Expression and Functional Assessment of a Truncated Cardiac Troponin T That Causes Hypertrophic Cardiomyopathy

Evidence for a Dominant Negative Action

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Abstract

Mutations in the β-myosin heavy chain gene are believed to cause hypertrophic cardiomyopathy (HCM) by acting as dominant negative alleles. In contrast, a truncated cardiac troponin T (TnT) that causes HCM implies that altered stoichiometry of contractile proteins may also cause cardiac hypertrophy. Wild-type and HCM-mutant (truncated) TnT were studied in a novel quail myotube expression system. Unexpectedly, antibody staining demonstrated incorporation of both forms of human cardiac TnT into the sarcomeres of quail myotubes. Functional studies of wild type and mutant transfected myotubes of normal appearance revealed that calcium-activated force of contraction was normal upon incorporation of wild type TnT, but greatly diminished for the mutant TnT. These findings indicate that HCM-causing mutations in TnT and β-myosin heavy chain share abnormalities in common, acting as dominant negative alleles that impair contractile performance. This diminished force output is the likely stimulus for hypertrophy in the human heart. (*J. Clin. Invest.* 1996. 98:2456–2461.) Key words: hypertrophic cardiomyopathy • troponin T • contractility • cardiac muscle • cardiac mechanics

Introduction

Genetic analyses have shown that hypertrophic cardiomyopathy (HCM)[1] is an autosomal dominant disorder of heart muscle that results from mutations in genes encoding sarcomeric proteins (1). Six different disease genes have been identified: the β-myosin heavy chain (2), the cardiac myosin essential light chain (3), the cardiac myosin regulatory light chain (3), α-tropomyosin (4), cardiac troponin T (4), and cardiac myosin-binding protein C genes (5, 6). That mutations in these diverse components of the sarcomere appear to produce the same phenotype suggests that the mutations share functional consequences that lead to a common mode of pathogenesis.

Many different mutations in the β-myosin heavy chain gene cause HCM, but all are missense mutations clustered in the head and head–rod junctional region of the peptide (5, 7). No null alleles have been shown to segregate with disease; one documented null allele was associated with no discernible phenotype (8). Therefore, by analogy to *unc-54* mutations in *C. elegans* (9), these data suggested that the dominant phenotype of the β-MHC mutations results from a dominant-negative effect. Functional analyses of mutant β-MHC peptides have supported this model; a mixture of mutant and wild-type peptides showed the mutant phenotype to be dominant when present at equal levels (10, 11). The consequence of these mutations is diminished power production by muscle cells expressing the mutant myosins, which likely creates a stimulus for hypertrophy (12).

The more recently identified mutations in cardiac troponin T (cTnT) and alpha-tropomyosin that have been demonstrated to cause HCM are missense mutations, with one exception (13). One of the TnT mutations results in abnormal splicing: the $Int15^{G\rightarrow A}$ mutation inactivates a 5′ splice donor site leading to either skipping of exon 15 or activation of a cryptic splice site; both aberrant cDNAs encode a truncated TnT peptide lacking the conserved carboxyl terminus. Based on analogy to a similar mutation previously found in *Drosophila melanogaster* which encodes no stable TnT peptide (14), the $Int15^{G\rightarrow A}$ mutation was predicted to result in a functional null (4). If this were the case, then the pathogenesis would likely be via a different mechanism than that of the myosin mutations, with important consequences for further investigation and potential intervention in this condition. Moreover, this finding suggested altered stoichiometry of sarcomere components might be of importance in secondary forms of cardiac hypertrophy (4).

Although the notion that the functional consequences of altered stoichiometry differ for TnT and myosin heavy chain has been discussed (4, 15), it has not been possible to test this hypothesis directly. As cardiac TnT is only expressed in cardiac muscle it has not been possible to determine whether the mutant protein is stably expressed in patients with the $Int15^{G\rightarrow A}$ mutation. Therefore, to assess whether this trun-

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1. *Abbreviations used in this paper:* cTnT, cardiac troponin T; HCM, hypertrophic cardiomyopathy; Int, intron.

