Molecular and Physiological Effects of Overexpressing Striated Muscle β-Tropomyosin in the Adult Murine Heart*

Mariappan Muthuchamy†, Ingrid L. Grupp§, Gunter Grupp§, Barbara A. O’Toole¶, Ann B. Kier*,*, Gregory P. Boivin*,†, Jon Neumann‡, and David F. Wieczorek‡‡

From the †Department of Molecular Genetics, Biochemistry, and Microbiology, the §Department of Pharmacology and Cell Biophysics, the ‡Department of Physiology and Biophysics and Medicine, and the ¶Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Tropomyosins comprise a family of actin-binding proteins that are central to the control of calcium-regulated striated muscle contraction. To understand the functional role of tropomyosin isoform differences in cardiac muscle, we generated transgenic mice that overexpress striated muscle-specific β-tropomyosin in the adult heart. Nine transgenic lines show a 150-fold increase in β-tropomyosin mRNA expression in the heart, along with a 34-fold increase in the associated protein. This increase in β-tropomyosin message and protein causes a concomitant decrease in the level of α-tropomyosin transcripts and their associated protein. There is a preferential formation of the αβ-heterodimer in the transgenic mouse myofibrils, and there are no detectable alterations in the expression of other contractile protein genes, including the endogenous β-tropomyosin isoform. When expression from the β-tropomyosin transgene is terminated, α-tropomyosin expression returns to normal levels. No structural changes were observed in these transgenic hearts nor in the associated sarcomeres. Interestingly, physiological analyses of these hearts using a work-performing model reveal a significant effect on diastolic function. As such, this study demonstrates that a coordinate regulatory mechanism exists between α- and β-tropomyosin gene expression in the murine heart, which results in a functional correlation between α- and β-tropomyosin isoform content and cardiac performance.

A precise assembly of contractile proteins consisting of myosin, actin, and associated molecules (i.e., tropomyosin (TM)1, troponin (Tn), and α-actinin) is required for a functional striated muscle sarcomere. Many myofibrillar proteins exist in multiple isoforms, and major changes in their expression occur during myogenesis. Differences in contractile and regulatory function among isoforms have been determined for mammalian myosin, TM, and Tn-T (1–4). For example, in different skeletal muscle fiber types, the expression of particular TM and Tn-T isoforms correlates with the Ca2+ regulation of sarcomeric tension. Investigations on muscle assembly and function also serve as a paradigm for understanding development and differentiation of many biological systems. For example, genetic mutants of Caenorhabditis elegans, Drosophila melanogaster, and axolotl Ambystoma mexicanum illustrate the importance of contractile protein genes for myofibrillar structure, assembly, and function (5–8). Insufficient maintenance of functional myofibrils can lead to cardiac failure and mutations in ventricular myosin heavy chain (β-MHC), α-TM, and cardiac Tn-T cause familial hypertrophic cardiomyopathy (9, 10).

Investigations on the functional roles of contractile protein isoforms, such as striated muscle-specific α- and β-TM are essential for understanding the physiology of the striated muscle sarcomere. TM, which is encoded within a multigene family (11–17), is a coiled-coil dimer that stabilizes actin filaments and is central to the control of striated muscle contraction. In association with the Tn complex, the function of TM in skeletal and cardiac muscle is to regulate the calcium-sensitive interaction of actin and myosin. The TM-Tn complex inhibits actomyosin ATPase activity under resting intracellular calcium ion concentrations. In response to Ca2+ release by the sarcoplasmic reticulum, Tn-C binds additional calcium ions and a conformational change is transmitted through the Tn-bound TM complex. This movement coordinately releases the inhibition of actomyosin ATPase activity and results in sarcomeric contraction.

Although there is significant amino acid similarity between the sarcomeric α- and β-TM isoforms (87.5% identity), differences in the ratio of these isoforms have been observed between fast and slow contracting striated muscles (18), thereby suggesting a functional difference between the isoforms. Fast contracting skeletal and cardiac muscles contain more αβ-homodimers, and slow contracting muscles express more αβ-homodimers. Nevertheless, additional studies have demonstrated that the αβ-heterodimer is preferentially formed in most muscles (2, 4, 19), although the biological significance of this remains unclear. Functional differences of these protein isoforms and their interactions with other molecules like the Tn complex have been studied (1, 4, 20, 21). Preliminary studies by Thomas and Smillie (22) indicate that strength of binding the αα- and αβ-TMs to Tn-T are equivalent, whereas the strength of binding ββ-TM to Tn-T is less. Also, biochemical studies suggest the αα-homodimer exhibits higher tropomyosin-actin-S1 ATPase activity and greater Ca2+-sensitive release of Tn inhibition than either the ββ-homodimer or αβ-heterodimers. Further
support that specific combinations of these isoforms have different contractile properties is found during development of chicken and rabbit skeletal muscle (18, 23).

Despite the biochemical evidence, the relationship between TM isoforms and contractile behavior of the heart has not been delineated. Previous studies by Izumo et al. (24) have demonstrated that β-TMstr transcripts and other fetal program genes (skeletal α-actin, ANF, etc.) are reinduced in the adult rat heart during pressure-overloaded hypertrophy. Whether the isoform changes are directly responsible for and sufficient to cause alterations in cardiac behavior remains unresolved. With the advent of gene-targeting and transgenic animal technologies, it is now possible to target a specific gene/isoform for overexpression in murine cardiac tissue (25). Both striated muscle-specific α- and β-TM transcripts are increased during embryonic cardiac development (days 11–19), with the ratio of these mRNAs changing from 5:1 to 60:1 during the embryonic to adult transition. To understand the functional role of striated muscle-specific α- and β-TM isoforms, we have generated transgenic mice that overexpress β-TMstr specifically in the adult heart. By utilizing this approach, we could address whether any compensatory mechanism in cardiac musculature is activated in response to the overproduction of β-TMstr mRNA or protein and whether differences in the α:β-TM isoform ratios could morphologically and/or physiologically change the contractile behavior of the heart. Nine independent transgenic lines were generated, which show a 150-fold increase in β-TMstr mRNA expression in the heart, along with a 34-fold increase in the associated protein. Expression was restricted to the cardiac compartment. The data show that the increase in β-TMstr messages and protein in the transgenic (TG) mouse heart also causes a concomitant decrease in the levels of α-TMstr transcripts and their associated protein. There is also a preferential formation of αβ-heterodimers that occurs in the TG mouse hearts. No detectable alterations in the expression of any other contractile protein genes are found in these transgenic mice. Morphological analyses indicate that there are no structural changes in the heart nor in the sarcomere that is associated with the increase in β-TMstr expression. Interestingly, physiological analyses of these transgenic hearts reveal that functional parameters associated with myocardial contractility appear normal; however, there is a significant delay in the time of relaxation and a decrease in the maximum rate of relaxation in the left ventricle.

