrated that the alternative splicing patterns of exons 4, 5, and 7 are similar in the four chambers of the adult dog heart. B, the splicing patterns in the embryonic dog heart are different from that in the adult. The low molecular weight cTnT bands seen in the adult heart due to the exclusion of exon 7 were not observed in the neonatal dog cardiac muscle. The cTnT splicing patterns in the neonatal atria and ventricles were also different where exon 4 exclusion only occurred in the atria. When the cTnT gene is expressed in the neonatal skeletal muscle, the splicing of exon 4 and 5 was the same as that observed in the neonatal atria, but exon 7 was spliced out.
Function of the isolated transgenic cardiomyocytes was analyzed by contraction paced at 5 Hz. This frequency of contraction is close to the rate of mouse heart in vivo. Transgenic mouse cardiomyocytes expressing embryonic or exon 7-deleted cTnT exhibited shortening amplitudes similar to that of wild type mouse cardiomyocytes (p = 0.60, Fig. 10D). Isoproterenol had similar positive inotropic effects on the transgenic and wild type cardiomyocytes (Fig. 10D). Although the shortening amplitude of cardiomyocytes in the absence of external loading did not reflect the aberrance in cTnT structure, changes in several contractile parameters are found in the transgenic cardiomyocytes in comparison with the wild type control (Table III). An alignment of the normalized shortening traces of cardiomyocytes from wild type (WT), transgenic mice expressing embryonic (Emb), and exon 7-deleted (ΔE7) cTnT is shown in Fig. 11A to outline the contractile parameters analyzed. Fig. 11B demonstrates that the maximum shortening velocity (V_{max}) of the transgenic mouse cardiomyocytes expressing Emb cTnT was significantly slower than that of the wild type control (p < 0.01) and the ΔE7 transgenic cardiomyocytes. The change in shortening V_{max} is in agreement with a prolonged time to peak shortening. Fig. 11C shows that the maximum re-lengthening velocity of transgenic cardiomyocytes expressing Emb cTnT was also decreased (p < 0.01), in agreement with the longer time from peak shortening to 75% re-lengthening (p < 0.01). This reduced contractile velocity and prolonged time parameters resulting from the expression of Emb cTnT may reflect a negative functional effect of the embryonic cTnT in the adult cardiac muscle. Pacing at 1 and 2 Hz, isoproterenol produced increased shortening and re-lengthening of V_{max} in all three groups (data not shown). Pacing at 5 Hz, isoproterenol treatment resulted in increased shortening and re-lengthening velocity with shortened time parameters in the WT and Emb cTnT transgenic mouse cardiomyocytes (Fig. 12). In contrast, the ΔE7 transgenic cardiomyocytes did not show this positive inotropic response to isoproterenol stimulation. In fact, isoproterenol treatment produced slower V_{max} of shortening and re-lengthening with prolonged time parameters in the transgenic cardiomyocytes expressing ΔE7 cTnT (Fig. 12, B and C). Together with the previously reported effect of TnT isoform expression on isoproterenol-stimulated phosphorylation of cardiac TnI (27), the negative effect of ΔE7 cTnT on the inotropic stimulation of isoproterenol at a near physiological frequency of contraction may indicate the pathogenic role of the deletion of exon 7 segment from the cTnT polypeptide chain.

**DISCUSSION**

We report here the finding and characterization of multiple aberrantly spliced cTnT variants in several mammalian species with a correlation to the development of DCM and heart failure. The observations below suggest the significance of this study.