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cated TnT can assemble into the thin filament, a novel expression system and functional assay was devised. Wild type and truncated human cTnT were expressed in quail myotubes, which were examined microscopically as well as assayed for calcium activated force production. Surprisingly, both the wild type and truncated cTnT were incorporated into the thin filaments of sarcomeres. However, while incorporation of wild type cTnT resulted in myotubes that generated normal levels of calcium activated force per cross-sectional area, the truncated cTnT greatly inhibited force generation. In experiments where both wild type and mutant human cTnT were co-expressed, maximum calcium activated force per cross-sectional area was 10–100% of control levels. Thus the truncated cTnT does not function as a null protein, but rather as a dominant-negative leading to reduction in the level of calcium activated force production. The mechanism by which HCM is induced is therefore analogous to the myosin heavy chain mutations that have been characterized; namely, diminished force and/or power output at a given level of activation which likely reflexly elicits a genetic program of hypertrophy.

Methods

Expression constructs. Human cardiac TnT cDNA was obtained by RT-PCR of mRNA from normal heart tissue. To include the transcription start site, and to facilitate sub-cloning, a linker consisting of residues 5–43 with an EcoRI site added at the 5′ end was ligated to the amplified cDNA cleaved at position 43 by digestion with RsaI. An EcoRI–BglII fragment, containing residues 5–900 of the cDNA was then subcloned into the pSG5 expression vector (Stratagene Inc., La Jolla, CA) and the nucleotide sequence of the insert confirmed. The mutant cDNA with exon 15 skipped was similarly amplified from an affected member of Family AU bearing the $Int15^{G\rightarrow A}$ mutation. A PstI–BglII fragment of the mutant cDNA (residues 764–900) was substituted for the corresponding fragment in the wild-type subclone and the nucleotide sequence confirmed. Integrity of both wild-type and mutant cDNA subclones in pSG5 was confirmed by expression in COS 7 cells followed by western blot analysis of protein extracts.

For the myocyte transfection experiments an eukaryotic expression vector (denoted pMLC) was derived from pRC/RSV (Invitrogen Corp., San Diego, CA) by substituting the chicken myosin essential light chain promoter; this vector contains a neomycin resistance gene as a selectable marker. Each TnT cDNA subclone was transferred to the pMLC vector; the inserts thus contained the full TnT coding sequence, and the polyadenylation signal from the PSG5 vector (see Fig. 1). The resultant clones were denoted pMLC.WT (wild-type) and pMLC.AuS (for the truncated cDNA lacking exon 15).

Myocyte transfection. Primary quail myocytes were isolated from the pectoralis muscles of day 8 quail embryos. Cells were plated onto a 35-mm collagen- and MATRIGEL-coated dish at a density of 1×10^6 cells. 24 h later, the cells were transfected, by calcium phosphate co-precipitation, with either 2.5 μg of the pMLC plasmid with no insert (control), pMLC.WT (wild-type) or pMLC.AuS (mutant). For experiments involving co-transfection of wild-type and mutant TnT, 1.25 μg of each of the pMLC.WT and pMLV.AuS plasmids were used in transfections. Selection in the presence of G418 and BuDR (which has been shown to block differentiation) took place for seven days. BuDR was then withdrawn and cells began to fuse. 8 d after withdrawal of BuDR, differentiated myotubes had formed and were examined by immunostaining and were prepared for measurements of force production.