EXPERIMENTAL PROCEDURES

Construction of αMHC/β-TMstr Transgenic Construct—A 1.1-kb NcoI-KpnI fragment, containing the complete coding region of the mouse β-TMstr cDNA and its 3′ untranslated sequence (3′-UTR) was ligated to the SalI site of the cardiac α-MHC promoter (26). A 240-bp NotI-HindII fragment containing the SV40 poly(A) signal was ligated to the 3′-end of α-MHC/β-TM construct to insure correct 3′-processing of the transgene.

Production of Transgenic Mice—The DNA used in the microinjection was released from the transgene vector by digestion with SacI/HindII, to generate the linear 6.8-kb fragment. This was first isolated on a low melting point agarose gel followed by purification in a cesium chloride gradient. The resulting DNA was suspended in 5 ml Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 2 μg/ml. Single cell embryos derived from superovulated FVB/N females were used in the microinjection. Purified DNA was microinjected into the pronuclei, and the surviving embryos were implanted into pseudopregnant foster mothers (27). Founder mice were identified using PCR as described (28). PCR primers corresponding to nucleotide sequences within the second intron of the MHC promoter and the β-TMstr DNA were annealed to genomic DNA isolated from ear clips and produced a 234-bp fragment in the transgenic mouse tissue using PCR; this PCR product was resolved in 1.5% agarose gels. Stable transgenic lines were raised by breeding the founder TG mice with nontransgenic (NTG) cohorts. Genomic Southern Blotting and Copy Number—Genomic DNA was isolated from tail clips and digested with EcoRI to yield a unique 6.8-kb fragment when the construct is oriented in a head to tail fashion. The DNA was electrophoresed in 0.7% agarose and transferred to a nitrocellulose filter. The copy number of the transgene was determined by quantitative nucleic acid blot analysis. 2.5 μg of EcoRI-digested DNA was applied to a nylon membrane as described (GeneScreen protocol). A set of blots was generated by using serial dilution of purified α-MHC/β-TMstr fragment as a standard. In each case, the blot was probed with a 32P-labeled SV40-containing fragment. Quantitation was carried out on an Imagequant PhosphorImager V 3.0 (Molecular Dynamics, Sunnyvale, CA).

S1 Nuclease Mapping—Total RNA was isolated from NTG and TG mouse hearts by using RNAzol (Cinna Biotex, Friendswood, TX). RNA-DNA hybridization by S1 nuclease-mapping analysis was performed under the conditions used previously (29, 30). The single-stranded DNA probes used for hybridization were generated by digestion with appropriate restriction endonucleases and labeled at the 3′-end with [α-32P]deoxycytidine triphosphate (Life Technologies, Inc.). The DNA strands were separated, and the strand complementary to the mRNA was purified. A double-stranded gyceraldehyde-3-phosphate dehydrogenase probe was prepared by reverse transcriptase-PCR using mouse heart RNA in which the 5′-primer was end-labeled during the PCR reaction. Primers were as follows: 5′-primer, 5′-CTACACTGAGGACCAGGTTGTCGAGGAAGCAGTTCGCG-3′; 3′-primer, 5′-GCCCTTTCTGCTAGTCTGCTTGGC-3′. Total RNA (25 μg) was hybridized to the probes (8 × 105 cpm) for 16 h, and the S1 nuclease reaction was done as described (25). The protected nucleotide fragment was separated on a 5% polyacrylamide, 8 M urea sequencing gel and visualized by autoradiography. The dried S1 gel was exposed to a PhosphorImager plate, and the radioactivity of desired fragments in the S1 nuclease analysis was quantitated with a PhosphorImager system. Quantification was performed by the volume integration method, subtracting appropriate positions of the RNA control sample lane as background. Values derived from two different S1 gels were used to quantitate the relative levels of α- and β-TMstr transcript levels, and the means ± the standard error of the mean (S.E.) were calculated.

Northern Slot-Blot Hybridization—Serial diluted RNA was blotted onto nylon membrane (GeneScreen) as described in the GeneScreen protocol. Several blots were prepared and hybridized with β-TMstr, α- and β-MHC, α-cardiac actin, α-skeletal actin, Tn-C, and Tn-T transcripts using specific oligonucleotide probes. Following hybridization, the filters were washed as described in the protocol and exposed to film.