Abnormal Splicing of cTnT mRNA Occurs in Both Avian and Mammalian Hearts—Most mammals investigated to date express only one class of cTnT in the adult heart. Alternative splicing of exon 4 occurs in a few mammalian hearts such as rabbit, rat, mouse, and bovine (Fig. 1). Exon 4 encodes only 4 or 5 amino acids in the NH2-terminal-variable region (Figs. 3 and 7), and its exclusion produces a relatively small difference in TnT structure (Table I) and function (11, 12, 28). With the exception of the bovine, mammals expressing the exon 4-excluded variant do so at a low level. The exclusion of exon 4 from

![**Fig. 10.**](image)

*Adult transgenic mouse cardiac myocytes expressing the embryonic isoform or ΔE7 cTnT.* A, SDS-PAGE and Western blots using anti-cTnT mAb CT3 on total cardiac muscle and Triton X-100 washed myofibrils (MF) from the transgenic mice (TG) confirmed the expression and myofilament incorporation of the exogenous embryonic (Emb) or exon 7-excluded (ΔE7) cTnT in the adult heart. MW, molecular weight. B, Western blots using anti-TnI mAb TnI-1 and anti-Tm mAb CH1 demonstrate no detectable change in cardiac TnI or Tm expression in the Emb or ΔE7 TG mouse cardiac muscle compared with the wild type (WT). C, cardiomyocytes isolated from wild type and transgenic mice showed similar size and striation pattern. D, the transgenic and wild type mouse cardiac myocytes showed similar shortening amplitude (mean ± S.E.) during paced baseline contraction at 5 Hz (open columns). Isoproterenol treatment (filled columns) resulted in significant increases in both WT and TG mouse cardiac myocytes (**, p < 0.01 as compared with the baseline contraction).

**Table III**

<table>
<thead>
<tr>
<th></th>
<th>Number of cells</th>
<th>Percent shortening</th>
<th>Shortening V_{max}</th>
<th>Time to peak shortening</th>
<th>Re-lengthening V_{max}</th>
<th>Time to 75% re-lengthening</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µm/s</td>
<td>ms</td>
<td>µm/s</td>
<td>ms</td>
<td>µm/s</td>
</tr>
<tr>
<td>WT</td>
<td>14</td>
<td>8.4 ± 0.07</td>
<td>436.2 ± 0.2</td>
<td>65.1 ± 0.3</td>
<td>371.1 ± 0.2</td>
<td>104.4 ± 0.4</td>
</tr>
<tr>
<td>Emb TG</td>
<td>19</td>
<td>8.1 ± 0.07</td>
<td>368.4 ± 0.1^b</td>
<td>71.2 ± 0.2</td>
<td>303.4 ± 0.2^b</td>
<td>115.4 ± 0.2^b</td>
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<tr>
<td>ΔE7 TG</td>
<td>14</td>
<td>8.5 ± 0.06</td>
<td>433.2 ± 0.2</td>
<td>64.1 ± 0.2</td>
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<td>99.4 ± 0.2</td>
</tr>
<tr>
<td>WT + ISO</td>
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<td>10.9 ± 0.08^b</td>
<td>574.3 ± 4.4</td>
<td>70.3 ± 0.6</td>
<td>517.2 ± 3.9^c</td>
<td>111.0 ± 0.6</td>
</tr>
<tr>
<td>Emb TG + ISO</td>
<td>7</td>
<td>12.0 ± 0.11^c</td>
<td>574.1 ± 8.2</td>
<td>75.5 ± 0.5</td>
<td>565.5 ± 11.1^d</td>
<td>117.4 ± 0.6</td>
</tr>
<tr>
<td>ΔE7 TG + ISO</td>
<td>9</td>
<td>11.6 ± 0.11^d</td>
<td>491.0 ± 4.0^d</td>
<td>67.7 ± 0.4^d</td>
<td>436.7 ± 0.4^d</td>
<td>106.2 ± 0.4^d</td>
</tr>
</tbody>
</table>

^a Isoproterenol stimulated shortening is significantly different from baseline shortening (p < 0.01).

^b Emb TG mice are significantly different from WT (p < 0.01).

^c Isoproterenol stimulated is significantly different from baseline (p < 0.05).