Immunostaining. A monoclonal anti-Troponin T (mouse IgG1 isotype, Clone JLT-12; Sigma Immuno Chemicals, Sigma Chemical Company, St. Louis, MO) was used in immunoflourescence assays to detect human cardiac troponin T expression in quail myotubes. Cultures were rinsed with PBS and fixed for 3 minutes in 2% formaldehyde. The cells were then permeabilized and soluble proteins extracted using 0.5% Triton X-100 in PBS. The primary antibody was incubated in a humidified chamber at 37°C for 90 min, and the secondary antibody (an affinity-purified rhodamine-conjugated goat anti–mouse IgG; Jackson ImmunoResearch Laboratories Inc., Avondale, PA) was incubated for 60 min. Preparations were examined and photographed by a Leitz DMR microscope (Leica, Wetzlar, Germany).

Functional assessment. Myotubes were isolated from culture dishes of control, wild-type and mutant transfection groups by replacing medium with permeabilizing solution (170 mM potassium propionate, 5 mM EGTA, 2.5 mM MgCl2, 2.5 mM ATP, 10 mM imidazole, 0.2 mM PMSF, pH 7.0) at 4°C overnight. The cultures were subsequently stored in 170 mM potassium propionate, 5 mM EGTA, 2.5 mM MgCl2, 2.5 mM ATP, 10 mM imidazole, 1 mM NaN3, 2.5 mM glutathione, 50% glycerol, pH 7.0) at −20°C. A single permeabilized myotube was mounted with miniature aluminum "T-clips" between a post and a force transducer (Series 400A Force Transducer System; Cambridge Technology, Inc., Watertown, MA) in order to measure the isometric force. The entire set-up was mounted on the stage of a Leitz Fluovert equipped with interference contrast objectives for direct observation of sarcomere patterns and for the determination of cross-sectional area (based on measuring the width and depth of the myotube). Ca^{2+}-containing experimental solutions for force measurements were either relaxing levels (10^{-8} M) or maximal activation ($10^{-4.3}$ or $10^{-4.0}$ M).

Western blots. Protein extracts from (pooled) permeabilized myotubes were analyzed by Western blotting. One-dimensional denaturing SDS-PAGE (10% polyacrylamide) was followed by electrophoretic transfer to Immobilon P (Millipore Corp. Bedford, MA) membrane. The resulting transfers were probed with either of two anti-TnT mouse monoclonal antibodies (clone JLT-12; Sigma Chemical Co. [does not react with quail TnT] or clone T1/61; Serotec Ltd., Oxford, UK [recognizes all TnT isoforms]), followed by a secondary antibody conjugated with horseradish peroxidase. Visualization was via diaminobenzidene staining (Sigma Chemical Co.).

Results

Expression of human cardiac TnT on a quail sarcomere background. A primary quail myocyte culture was used in which both sarcomere incorporation and function of cloned human cardiac TnT cDNAs could be assessed. Primary quail myocytes were chosen because they survive long enough in primary culture to allow selection before the formation of myotubes and because the mature myotubes display an orderly sarcomere structure. To achieve differing levels of expression of the human cardiac TnT peptides, vectors with two different promoters were created: a chicken myosin essential light chain (MLC) promoter (Fig. 1) to drive physiologic levels of expression and a CMV promoter to drive higher levels of expression. To determine whether the HCM-causing truncated TnT could be incorporated into the sarcomere, transfection and selection conditions were designed to ensure uniform and near-complete replacement of the native quail TnT with the transfected human TnT. To document the relative levels of the different TnT peptides, one dimensional SDS-PAGE and Western blotting was performed (Fig. 2 *a*); the cardiac and endogenous quail TnTs could be distinguished by position on the blot and by differential antibody reactivity. Two different antibodies were used to probe the blots; a TnT antibody that recognized only the human cardiac TnT (Sigma Chemical Co.) and an avian TnT antibody that cross-reacted with the human cardiac TnT (Serotec Ltd.). In transfection experiments with both the pMLC and pCMV vectors the human cardiac TnT was shown