Western Blot Analysis—Myofibrillar protein fractions were prepared as described previously (2) with all solutions supplemented with 2 μg/ml of leupeptin and 1 mM phenylmethylsulfonyl fluoride. The protein amount for each sample was determined by using the Bio-Rad protein assay method. Equal amounts of protein (5 μg) were separated on two 10% SDS-polyacrylamide gels. One gel was stained with Coomassie Blue to ensure equal protein loadings for each sample, and the other gel was transferred to nitrocellulose filters by using a Bio-Rad trans blot apparatus. The filters were incubated with CH1 monoclonal antibody (Ref. 31; gift from Dr. J. Lin and obtained from Developmental Studies Hybridoma Bank, Iowa City, IA) at 1:1000 dilution for 1 h at room temperature. (Another monoclonal antibody, CH106, specific for chicken striated muscle β-TM, does not recognize mouse β-TMstr protein.) After washing in phosphate-buffered saline, filters were incubated with secondary antibody (anti-mouse rabbit IgG conjugated with peroxidase), followed by the enzymatic reaction using 4-chloronaphthol (Sigma) as substrate. For quantitative Western blots, the following reaction was utilized: 50 μl of the secondary antibody, 5S-labeled anti-mouse IgG (Amer sham Corp.), was used at a specific activity of 1 μCi/10 μl of blocking buffer. The intensity of the bands was quantitated with PhosphorImager analysis. Western blot analysis of myofibrillar proteins followed by quantitation was performed four times with each sample, and mean values ± S.E. were calculated.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed according to the method of O’Farrell (32) by Kendrick Labs, Inc. (Madison, WI) as follows: isoelectric focusing, with dithiothreitol omitted from the lysis buffer preventing dissociation of TM to its subunits, was carried out in glass tubes of inner diameter 2.0 mm, using 2% BDH, pH 4–8, ampholines, for 8600 V-h. Electrophoresis in the second dimension, SDS-polyacrylamide gel electrophoresis, was then performed on identically prepared first dimension tube gels in each of two ways: first, with dithiothreitol omitted from the equilibration buffer.
tion buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 62.5 mM Tris, pH 6.8) so that TM dimers were resolved; and second, with dithiothreitol present so that the subunit composition of the TM species was resolved by the two-dimensional procedure. The two types of electrophoresis were designated oxidized-oxidized and oxidized-reduced, respectively. After slab gel electrophoresis, the gel was transblotted onto nitrocellulose membrane using a Bio-Rad Western blot analysis using CH1 antibody and was carried out as described.

Induction of Hypothyroidism in Mice—Hypothyroidism was induced in the mice by feeding the animals a 0.15% 5-propyl-2-thiouracil (PTU)-containing diet (Teklad Premier, Madison, WI) for 4 weeks. Hypothyroidism was confirmed by determining T4 serum levels via radioimmunoassay and measuring samples both before and after PTU treatment.

Preparation of Heart Sections for Electron Microscopy Analysis—The cardiac tissue was fixed in 2% glutaraldehyde for 4 h and then transferred into cacodylate buffer. Tissues were postfixed with osmium tetroxide. Thick sections (1 μm) were prepared and stained with toluidine blue. Thin sections (70–80 Å) were stained with lead acetate. Examination of sections and photographs were taken with a Hitachi H600 microscope.

Mouse Heart Perfusion and Physiological Studies—The perfusion apparatus used on the mouse hearts has been described previously (33, 34). All physiological parameters measuring cardiac performance were recorded simultaneously on a six-channel P7 Grass polygraph. Intraventricular pressure, aortic pressure, and heart rate recordings were channeled from their respective direct amplifiers from the Grass polygraph, digitized via a TL-1 DMA interface board (Axon Instruments, Foster City, CA) and analyzed using computer software custom-designed for an IBM-compatible personal computer. The amplified and digitized signal from the intraventricular pressure transducer was constantly displayed; analyzing this data provided the rate of contraction and relaxation (+dP/dt and −dP/dt, respectively). The printed output from the data analysis program indicated the most negative and most positive peak pressure and the end-diastolic pressure. Time to peak pressure and half-relaxation time (RT½) were also derived from these data. Individual points of the record were summarized as means ± S.D., and the statistical difference was estimated by a t test.

Isoproterenol was added to the Krebs-Henseleit solution entering the heart using micropump infusion pumps (multispeed infusion pump, model 600, Harvard Apparatus). These pumps could be operated separately or together so that drug concentration delivery could be modulated easily. Each isoproterenol concentration was infused for 3 min into the venous return line before the volume control in order not to alter venous return. In all hearts, the infusions were initiated at similar preloads, afterloads, and heart rates.

RESULTS

Cardiac Specific Expression of the β-TMstr Transgene—To elucidate direct noncorrelative relations between sarcomeric TM isoforms and contractile behavior of the heart, we developed a transgenic mouse model to overexpress the striated muscle-specific β-TM isoform in the heart. The construct used to generate transgenic mice contained a 5.5-kb mouse genomic TM isoforms and contractile behavior of the heart, we developed a transgenic mouse model to overexpress the striated muscle-specific β-TM isoform in the heart. The construct used to generate transgenic mice contained a 5.5-kb mouse genomic DNA fragment encompassing the promoter of the α-cardinical MHC gene (26), ligated to the coding sequence for the mouse β-TMstr cDNA and its 3′-UTR (Fig. 1A). The α-MHC promoter has been demonstrated to be cardiac specific in transgenic mice when ligated to either a CAT reporter gene (26), or the β2-adrenergic receptor construct (35). Also, this promoter drives the transgene expression to levels similar to those obtained from the endogenous α-MHC, which is expressed at low levels in the embryonic mouse heart and becomes very active in adult atria and ventricles (26, 33). To insure correct transcript processing, we included the late SV40 polyadenylation and termination signal sequences in the 3′-end of the transgene construct. Transgenic mice were generated as described under “Experimental Procedures.” Nine founder mice were identified as carrying the transgene (data not shown), and none of these founder mice or their progeny demonstrate any gross phenotypic alterations or reduced viability.

We measured the expression of the construct using S1 nuclease and Northern slot-blot analyses following RNA isolation of hearts obtained from TG and NTG littermates. As depicted in Fig. 1B, the α-TM probe, 363 nucleotides long, was end-labeled at codon 184 and extended into the 3′-UTR of the striated muscle cDNA sequence. The β-TM probe, a 299-nucleotide-long PstI-BglII fragment, was end-labeled at codon 144 and extended into codon 244, encompassing the striated muscle-specific exon 6B. To quantitate the relative levels of α- and β-TM transcripts, S1 nuclease protection analysis was done in a single reaction in which equivalent amounts of specific activity from both α- and β-TM probes were hybridized to an equal amount of RNA (25 μg) from TG or NTG mouse hearts. Hybridization products were then digested with S1 nuclease and analyzed as described (25). As seen in Fig. 1B, full protection of the β-TM probe (299 bp) is readily detectable in all TG heart RNA samples. Upon longer exposure, very low levels of the endogenous β-TMstr band are detectable in the NTG samples.