^d Isoproterenol stimulated TG is significantly different from isoproterenol stimulated WT (p < 0.05).
the adult human cTnT is responsible for the low molecular weight TnT-4 found in hypertrophic and failing hearts (14), supporting the functional importance of the NH2-terminal structure of TnT. The alternative splicing of exon 5 in cTnT is tightly regulated during development such that it is normally excluded from all adult cTnT (26). No other alternately spliced NH2-terminal exons are normally found in cTnT. We have previously shown that the aberrant splice-out of the exon 8-encoded 12 amino acids from turkey cTnT may correlate to the development of DCM (13), further demonstrating the pathogenic role of cTnT NH2-terminal structure variation. In the present study, we report that the monkey, cat, pig, guinea pig, and dog hearts express two or more cTnT variants due to aberrant splicing of exons 5, 6, and 7 (Figs. 2 and 3 and Table I). The results demonstrate that abnormal splicing of cTnT also occurs in mammalian hearts. In addition to the splicing pathway observed in the turkey cTnT, abnormal inclusion of the embryonic exon 5 in the adult cTnT and exclusion of the large exon 6 were found for the first time. Exons 4, 5, 6, and 7 (mammalian exon 7 is equivalent to the avian exon 8) all encode amino acids within the NH2-terminal domain of TnT (10). The aberrant splicing of these NH2-terminal exons results in significant changes in molecular weights and pl of cTnT (Table I). It has been shown by a number of previous studies that structural changes in this region of TnT have significant functional effects (11, 12).

The Correlation of Aberrant cTnT Splicing with Dilated Cardiomyopathy—It is a striking fact that three of the six species observed to have abnormal splicing in cTnT have been described as genetic models of spontaneous DCM and heart failure. The turkey (29–31), cat (32–34), and dog (35–37) all exhibit a high instance of inherited DCM. Although the monkey heart has not been extensively studied, it is worth noting that spontaneous cardiomyopathies in pig and monkey have been documented (38, 39). Pig and guinea pig are also readily inducible models of heart failure (40). Therefore, the correlation between the aberrant splicing of the NH2-terminal region of cTnT, which has been established as a modulatory domain of the molecule (10, 41), with DCM is unlikely to be a coincidence. It is important to note that, except for the Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermore, Western blot analysis on ventricular muscle from Doberman pincher hearts with end-stage DCM, the heart samples investigated in the present study were all acquired from individuals with no clinical evidence of heart disease, indicating the aberrant splicing was a primary change. Furthermor...
regulated, and the aberrant exclusion of exon 8 is constitutive in turkey cardiac and skeletal muscles (13). In contrast, in the canine cTnT the expression of exon 5 is no longer tightly regulated during development, and the exclusion of exon 7 is not a constitutive event (Fig. 9). The differential splicing of canine cTnT mRNA in embryonic cardiac, embryonic skeletal, and adult cardiac muscles indicates that the abnormal exclusion of exon 7 is unlikely based on cis-mutations in the cTnT gene structure as proposed for the turkey cTnT exon 8. This is in agreement with the apparently normal genomic structure in the corresponding region of the canine cTnT gene (Fig. 8). On the other hand, the developmental regulation and tissue specificity of canine cTnT exon 7 splicing suggest abnormalities in cellular trans-regulatory factors.

Although the adult canine cTnT retained an abnormally high level of the inclusion of the embryonic exon 5, it was downregulated slightly during postnatal development (Fig. 9B). This splicing pattern suggests that the developmental regulation was not completely lost but significantly weakened. Because no change in the purine-rich positive signal sequence in exon 5 was noted (42) and the intron splicing boundaries remained conserved (Fig. 8), this quantitative loss of exon 5 regulation during development supports abnormalities of trans-acting factors. We have previously shown that the developmentally regulated splicing of exon 5 is normally synchronized in cardiac and skeletal muscles (26). Therefore, the desynchronized splicing of cTnT exon 5 in canine atrial, ventricular, and skeletal muscles supports the role of tissue-specific trans-regulatory factors. It is less likely that two concurrent mutations in the same gene resulting in abnormalities in the splicing of exons 5 and 7. In contrast, the opposite changes in exon 5 and exon 7 splicing are better explained by an imbalance between the positive and negative trans-acting splicing factors (43). It is worth noting that the inclusion of exon 4 is favored in the embryonic dog muscles, especially the ventricles, as compared with the adult heart (Fig. 9B). Considering that the splicing of exon 4 is normally a non-developmental regulated event, the differential inclusion of exon 4 in the different dog muscle tissues during development also supports the presence of abnormalities in trans-acting splicing factors.