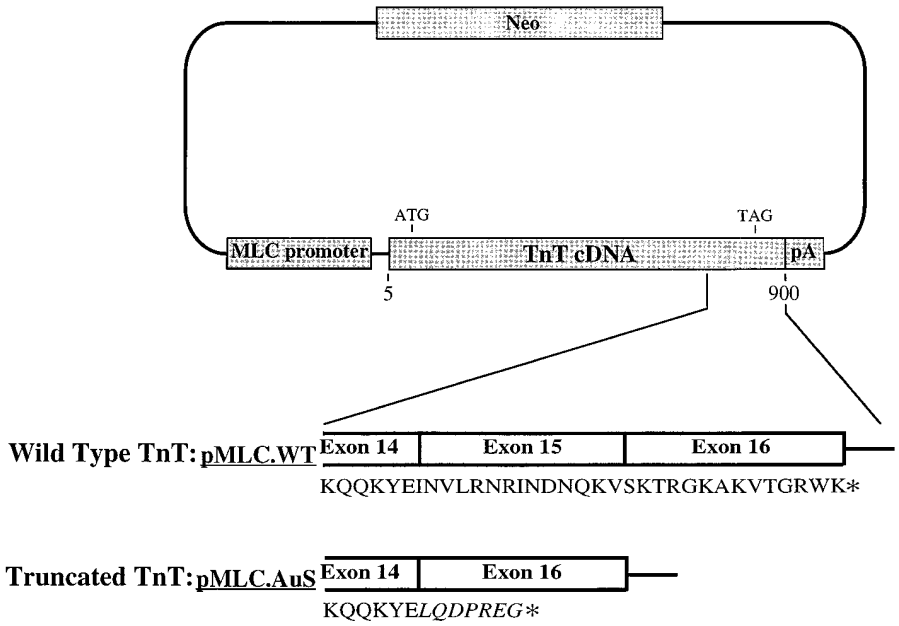

Figure 1. Structure of the human cardiac troponinT expression vectors. One vector, designated TnT:pMLC.WT encoded the wild-type cardiac troponinT cDNA and the other vector, designated TnT: pMLC.AuS, encoded a mutant, truncated cardiac troponin T that lacks exon 15 and reads exon 16 in the wrong frame. A full-length human cardiac troponinT (TnT) cDNA was cloned 3′ of the myosin light chain promoter element (MLC promoter). The cardiac troponinT cDNA extends from residue 5 to 900 of the cDNA sequence described previously (4), and contains an initiation methionine codon (ATG), a termination codon (TAG) and a polyA addition signal (pA). The amino acid sequences (indicated by the single letter designation; an asterisk indicates stop codon) encoded by exons 14, 15 (wild type only) and 16 are shown for each vector. Both vectors contain a gene (Neo) encoding a protein that confers resistance to the drug G418 to mammalian tissue culture cells.

to be the predominant TnT expressed in the myotubes. Replacement was such that endogenous TnT levels were undetectable by Western blotting of permeabilized myotube extracts for either the pMLC (Fig. 2 *a*) or pCMV (not shown) driven constructs. For myotubes expressing either wild type or truncated human cTnT, one dimensional SDS-PAGE revealed normal troponin I and troponin C stoichiometry (not shown), indicating that the troponin complex was assembled.

Sarcomere assembly with wild-type and truncated TnT. Under these conditions the ability of the transfected TnT peptides to assemble into sarcomeres was assessed by immunostaining of individual myotubes. An antibody to rat skeletal muscle TnT (JLT12; Sigma Chemical Co.) did not recognize the native quail TnT sufficiently to reveal sarcomeres (Fig. 2 *b*). Immunostaining of myotubes transfected with wild type human cardiac TnT revealed an orderly sarcomere structure (Fig. 2 *b*); this normal sarcomere appearance was achieved with both the MLC and CMV promoters. Transfection with the truncated TnT plasmid with the MLC promoter also produced a majority of myotubes with an orderly sarcomere pattern which were indistinguishable from wild-type transfected cells (Fig. 2 *b*). However, ~ 21% of mutant-transfected cells displayed regions of disordered architecture with collapse of sarcomeres (Fig. 2 *b*). In experiments that involved cotransfection of vectors driving expression of wild type and mutant human cTnT (under the MLC promoter), ~ 14% of the myotubes displayed regional disorder. In cells transfected with the truncated cTnT driven by the CMV promoter sarcomere collapse was almost universal.