Full protection of the α-TM probe (363 nucleotides) is clearly visible in all cardiac muscle samples. The data also clearly demonstrate there is a concomitant decrease in the α-TMstr message levels in the TG cardiac RNAs as compared with those of the NTG samples (full protection of the 363-nucleotide band). (The NTG 28 sample spilled during loading of the gel; independent experiments with additional NTG 28 RNA samples showed that the α-TM transcript levels are equivalent to other NTG samples (data not shown)). Also, additional experiments using glyceraldehyde-3-phosphate dehydrogenase as control probes have demonstrated equivalent amounts of total RNA were analyzed among the NTG and TG samples. A PhosphorImager analysis was conducted to quantitate the α- and β-TMstr transcript levels. The intensities of the bands at 363 and 299 bp were measured, and the relative ratio was calculated.

There is an increase of β-TM mRNA amount in the different TG lines (58.0 ± 6.5 units) when compared with their NTG littermate (0.4 ± 0.01 units). These values represent a 150-fold increase in β-TMstr mRNA expression in the heart. The αβ striated muscle TM transcript ratio in the hearts of TG mice is 0.3:1, whereas this ratio is 58:1 in the NTG hearts. This value for the NTG αβ ratio corroborates our earlier published value of 60:1 in the adult mouse heart (25). Further, there is a 3-fold increase in the total amount of α-TM plus β-TM transcripts in the TG versus NTG mouse hearts. Northern slot-blot analyses confirmed these results; additional experiments addressing the expression of the α-MHC/β-TMstr transcript in different tissues showed that the expression of the construct is restricted to heart tissue (data not shown). This result is in agreement with previous studies demonstrating the cardiac-specific expression exhibited by this α-MHC promoter (26, 35).

The observed decrease in the α-TMstr isoform following overexpression of the β-TMstr strongly suggests a mechanism for coordinately regulating TM mRNA levels. We addressed whether the endogenous β-TM expression is altered by the transgene construct: using Northern slot-blot analysis with an oligonucleotide probe specific to the β-TMstr 5′-UTR, which is lacking in our construct (see “Experimental Procedures”). Results demonstrate that the α-MHC/β-TMstr transgene did not transactivate expression of the endogenous β-TM gene, as similar low levels of β-TMstr mRNA were detected in TG and NTG mouse heart samples (data not shown). We also checked if the α-MHC promoter or the SV40 fragment influenced expression of TM in the transgenic mouse. RNA from the α-5.5 transgenic mouse (gift from Dr. J. Robbins; Ref. 26), which contains the α-MHC promoter ligated to the chloramphenical acetyltransferase gene and the SV40 poly(A) signal, was quantitated for α- and β-TM transcripts by S1 nuclease mapping as described previously. No changes are found in the levels of the α- and β-TMstr messages in the α-5.5 transgenic mouse heart when compared with control mice (Fig. 1C).
We further examined whether there were molecular compensatory reactions in the expression of other contractile protein genes (α- and β-MHC, α-cardiac and α-skeletal actin, Tn-C, and Tn-T). Interestingly, results from Northern slot-blot analyses demonstrate that there are no dramatic quantitative differences in the expression of transcripts from these genes in TG hearts as compared with their NTG littermates (data not shown). This result is in agreement with the recent finding that
overexpression of skeletal Tn-C in cardiac muscle also does not alter the expression of other contractile protein genes, including the endogenous cardiac Tn-C (36).

Differences in the levels of β-TMstr transcripts among the nine transgenic lines were observed with TG line 1, showing the least expression, and several TG lines exhibiting much greater expression. Variability in expression of the transgene among the TG lines may be due to differences in the copy number of the integrated transgene construct and/or variability in the site of integration. To determine the integration and copy number of the microinjected DNA, we conducted a genomic Southern blot analysis. Results demonstrate that the expected size band (6.8 kb) is present in the genomic DNA from all TG lanes when hybridized with a radiolabeled SV40 fragment (Fig. 2). A nucleic acid slot-blot analysis confirms that the different levels of expression of the β-TMstr transgene are partially due to differences in the copy number among the lines, with these values ranging from 4 copies (TG line 1) to 56 copies (TG line 10) (data not shown).

TM Protein Production in the TG Mouse Hearts—To examine the production of exogenous β-TMstr protein in the hearts of transgenic mice, myofibrillar protein fractions from TG and NTG hearts were run on 10% SDS-polyacrylamide gels and either stained with Coomassie Blue or subjected to Western blot analysis. Coomassie Blue staining of myofibrillar proteins shows that equal amounts of the proteins are present in all lanes and also that there is an increased level of β-TM protein in all of the TG samples (data not shown). As seen in Western blot analysis (Fig. 3A), the NTG sample only contains the α-TM protein, whereas both α- and β-TM are clearly visible in all TG samples. Interestingly, in agreement with the RNA analyses, the increase in β-TM protein in the TG mice has also resulted in a concomitant decrease in α-TM production. A PhosphorImager quantitative analysis shows that 57.8 ± 8.4% of the total cardiac TM content is the β-TM isoform in these TG hearts (Fig. 3B). The NTG sample has very low levels of β-TM protein (a faint signal can be seen upon longer exposure). These values represent a 34-fold increase in β-TM protein production. Furthermore, the quantitative analysis demonstrates that only 42.2 ± 7.1% of the TM content is the α-TM isoform in the TG myofibrils. It is interesting to note that β-TM expression in TG line 7 is slightly greater than in TG line 10 even though the copy number of the transgene is 2.5 times less (20 copies versus 56 copies); a potential reason for this increased expression in TG line 7 could be the positional effects of the insertion. Nevertheless, a constant finding was that the total amount of TM in the hearts of the TG mice remains nearly constant (Fig. 3B). This data suggests there may be a regulatory mechanism that exists controlling the amount of contractile protein incorporated into the functional sarcomere. It is interesting to note that similar results are obtained with the overexpression of myosin light chains using the same promoter.

