Striated muscle tropomyosin (TM) interacts with actin and the troponin complex to regulate calcium-mediated muscle contraction. Previous work by our laboratory established that α- and β-TM isoforms elicit physiological differences in sarcemeric performance. Heart myofilaments containing β-TM exhibit an increased sensitivity to calcium that is associated with a decrease in the rate of relaxation and a prolonged time of relaxation. To address whether the carboxyl-terminal, troponin T binding domain of β-TM is responsible for these physiological alterations, we exchanged the 27 terminal amino acids of α-TM (amino acids 258–284) for the corresponding region in β-TM. Hearts of transgenic mice that express this chimeric TM protein exhibit significant decreases in their rates of contraction and relaxation when assessed by ex vivo work-performing cardiac analyses. There are increases in the time to peak pressure and a dramatic increase in end diastolic pressure. In myofilaments, this chimeric protein induces depression of maximum tension and ATPase rate, together with a significant decrease in sensitivity to calcium. Our data are the first to demonstrate that the TM isoform-specific carboxyl terminus is a critical determinant of sarcemeric performance and calcium sensitivity in both the whole heart and in isolated myofilaments.

Tropomyosin (TM), a α-helical coiled-coil dimer, plays an essential role in the regulation of contraction and relaxation of the sarcemere. TM regulates sarcemeric performance through its binding to actin and the troponin complex. During muscle relaxation when cytoplasmic levels of calcium are low, tropomyosin blocks the myosin-binding site on the filamentous striated muscle actin. Upon stimulation, calcium is released into the cytosol and the troponin complex to regulate calcium-mediating muscle contraction. TM interacts with actin and the troponin complex, which causes a conformational change in the position of TM on actin, thereby exposing the myosin-binding site. Myosin binds to actin and triggers muscle contractile activity until the stimulus ceases, and calcium is re-sequestered into the sarcoplasmic reticulum.

Recent studies (1–3) in our laboratory have demonstrated that there are functional differences among the three highly conserved striated muscle TM isoforms. The α-TM isoform is the predominant isoform in both skeletal and cardiac muscle. In the adult murine heart, this isoform constitutes ~98% of the total TM. The remaining 2% of TM is β-TM, which is 86% identical to α-TM. In the skeletal muscle, β-TM expression is much greater, with levels dependent upon specific muscle. TPM 3 (TM 30sk, γ-TM) is not expressed in the murine heart but is found in slow-twitch skeletal muscle, such as the soleus where it composes ~30% of all the TMs (4). The TPM 3 isoform is 93% identical to α-TM and is 86% identical to β-TM. Studies in our laboratory using transgenic mice that express β-TM in the heart demonstrate that β-TM increases the sensitivity of the cardiac myofilaments to calcium (5). This increase leads to a delay in the relaxation rate of the heart, and these hearts do not relax completely (1). When β-TM is expressed at high levels in the heart, the mice die due to defects in both contraction and relaxation (2). Additional studies (6, 7) demonstrate that overexpression of wild-type β-TM in the heart does not lead to any morphological or physiological alterations in cardiac performance.

The regions within TM that associate with troponin T are amino acids 175–190 and 258–284. Experiments carried out in vitro show that removal of the TM carboxyl-terminal amino acids by digestion with carboxypeptidase results in the loss of both affinity for actin and the ability to polymerize (8–10). Their removal also impairs the cooperative interaction of TM with the myosin S1 region (11, 12). Substitution of non-muscle α-TM for muscle carboxyl-terminal amino acids affects TM-actin and TM-troponin affinities (13). The focus of the present investigation was to selectively modify the isoform of the α-TM carboxyl terminus to provide in vivo information about the role of this domain in the mechanism of muscle contraction and its interaction with actin and the troponin complex.

To address this, we substituted the carboxyl-terminal amino acids of α-TM for that of the striated muscle β-TM isoform. This substitution maintains the conserved length of 284 amino acids for TM molecules, whereas we could assess the importance of this.
region in TM function. By using a transgenic mouse approach, we generated a DNA construct that encodes a TM molecule consisting of \( \alpha \)-TM sequence from amino acids 1 to 257 and \( \beta \)-TM sequence from amino acids 258 to 284. This chimeric TM is expressed only in cardiac myocytes and is incorporated into the myofilaments. The incorporation of this protein did not result in any morphological or pathological alterations. However, when amino acids 258-284 of \( \beta \)-TM substrate for their corresponding \( \alpha \)-TM counterparts, there are significant decreases in rates of contraction and relaxation when assayed by well-perfused mouse heart analysis. There are increases in the time to peak pressure, and a tendency toward an increased time of half-relaxation. Intraventricular pressure was significantly decreased, and there was a dramatic increase in end diastolic pressure. Maximum developed tension and ATPase activity remained significantly depressed, and there was a dramatic increase in end ligated to join at the amino acid 257