Contractile properties of myotubes with wild-type and truncated cardiac TnT. After permeabilization, individual myotubes were isolated and mounted for analysis of the contractile response to activating levels of calcium (Fig. 3). Maximum isometric force was not different between control myotubes transfected with vector alone (1.45±0.18 kg/cm^2) and myotubes transfected with wild-type human cardiac TnT (1.32± 0.05 kg/cm^2). Thus the substitution of the human cardiac TnT for the native quail TnT did not detectably alter the maximal force of contraction. However, myotubes expressing the truncated TnT driven by the MLC promoter, and selected for intact sarcomere appearance, generated considerably reduced maximum isometric force (0.30±0.08 kg/cm^2). Myotubes with any visible disruption of sarcomere architecture generated no sustained force (Fig. 3 *b*). In an experiment that involved transfection of vectors driving expression of both wild type and mutant human cTnT, the maximal calcium activated force varied greatly among myotubes (Table I) and was over a range of 10–100% of control force levels. While it was not technically possible to quantify the relative amounts of human cardiac wild type and the human cardiac mutant TnT incorporated into each myotube, Western blots of pooled myotubes revealed nearly equal levels of wild-type and mutant cTnT in the overall myotube population (Fig. 2 *a*, lane *6*).

Discussion

The assay described here has been designed to allow selection and analysis of transfected myocytes in which the quail TnT has been effectively replaced by the human cardiac TnT. These analyses demonstrate that, in contrast to the analogous *Drosophila* mutation, the truncated cardiac TnT that causes HCM does not act as a null allele; the mutant TnT is sufficiently functional to be incorporated into troponin complexes within the sarcomere. Sarcomeres containing the truncated TnT produce a much diminished maximum force of contraction. This abnormality is specific to the mutant cardiac TnT; the wild-type human cardiac TnT produces contractile responses indistinguishable from the native quail myocytes transfected with vector alone. We conclude that the Int15$^{G\rightarrow A}$ mutation acts as a dominant negative allele, blocking full calcium activation of the thin filament.