Previous investigations conducted by our laboratory (17, 25) and others (24) found that striated muscle α-TM is the primary mRNA isoform expressed in rodent cardiac musculature. The results shown in Fig. 3 demonstrate that TM composition in wild-type cardiac musculature is essentially α-TM. However, the dimeric species of TM protein with respect to its α and β subunits have not been examined in the heart. To ascertain the distribution of α- and β-TM dimeric species in control and transgenic mice, myofibrillar proteins from NTG and TG hearts were subject to two-dimensional gel electrophoretic analysis, followed by immunoblotting of the transfer membrane with the striated muscle TM antibody. A minor modification of the two-dimensional electrophoretic procedure of O’Farrell (32) was developed to resolve the different TM dimers and determine their subunit composition (see “Experimental Procedures”). Heart myofibrillar protein samples from NTG mice show that a diffuse area, labeled α2, is reactive with the TM antibody (Fig. 4A). As shown in the oxidized-reduced electrophoretic conditions, the α2-TM dimer is resolved into the α subunit (Fig. 4B). The protein of this spot with respect to isoelectric focusing (pl value of 5.1) and SDS mobility strongly suggests this protein is the α2-dimer. In concert with previous studies on TM composition in skeletal musculature (where multiple α-TM isoform species have been described; Ref. 2), the results suggest multiple α isoform species are also present in the heart. This production of several α-TM protein species in cardiac musculature is also supported by the heterogeneity found at the mRNA level (17, 25).

The results from Fig. 3 show that in the TG mice, both α- and β-TM proteins are present in the myofibrils. When the TM subunit composition of TG hearts is examined, a cluster of spots is detected in polyacrylamide gels run under oxidized-oxidized conditions (Fig. 4C). The position of these spots with respect to isoelectric focusing (pl value of 5.0) and SDS mobility is consistent with the formation of α2-heterodimers (2). Detection of αβ-TM species at this position has been found previously in various skeletal muscles and is partially attributable to heterogeneity in the production of TM isoforms (2, 17, 25). Resolution of these spots into α and β subunits in TG samples subject to oxidized-reduced polyacrylamide gel electrophoresis (Fig. 4D) also strongly suggests the composition of αβ-heterodimers in the TG cardiac myofibrils.

Shutting Off the α-MHC/β-TMstr Transgene Restores α-TMstr Expression to Normal—The α- and β-MHC genes have been reported to be hormonally regulated throughout development (26, 33, 37–41). Subramaniam et al. (26) have shown that with an α-MHC-5.5 transgene CAT construct, expression in transgenic mice is regulated by thyroid hormone in a manner that reflects regulation of the endogenous α-MHC gene. Since we used the same promoter in our construct, we hypothesized that shutting off the promoter (and subsequent exogenous β-TMstr isoform expression) by inducing hypothyroidism in the transgenic mouse would return the levels of endogenous α-TMstr isoform production to normal. Previous studies demonstrated that expression of endogenous α- and β-TMstr isoforms are not

\[\text{\footnotesize \cite{J. Robbins, personal communication.}}\]
affected by thyroid hormone in rat cardiac muscle (24). Hypothyroidism was induced in the mice and confirmed by standard radioimmunoassay. In the hypothyroid mice, serum thyroxine levels dropped significantly (15-fold less than normal levels). Normal serum thyroxine levels were confirmed in control (euthyroid) mice fed a normal diet. Neonatal and adult TG and littermate NTG controls were determined by S1 nuclease analysis followed by PhosphorImager quantitation. Further, to insure that equal amounts of RNA were used in this assay, a glyceraldehyde-3-phosphate dehydrogenase probe was also included along with the α- and β-TM probes. Results demonstrate that there is no detectable β-TMstr band (299 nucleotides) in the transgenic hearts of the hypothyroid mice (Fig. 5A, lanes 1 and 5), indicating that the transgenic β-TM expression is completely down-regulated. However, in euthyroid transgenic mice (Fig. 5A, lanes 2 and 6), the overproduction of β-TMstr message is quite clear, as we had demonstrated earlier (Fig. 1B). Interestingly, the levels of α-TMstr isoform expression in these hypothyroid TG hearts are increased above euthyroid TG levels (compare the 363-nucleotide band of lanes 4 and 8 with lanes 2 and 6). In addition to demonstrating that expression of the TM transgene construct can be regulated by altering thyroid hormone levels, this analysis also shows that endogenous α- and β-TMstr isoform expression in the murine system is not affected by the hypothyroid state; this can be seen by the relatively equal amounts of mRNA present in euthyroid (lanes 1 and 5) and hypothyroid (lanes 3 and 7) NTG hearts.

Since results demonstrate there is an increase in α-TMstr transcript levels in response to hypothyroidism by the TG mice, we addressed whether this change would be reflected at the translational level. Using Western blot analysis, the levels of α- and β-TMstr protein in the TG and NTG hypothyroid mouse ventricles were examined. In results similar to those observed at the mRNA level, β-TM protein is down-regulated in the hypothyroid TG mouse hearts (Fig. 5B, lanes 4 and 8). In hypothyroid TG mice, the level of α-TMstr protein has been up-regulated and is present in quantities similar to levels found in NTG hypothyroid mice (lanes 3 and 4 and lanes 7 and 8). In addition, there are no detectable changes in the α-TMstr protein levels between euthyroid and hypothyroid NTG hearts (lanes 1 and 3 and lanes 5 and 7), indicating that thyroid hormone does not have any major influences on striated muscle TM protein expression. Since there is no effect of thyroxine on endogenous α- and β-TMstr isoform expression in murine hearts, we can conclude from our results that these two genes coordinately regulate their expression in the mouse heart, as previously suggested to occur in chicken pectoralis muscle (42).