**Functional Effect of the Aberrantly Spliced cTnT Variants**—Alternative splicing of exons 4 and 5 in cTnT has been shown to affect the Ca$^{2+}$ sensitivity and cooperativity of the myofilament (12). A recent study demonstrated the effects of the four human cTnT variants produced by combinations of alternative splicing of exons 4 and 5 on Ca$^{2+}$ regulation and inhibition of force development (11). These investigators demonstrated that inclusion of exon 5 in cTnT reduced the ability of troponin to inhibit actomyosin ATPase and resulted in less relaxation of *in vitro* reconstituted muscle fibers. By analyzing transgenic

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**FIG. 12.** Isoproterenol-induced changes in contractile parameters of the transgenic mouse cardiomyocytes. *A*, representative normalized shortening traces of cardiomyocytes from wild type (WT) and transgenic (TG) mice expressing embryonic (Emb) or exon 7-deleted (ΔE7) cTnT upon 10 nM isoproterenol treatment are shown. *B*, isoproterenol treatment resulted in increased maximum shortening velocity with shortened time parameters in the WT and Emb cTnT transgenic mouse cardiomyocytes. In contrast, isoproterenol treatment produced slower $V_{\text{max}}$ of shortening with prolonged time to peak shortening in the transgenic cardiomyocytes expressing ΔE7 cTnT. *C*, isoproterenol treatment resulted in increased maximum re-lengthening velocity with a shortened time parameter in the WT and Emb cTnT transgenic mouse cardiomyocytes but slower $V_{\text{max}}$ of re-lengthening with prolonged time parameter in the transgenic cardiomyocytes expressing ΔE7 cTnT. *a*, significantly different from baseline, $p < 0.05$; *b*, TG significantly different from WT, $p < 0.05$. Data are presented as mean ± S.D.
mouse cardiomyocytes, our results demonstrate that the expression of embryonic cTnT containing the exon 5 sequence produced slower $V_{\text{max}}$ of shortening and re-lengthening (Fig. 11), consistent with a reduced regulatory activity. These data support the notion that the abnormal expression of high levels of embryonic cTnT in the adult heart may have a negative effect on cardiac function and, therefore, constitutes a pathogenic factor in the development of DCM and heart failure.

The mammalian cTnT exon 7 abnormally excluded from the DCM canine cTnT is equivalent to the avian cTnT exon 8 that has been found in the turkey model of DCM (13). Exclusion of the 12 amino acids encoded by avian exon 8 resulted in significant changes in the molecular conformation of cTnT, interactions with TnI and Tm, and Ca$^{2+}$ sensitivity of the thin filament (13). The lack of a significant difference in the shortening amplitudes between the ΔE7 TG and WT mouse cardiomyocytes (Fig. 10) is consistent with the previous result that reconstituted thin filaments containing exon 8-deleted turkey cTnT exhibited no difference in maximal ATPase activity as compared with wild type control (13). The potential change in Ca$^{2+}$ sensitivity by the exclusion of exon 7 from the mammalian cTnT was not detected in the analysis of intact cardiomyocytes. This is likely due to the limitation of data sampling rate during the fast initial phase of cell shortening (Fig. 11). Nevertheless, we show that the transgenic mouse cardiomyocytes expressing ΔE7 cTnT failed to increase shortening and re-lengthening velocity upon isoproterenol stimulation (Fig. 12). This effect of exon 7 deletion is consistent with the diminished response of DCM turkey cardiac muscle to β-adrenergic stimulation (46). Therefore, the abnormal exclusion of exon 7 in canine cTnT may also constitute a negative effect on myocardial function, especially in limiting the positive inotropic po-