Under the study conditions used, the replacement of the

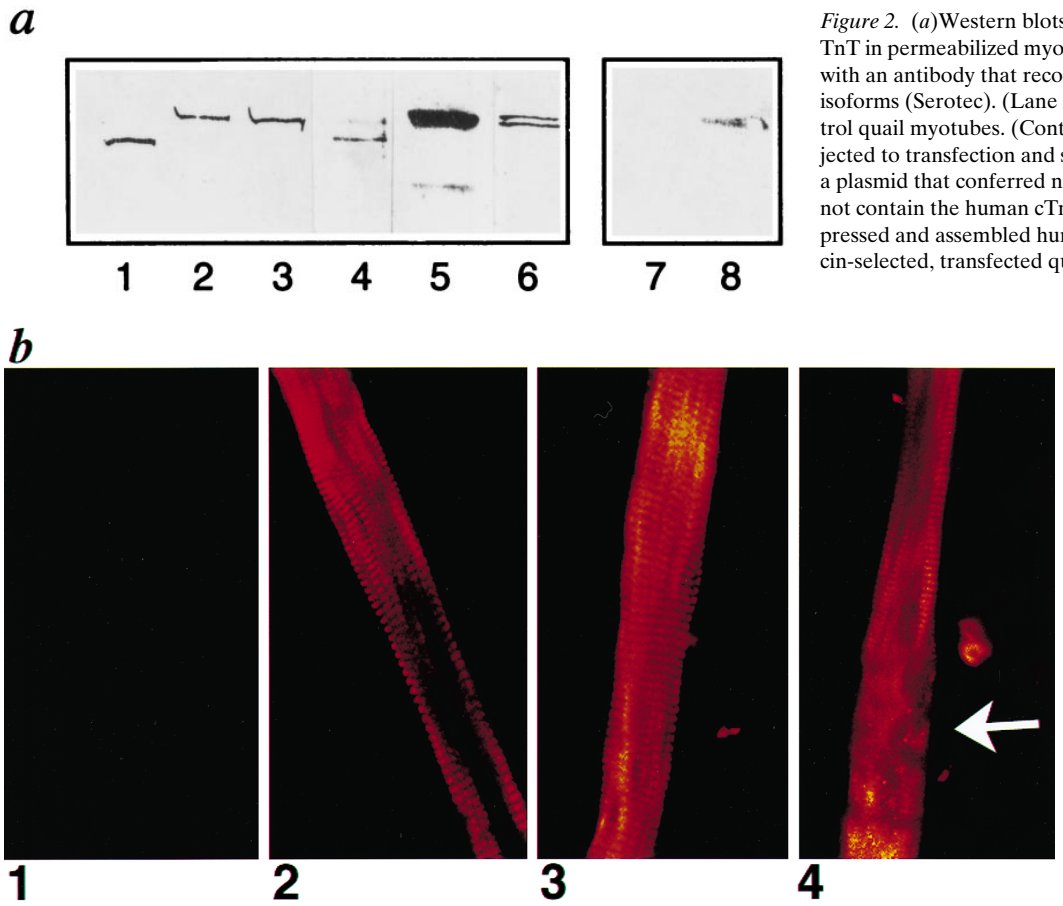

Figure 2. (*a*)Western blots showing relative levels of TnT in permeabilized myotubes. Lanes *1–6* are probed with an antibody that recognizes all vertebrate TnT isoforms (Serotec). (Lane *1*) Native quail TnT in control quail myotubes. (Control myotubes were subjected to transfection and selection protocols, but with a plasmid that conferred neomycin resistance but did not contain the human cTnT cDNA.) (Lane *2*) Expressed and assembled human cardiac TnT in neomycin-selected, transfected quail myotubes (transfected with pMLC.WT). Note loss of endogenous TnT incorporation. (Lane *3*) Expressed and assembled truncated (HCM mutant) human cardiac TnT in neomyocin-selected, transfected quail myotubes (transfected with pMLC.AuS). Note loss of endogenous TnT incorporation. (Lane *4*) Co-expression and incorporation of endogenous quail TnT (lower band) and human cTnT (faint upper band) in myotubes after transfection of wild type human cTnT expressing plasmid (pMLC.WT) into cultures in the absence of neomycin selection. (Lane *5*) Purified human cardiac TnT (lower band is proteolytic breakdown product). (Lane *6*) Co-expression and incorporation of wild-type (upper band) and mutant (lower band) human cTnT in myotubes after co-transfection of wild type and mutant human cTnT expressing plasmids (pMLC.WT and pMLC.AuS) into neomyocin-selected quail myotubes. Lanes *7* and *8* are probed with an antibody that recognizes human cardiac, but not quail, TnT isoforms (Sigma Chemical Co.). (Lane *7*) Homogenate of quail myotubes. (Lane *8*) Purified human cardiac TnT. (*b*) Demonstration of incorporation of wild type and mutant TnT into sarcomeres. All myotubes were probed with an antibody that recognizes human cardiac, but not quail, TnT isoforms (Sigma Chemical Co.). (*b1*) Control quail myotubes, unstained because of lack of antibody reactivity. (Control myotubes were subjected to transfection and selection protocols, but with a plasmid that conferred neomycin resistance but did not contain the human cTnT cDNA.) A force record from such a myotube is shown in Fig. 3 *a1*. (*b2*) A quail myotube demonstrating expressed (MLC promoter) and assembled human cardiac TnT from a neomycin-selected, transfected culture. Note the regular sarcomeric pattern which is seen in 100% of myotubes expressing the wild type human cTnT. A force record from such a myotube is shown in Fig. 3 *a2*. (*b3*) A quail myotube demonstrating expressed (MLC promoter) and assembled mutant (truncated) human cardiac TnT from a neomycin-selected, transfected culture. Note the regular sarcomeric pattern, which is seen in ~ 79% of myotubes expressing the truncated cTnT. A force record from such a myotube is shown in Fig. 3 *a3*. (*b4*) An example of quail myotube demonstrating expressed (MLC promoter) and assembled mutant (truncated) human cardiac TnT but with focal disruption (*arrow*) of the regular sarcomeric pattern. Focal disruptions are seen in ~ 21% of the myotubes expressing the truncated cTnT. A force record from such a myotube is shown in Fig. 3 *a4*.