Histological and Physiological Analysis of the Transgenic Heart—A morphological analysis was conducted on the TG mice to determine whether the overexpression of β-TMstr would affect organ or cellular structures. The focus of this analysis was on cardiac tissue since RNA analysis indicated the expression of the transgene was restricted to the heart. Neonatal and adult TG and littermate NTG controls were sacrificed, and a detailed histological analysis was conducted. Results from over 35 TG and 35 NTG mice showed that the TG mice had similar heart-to-body weight ratios relative to their
control littermates, with no gross or histologic evidence of cardiac hypertrophy. Light microscopic analysis of hematoxylin-and eosin-stained neonatal and adult heart sections reveals there is no evidence of myocyte necrosis, fibrosis, or any other pathological or morphological changes in the TG ventricles or atria when compared with the NTG controls (data not shown).

A detailed analysis of the sarcomeric structure of the myocardial tissue was conducted using electron microscopy and immunohistochemistry. Adult cardiac tissue from TG and NTG mice was examined by transmission electron microscopy for anomalies of sarcomeric structure or other morphological alterations. Results demonstrate there are no differences in cardiac myocyte structures or organization in the TG mouse hearts (Fig. 6). Additional studies on other mice at various magnifications confirmed this finding. In immunohistochemical analyses, sections of TG hearts were immunostained using a striated muscle-specific TM monoclonal antibody. There are no apparent differences in the staining pattern between TG and NTG sections, and there is no nonspecific binding of the TM antibody to structures other than the myofilaments (data not shown). Thus, the results from the histological analyses demonstrate that overexpression of β-TMstr isoform in these TG mouse does not lead to morphological or pathological alterations in cardiac muscle structure.

Studies on rabbit fast skeletal muscle fibers demonstrate that different Tn-TM combinations in the relaxed and active states and their affinities for calcium appear to be a significant determinant of myofiber contractile properties in vivo (18). In our investigation, we have developed an in vivo system where the sarcomeric TM is mostly αβ-heterodimers instead of the usual α2-homodimers. As such, one might expect changes in the cardiac performance. To determine whether any such changes occur in the TG mice, we implemented the isolated work-performing mouse heart preparation. This model is a powerful technological tool used to elucidate the quality of cardiovascular and contractile parameters in individual mouse hearts (33, 34, 43, 44). The advantage of this method is the observation of contractile and relaxation parameters of individual mouse hearts under identical minimal afterloads (aortic pressure of 50 mm Hg) and identical preloads (cardiac output 5 ml/min), and similar heart rates. The contractile parameters of seven TG mouse hearts were age (2-4 weeks old) and sex matched with seven NTG littermate controls (2 hearts each from TG lines 7, 10, and 28 and one heart from line 12). These results are summarized in Table I. It is clear that there are only a few significant differences in cardiac performance that exist between TG and NTG mice. Most of the physiological measurements are similar between the two groups, including heart rate, mean aortic pressure, cardiac output, and intraventricular pressure. When the minimal pre- and afterload was applied, both TG and NTG hearts showed similar systolic, diastolic, and end-diastolic intraventricular pressures. However, there were some differences in the additional quantitative measurements of contractile performance. Although the contractile parameters (maximum rate of contraction and time to peak pressure) of TG hearts showed no significant differences from the NTG values, the relaxation parameters (maximum rate of relaxation and time to half relaxation) of the TG hearts were significantly reduced or prolonged, respectively, compared with the NTG hearts (Table I).

This effect on relaxation performance disappeared when the Starling forces were increased: an afterload increase from a normal value of 50 mm Hg mean aortic pressure to 62 mm Hg and/or an increase in the preload from a normal value of 5 ml/min cardiac output to 7 ml/min. Consequently, at maximally tolerated workload, contractile and relaxation parameters were indistinguishable between NTG and TG hearts. Similar results could also be accomplished by exposing the TG hearts to increasing concentrations of the β-adrenergic stimulant isoprotanol (Table I). When 4 nM isoprotanol was administered to the working hearts via the Krebs-Henseleit solution perfusate (a concentration far below the ED50 values of 12-40 nM), the differences in relaxation parameters were completely removed. In summary, results from the physiological analysis show that there is a functional alteration in cardiac muscle performance in the TG mouse. The primary function that is effected is an inability of these hearts to relax fully coupled with a prolonged relaxation phase. As such, these results demonstrate there is a
The functional correlation between the α- and β-TMstr isoform content and cardiac performance.

**DISCUSSION**

Striated muscle contraction involves the interaction of Tn with TM on the muscle thin filament. Numerous biochemical studies have provided insight into the importance of interactions among thin filament proteins such as actin, Tn-T, and TM. The existence of muscle fiber types has been correlated with various contractile protein gene isoforms. However, understanding of the structure-function relationship of specific isoforms remains largely unresolved. To gain new insights into the functional role of sarcomeric TMs in murine cardiac muscle, we generated transgenic mice overexpressing striated muscle-specific β-TM in adult hearts and addressed the response of the myocardium at the molecular, morphological, and physiological levels. Results demonstrate that overexpression of the striated muscle β-TM isoform alters cardiac muscle performance by decreasing the maximum rate of relaxation and increasing the time needed to complete the relaxation phase of sarcomeric function. Also, this overexpression of β-TMstr activates a compensatory mechanism to reduce the production of α-TMstr mRNA and protein, thus limiting the total amount of

**FIG. 5.** RNA (A) and myofibrillar protein (B) analysis of normal and PTU-treated NTG and TG mouse hearts. A, an S1 nuclease analysis of RNA from normal (euthyroid) NTG hearts (lanes 1 and 5), TG hearts (lanes 2 and 6), PTU-treated (hypothyroid) NTG hearts (lanes 3 and 7), and PTU-treated TG hearts (lanes 4 and 8). This analysis was performed by using α- and β-TMstr and glyceraldehyde-3-phosphate dehydrogenase probes as described (see "Results" and Fig. 1). Lanes 2 and 4 represent TG line 10; lanes 6 and 8 represent TG line 13. B, Western blot analysis of myofibrillar proteins using the CH1 antibody was conducted as described in the "Results." The sample order is similar to A. The migrating positions of α- and β-TMstr proteins are marked on the left; the standard molecular mass markers are on the right.