The evolutionary fixation of these potentially pathogenic cTnT alternative splicing pathways in these avian and mammalian species remains to be investigated. One hypothesis to be tested is that the thin filament heterogeneity in the cardiac muscle might produce a functional advantage as in the fast skeletal muscle that normally expresses multiple TnT isoforms (25). However, this advantage would be based on a higher energetic cost leading to the subsequent development of cardiomypathy. This short term benefit during the reproductive age may confer a selection value to allow the fixation of this trait in the species, whereas the selection against post-reproductive individuals through the late onset of DCM and heart failure reduces competition for resources and adds to the selection value.

**The Potential Pathogenic Role of Myocardial Heterogeneity**—

Previous studies have shown that the switch in the alternatively spliced isoforms of human cTnT relates to the development of heart failure (14, 15). Although the TnT-T4 expression in human cTnT (14, 15) could be secondary and compensatory to the heart failure conditions, it lends support to the role of the cTnT NH$_2$-terminal variation in modulating myocardial function. The aberrant splicing of exon 8 in turkey cTnT and exons 5 and 7 or 6 in dog and guinea pig cTnT occurs as primary changes, indicating a cause or precondition for the development of DCM and heart failure in these animals (13, 32–37). With the much larger structural and functional changes resulting from the abnormal splicing of exons 5 (11) and 7 (13), the cat and dog cTnT variants are likely to have greater functional effects than the human TnT-4. This prediction is supported by the recent study on human cTnT isoforms (11) and our analysis of transgenic mouse cardiomyocytes overexpressing Emb or ΔE7 cTnT to largely replace the endogenous wild type adult cTnT. The integrated mechanism for the cTnT splicing variants to cause DCM deserves further investigation. The aberrant splicing occurs in the modulatory NH$_2$-terminal variable region of TnT (Fig. 3) and the alterations do not destroy the core structure (44, 45) and basic activity (13) of TnT. In an isolated system such as the reconstituted myofilaments, the exclusion of exon 8 from turkey cTnT even produced higher Ca$^{2+}$ sensitivity in comparison with the wild type control (13). Therefore, it seems not the loss of overall activity of cTnT that confers the pathogenic effect. We have proposed a hypothesis that cardiomyopathy may result from the heterogeneity generated due to the presence of more than one functional class of TnT in the cardiac muscle. During the activation and relaxation of cardiac muscle, multiple classes of troponin with varying Ca$^{2+}$ sensitivities and/or interactions with other thin filament proteins will act heterogeneously during the contractile cycle. It is well known that the myocardium needs to contract as a syncytium to maintain high efficiency. Compared with the normal cardiac thin filament containing only one class of cTnT, the thin filament containing multiple cTnT variants will be activated over a wider time frame with lowered peak activity. This desynchronizing effect will not only lower the contractile force of the cardiac muscle at peak activation but also prolong the time of relaxation. The myocardial heterogeneity is clearly harmful to the function of the heart by causing decreased energetic efficiency. In comparison to the two cTnT variants in the adult turkey heart (13), the expression of multiple low and high molecular weight cTnT variants in the dog and guinea pig hearts would produce multiple classes of thin filament regulatory units in the cardiac muscle, resulting in myocardial heterogeneity to a much greater degree. A dose correlation between TnT heterogeneity and myocardial pathogenesis is supported by the fact that, in contrast to the turkey DCM that often requires an induction by furazolidone (46), many canine breeds, such as the Doberman pincher (32), Boxers (36), Portuguese water dogs (47), and Irish wolfhounds (48), have high rates of spontaneous DCM and heart failure.

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