native TnT with the transfected mutant TnT is essentially complete; thus the myocytes studied are functionally homozygous for the mutant TnT peptide. This analysis is appropriate to demonstrate that the truncated TnT does not function as a null allele and to illustrate the impaired contractile function of the mutant peptide, but does not reproduce the heterozygote state. We do not know at present the proportion of mutant TnT incorporated into the sarcomeres in individuals with HCM, but most probably wild-type protein will constitute at least 50% of the total. The reduction of force production may be less marked in this situation. Indeed the profound loss of function in the 100% mutant TnT sarcomeres contrasts with the apparently preserved myocardial function of patients heterozygous for this TnT mutation. The effect on force production of expression of both wild-type and truncated TnT as assessed in the myotubes listed in Table I suggests that a mixture of wild type and mutant TnT will lead to diminished force production, the extent of which is likely dependent on the relative amounts of mutant TnT.

The mechanism whereby the truncated cardiac TnT peptide leads to diminished contractile force in the quail myotubes remains to be elucidated. Because of the dramatic loss of maximum force generated it has not yet been possible to explore the response to partially activating calcium concentrations to determine whether the response curve is simply depressed or if the pCa_{50} also is shifted. Given that sarcomeric structures appear normal at both the light and electron microscopic levels, and that there is uniform depression of contractile perfor-