**FIG. 6.** Electron microscopy analysis of TG mouse heart sections. Heart sections for EM were prepared as described under "Experimental Procedures." A representative picture of a TG mouse left ventricular myofilament structure is shown. Original magnification: ×31,500.
Overexpression of β-TMstr in the Murine Heart

**Table I**

|                | NTG (n = 5) | ED₃₀ | TG (n = 6) | ED₃₀ |
|----------------|------------|------|------------|------|
| +dP/dt (mm Hg/s) |            |      |            |      |
| 0.0             | 3394 ± 304 | +3419 ± 282 | 3625 ± 440 | +3844 ± 447 |
| 0.8             | 29 +3108 ± 444 | 3233 ± 296 | 3416 ± 330 | 3466 ± 414 |
| 4.0             | 33             | 330             | 330             | 330             |
| 8.0             | 33             | 330             | 330             | 330             |
| -dP/dt (mm Hg/s) |            |      |            |      |
| 0.0             | -2937 ± 550 | -3000 ± 567 | -3094 ± 572 | -3281 ± 527 |
| 0.0             | 38             | -2246 ± 321a | -2320 ± 299a | -2704 ± 490 |
| 4.0             | 40             | 0.493 ± 0.042 | 0.497 ± 0.039 | 0.468 ± 0.049 |
| 8.0             | 40             | 0.493 ± 0.042 | 0.497 ± 0.039 | 0.468 ± 0.049 |
| TPP (ms/mm Hg)  | 0.529 ± 0.072 | 0.514 ± 0.089 | 0.473 ± 0.036 | 0.453 ± 0.045 |
| RTₒ₂ (ms/mm Hg) | 0.460 ± 0.036 | 0.490 ± 0.039 | 0.471 ± 0.051 | 0.405 ± 0.067 |

Values are means ± S.D.; n, number of mice; ED, effective dose.

**a** p < 0.05.

**b** p < 0.001.

TM production. As shown by the administration of PTU to shut off the transgene promoter, this compensatory molecular mechanism is fully reversible. The exogenous β-TMstr protein that is produced preferentially associates to form an αβ-heterodimer, which data suggests becomes integrated into the cardiac muscle sarcomere. Interestingly, no morphological or pathological phenotype is associated with this increased expression of β-TM in cardiac muscle.

"Cross-talk" between α- and β-TM Genes—Muscle development and differentiation is associated with major isoform changes in the expression of contractile protein genes (45). Developmental changes in isoform expression are well documented for the myosin heavy chain and actin gene families (46–48). Gunning et al. (49) have demonstrated there is differential accumulation of α- and β-striated muscle TM mRNAs during the development of several skeletal muscles in the rat. We have recently shown that there is a steady increase in both α- and β-TM transcripts during early cardiogenesis in the murine system. However, in the adult, there is a drastic decrease in the expression of β-TMstr, whereas α-TMstr expression proceeds at a steady-state level (25). This constant level of α-TMstr transcript production and low level of β-TMstr mRNA is maintained even in response to variations in thyroid hormone (Ref. 24 and this paper). This is unlike the situation of the α- and β-MHC, which are antagonistically regulated in response to thyroid hormone (26, 33, 37–41). The forced expression of β-TMstr isoform in the transgenic mouse hearts results in a concomitant decrease in the level of endogenous α-TMstr isoform production without alterations in endogenous β-TMstr expression. Interestingly, when the β-TMstr transgene expression is down-regulated in the PTU-treated TG mouse, α-TMstr mRNA and protein levels return to near normal levels. Although the total amount of TM protein in the TG mouse hearts is similar to that in the NTG hearts, the results presented here demonstrate that there is a molecular mechanism that coordinately regulates the production and levels of the α- and β-TMstr isoforms. A similar type of feedback regulation in actin isoform production occurs in response to the expression of exogenous actin in vitro (50–53).

Previous studies demonstrate that the 3′-UTR of sarcomeric actin genes, which are highly conserved across species, may play an important role in their regulation of expression (54, 55). In vitro experiments coupling heterologous promoters to actin 3′-UTRs show that the expression of these chimeric constructs follows the expression patterns of the 3′-UTR regions, not the associated promoters. These results strongly suggest that this expression of actin isoforms is strongly influenced by their 3′-UTRs (56, 57). The mechanism through which the 3′-UTRs mediate actin expression is thought to be cis-regulated. Ras-tinejad and Blau (58) have recently shown that regulation by 3′-UTRs of certain differentiation-specific RNAs (α-TM, α-cardiac actin, and Tn-T), can operate in trans as well. Our results demonstrate that neither the α-MHC promoter nor the SV40 poly(A) fragment used in the transgene construct is responsible for the decreased level of α-TMstr isoform in the TG mice. Although both the endogenous α- and β-TMstr isoforms are generated by alternative splicing (13, 17), it is unlikely that the titration of splicing factors is responsible for the decrease in α-TM levels since the β-TM cDNA was used in the transgene construct. Thus, it is possible that the 3′-UTR of the β-TMstr may be involved in the down-regulation of α-TMstr isoform expression. It is tempting to speculate that this could be the reason why the relative ratio of αβ-TMstr muscle RNA in the TG mice is much lower than the protein value; portions of the overexpressed β-TMstr messages, particularly the 3′-UTR, may act as trans-acting regulators in a feedback loop that inhibits α-TMstr mRNA isoform production. However, we cannot rule out the possibility that translational control mechanisms or stability of the mRNA or protein may also influence TM production. Studies are in progress to elucidate the molecular basis of this coordinate regulation.