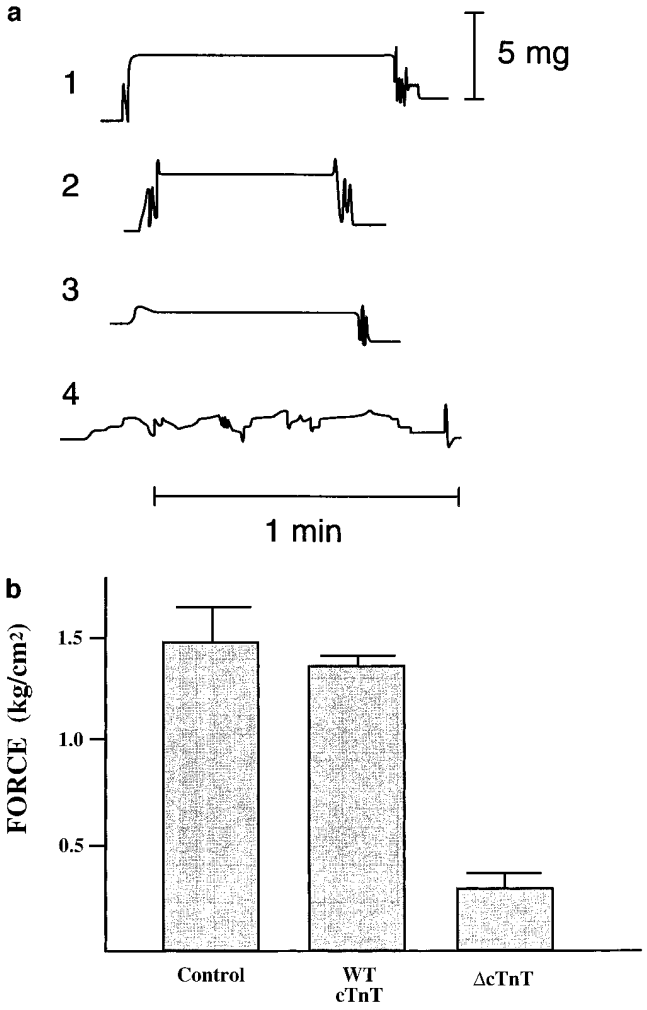

Figure 3. (*a*) Representative force records for myotubes containing (*1*) control (endogenous), (*2*) wild type human cTnT, (*3*) truncated human cTnT, and (*4*) truncated human cTnT (but with focal sarcomeric disruptions). All recordings were made at a calcium concentration of $10^{-4.3}$ M. (*b*) Bar graphs of mean maximal ($10^{-4.3}$ M Ca^{2+}) force per cross-sectional area for control and human cardiac troponin T-containing myotubes.

mance among myotubes (0.30±0.08 kg/cm^2), the truncated COOH terminus must interfere with the function of the troponin complex. The COOH terminus is in a region that is thought to confer Ca^{2+}-sensitive tropomyosin binding (16). The mutant protein does not abolish the ability of TnT to bind to tropomyosin or troponin subunits, but instead results in a troponin complex that continues to inhibit muscle contraction in the presence of calcium.

It is unclear whether or not the observation of regional disruption of sarcomeres in myotubes expressing high levels of the mutant (truncated) TnT is of physiological significance. There are at least three possibilities as to the origin of these disruptions. First they may arise because the truncated TnT is less soluble than the wild type and at high levels it aggregates. These putative aggregates could then interfere with sarcomere assembly. Secondly, the truncated TnT may initially assemble normally, but cause collapse of sarcomeres either as a result of abnormal contractile activity or because it interferes with proper turnover of the structures. Thirdly, there may in fact be a subtle primary assembly defect. In order to sort through these possibilities, a study examining the development of the sarcomeric disruptions at both the light and electron microscope levels must be undertaken. Thus, while it is tempting to suggest that one of the consequences of this HCM-causing mutation may be interference with sarcomere assembly, which could lead to the myofibrillar disarray characteristic of affected human hearts (17), this point remains to be demonstrated.

Table I. Maximal Force Generation in Myotubes Co-expressing Wild-type and Truncated Human Cardiac TnT

Myotube	Maximal force
	percent control
1	18.1
2	102.3
3	21.5
4	72.9
5	101.5
6	69.3
7	17.7
8	22.7
9	74.2
10	44.9
11	83.2
12	10.3

The Int15$^{G\rightarrow A}$ splice site mutation in cardiac TnT was selected for study because it was potentially a null allele, as opposed to the previously documented missense mutations that cause HCM, and so suggested a possible divergence in mechanisms leading to the HCM phenotype. The demonstration that this splice site mutation also acts as a dominant negative allele that interferes with contractile performance provides additional evidence that there may be a common mechanism among all HCM-causing mutations. The functional analyses performed to date suggest possible mechanisms that can now be investigated. Decreased cellular power output via decreased force and/or velocity of contraction may provide the primary stimulus to hypertrophy. For example, if cardiac performance is depressed, reflex pathways will increase sympathetic drive to elevate cytoplasmic calcium and return power towards normal; these may also activate proto-oncogenes and protein kinase C, leading to cellular hypertrophy (18, 19). At the cellular level stretch-sensitive transduction mechanisms that trigger a hypertrophic response may do so variably depending on local myocyte properties and hemodynamic factors, resulting in heterogeneous patterns of cellular hypertrophy (20). Dissection of the pathways leading to the hypertrophic phenotype will be easier if advantage can be taken of the shared features of the different HCM-causing mutations.

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