Overexpression of β-TMstr Does Not Cause Pathological Alterations—Overexpression of the β-TMstr isoform in the heart does not result in any gross morphological changes in cardiac muscle. Recent studies have demonstrated that missense mutations in α-TM, β-MHC, and cardiac Tn-T can trigger hypertrophic cardiomyopathy (30, 59). Previous work had demonstrated that β-TMstr mRNA expression can be reinduced in the pressure-overloaded hypertrophic rat heart (24). Since β-TM is one of the earliest contractile protein genes to be expressed during cardiogenesis, it was important to determine whether re-expression of β-TMstr could reinduce the production of other contractile protein isoforms whose expression in the adult rodent is often associated with cardiac hypertrophy. The results of this study demonstrate that aside from the down-regulation of α-TMstr, the overexpression of β-TMstr does not affect the expression of other contractile protein genes. This failure to trigger a change in the molecular regulation of other contractile protein genes is similar to the lack of response elicited from overexpression of the skeletal Tn-C isoform in the heart (36). Interestingly, overexpression of the wild type α-MHC exhibits limited, if any, cardiac abnormalities,3 a result similar to our findings with the α-MHC/β-TM transgenic mice. Thus, it appears that overexpression of contractile protein genes in vertebrates may lead to physiological changes in cardiac function.

3 L. Leinwand, personal communication.
but pathological abnormalities in the heart may only result from the expression/overexpression of contractile protein genes carrying missense or nonsense mutations.

Overexpression of β-TMstr Causes Physiological Alterations in Cardiac Function—Results from the physiological analysis strongly suggest that different functional properties are conferred on striated muscle by the α2 and αβ TM species. Contrary to the preliminary in vitro biochemical studies that demonstrate that rabbit striated muscle αα-homodimers exhibit greater Ca\(^{2+}\)-sensitivity release of Tn inhibition than αβ-heterodimers (22), the TG hearts that have primarily αβ-heterodimers show a decrease in the rate of relaxation, which reflects greater Ca\(^{2+}\)-sensitivity when compared with NTG hearts. A possible explanation for this discrepancy could be that our result reflects a physiological state existing in the whole organ, which is very similar to the in vivo situation, whereas the in vitro assay was performed with dephosphorylated rabbit striated muscle TM proteins. Also, in preliminary experiments, the pCa-force relation of Triton X-100-extracted bundles of fibers from TG hearts demonstrate a significant increased sensitivity to Ca\(^{2+}\).\(^4\)

It is interesting to note that small animals, such as adult rat, rabbit, guinea pig, and mouse have virtually no cardiac β-TM component, whereas larger species (i.e. pig, sheep, and human) have about 20% of their cardiac muscle TM in the β-form. It has also been suggested that increases in β-TM content are associated with a slower speed of striated muscle contraction (60). However, multiple contractile protein genes undergo only partial developmental isoform transitions in larger animals, thereby resulting in significant amounts of β-MHC, α-skeletal actin, and β-TMstr in the adult myocardium (60–65). As such, the slower contractile relaxation properties associated with larger animals may reflect multiple isoform differences. Our results show that when cardiac muscle TM is present as an αβ-heterodimer, there are no additional changes observed in the expression levels from other contractile protein genes. Further, it is well known that a decisive parameter determining contraction and relaxation of the cardiac muscle is not only the Ca\(^{2+}\) concentration per se but the calcium occupancy of Tn molecules. Thus, the contractile parameters of the sarcomere can also be altered by changing the calcium sensitivity of the regulatory proteins (i.e. Tn-C and associated proteins (i.e. TM and Tn-T) that influence the responsiveness of the myofilaments to calcium ions.

Measurements of contraction reflect cross-bridge turnover, whereas relaxation is more tightly associated with myocardial calcium handling (66). Interestingly, the two key components (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and phospholamban) that are involved in Ca\(^{2+}\) sequestration and cardiomyofilament relaxation do not exhibit changes in their expression in the β-TM TG mice (data not shown). Thus, it is reasonable to speculate that the increased production of β-TMstr protein in the TG mouse myocardium has a direct link to the relaxation function of the heart muscle. Interestingly, diastolic dysfunction is a frequently reported abnormality often associated with several cardiomyopathic conditions, including hypertrophy, ischemia, and dilated cardiomyopathy (67–69). Although no gross morphological abnormalities are detected with the overexpression of β-TMstr in the hearts of the TG mice, this work demonstrates a functional difference in diastole exists between the striated muscle α2 and αβ-TM dimers.

Hewett et al. (43) have demonstrated that a significant functional correlation exists between α-actin content and cardiac contractile function; increasing levels of α-skeletal actin in the cardiac muscle (which occurs genetically in BALB/c mice) enhances cardiac contractility when compared with α-cardiac actin. In this study, we have established a transgenic system that addresses functional questions regarding specific TM isoforms. Although the α- and β-TMstr proteins possess 88% amino acid sequence homology, it is not apparent how altering the αβ TM ratio might lead to functional differences in the myocardium. However, it is interesting to note that there are several amino acid differences between the α- and β-TMstr molecules positioned around amino acids 150–180, which is one of two putative Tn-T binding domains that attach TM to the Tn complex (70, 71). Perhaps, these differences contribute to a weaker binding of β-TM to the Tn complex, which subsequently affects the relaxation properties of the cardiac muscle.

Analyzing the functional significance and molecular regulatory mechanisms controlling contractile protein isoform expression is essential for understanding the development and differentiation of the mammalian cardiogenic system. By establishing transgenic overexpression and “knockout” animal models, we can address the functional and molecular significance of specific isoforms that exist within multigene and alternatively spliced gene families. By establishing a transgenic mouse that overexpresses the striated muscle β-TM mRNA and protein specifically in the heart, the importance of β-TMstr in muscle relaxation has been addressed in an in vivo system. In addition, we have begun to decipher the mechanisms controlling the molecular genetic regulation of α- and β-TM isoform production. Through further use of transgenic animal model systems, this knowledge can be extended to address the role of both wild-type and mutant transcripts and proteins in the development of normal and pathological conditions.

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\(^4\) J. Solaro, personal communication.
Molecular and Physiological Effects of Overexpressing Striated Muscle β-Tropomyosin in the Adult Murine Heart
Mariappan Muthuchamy, Ingrid L. Grupp, Gunter Grupp, Barbara A. O'Toole, Ann B. Kier, Gregory P. Boivin, Jon Neumann and David F. Wieczorek

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