**Experimental Procedures**

**Generation of \( \alpha \)-\( \beta \)-TM Transgenic Mice**—The mouse wild-type \( \alpha \)-TM and \( \beta \)-TM striated muscle-specific cDNA was generated using PCR primers that amplified from amino acids 1 to 257. The mouse wild-type \( \alpha \)-TM striated muscle cDNA was PCR-generated using primers that amplified amino acids 258 to 284 and through the entire 3’-untranslated region (UTR). These amplified fragments were isolated, purified, and blunt end ligated to join at the amino acid 257-258 junction. Nucleotide sequencing and comparison with the published sequences verified the amplified sequences. The chimeric \( \alpha \)-\( \beta \)-TM cDNA was ligated at the 5’ end to the 5.5-kb murine \( \alpha \)-myosin heavy chain (MHC) cardiac-specific promoter \( \beta \)-TM. The SV40 polyadenylation signal and termination sequence (0.24 kb) was ligated at the 3’ end of the minigene construct. The construct was linearized, purified, and microinjected into the pronuclei of fertilized ova to generate transgenic mice as described (1). Transgenic (TG) mice were produced using the FVB/N strain. Founder mice were identified by use of PCR primers corresponding to the \( \alpha \)-MHC 5’-UTR and \( \alpha \)-TM as the 5’ and 3’ primers, respectively. Genomic Southern blot analysis confirmed the founder mice. Six chimeric TM transgenic lines were established and distinguished from their non-transgenic (NTG) cohorts by PCR.

**RNA and Protein Analysis**—RNA was isolated from murine hearts using TrizReagent (Molecular Research Center, Cincinnati, OH). Total RNA (10 \( \mu \)g) from each transgenic line and control mouse ventricles was electrophoresed on 1% formaldehyde gels and transferred to nitrocellulose membrane. Hybridization was conducted with \( 3^2 P \)-radiolabeled cDNA fragments from the \( \alpha \)-TM and \( \beta \)-TM 3’-UTRs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). TM message levels were analyzed on ImageQuant PhosphorImager and normalized to GAPDH expression levels.

Myofilibrillar proteins were prepared from ventricular myocardium as described (1). Equal amounts of protein (25 \( \mu \)g) were run on two 10% SDS-PAGE gels. One gel was stained with Coomassie Blue to confirm equal protein loading, and proteins from the other gel were transferred to nitrocellulose for Western blot analysis. The filter was incubated with a striated muscle TM-specific antibody (CH1) (Sigma).

Two-dimensional gel electrophoresis was performed on myofilibrillar protein preparations from NTG and TG (line 14) hearts according to the method of O’Farrell (14). In brief, isoelectric focusing was carried out using 25 \( \mu \)g of the protein sample denatured in 300 \( \mu l \) of rehydration buffer (8 M urea, 2 mM tributylphosphine, 2% CHAPS, 0.2% Bio-Lytes (4,7-5.9)). Each sample was used to hydrate a 17-cm ReadyStrip pH 4.7-5.9 for 12 h. Isolelectric focusing was performed in three stages of applied potential difference as follows: 250 V for 15 min, 10,000 V for 1 h, followed by 8000 V for up to 6 h, until 40,000 V were achieved. Aqueous strips were then soaked in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 6.8, 20% glycerol) containing 130 mM Tris-HCl, 10 mM, followed by 130 mM Tris-HCl, 135 mM iodoacetamide for another 10 min. Strips were then applied to 10% acrylamide gels for SDS-PAGE, followed by transblotting onto polyvinylidene difluoride membrane. Western blot analysis using the striated muscle TM-specific CH1 antibody was conducted using a 1:5000 dilution. Binding of the primary antibody was detected by peroxidase-conjugated secondary antibody (1:5000) and enhanced by chemiluminescence (Amersham Biosciences).

**Histological Analysis**—Heart tissue was fixed in 10% neutral buffered formalin for 48 h. The hearts were dehydrated through a gradient of alcohols and xylene, followed by embedding in paraffin. Sections (5 \( \mu \)m) were prepared and stained with hematoxylin and eosin.

**Isolated Anterograde Perfused Heart Preparation**—Control and transgenic mice were anesthetized intraperitoneally with 100 mg/kg sodium nembutal and 1.5 units of heparin to prevent intracoronary microthrombi. The heart was rapidly excised, and the aorta was cannulated with a 20-gauge needle, followed by retrograde perfusion with a modified Krebs-Henseleit solution (mM): NaCl 118, CaCl\(_2\) 2.5, KCl 4.7, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, Na\(_2\)EDTA 0.5, NaHCO\(_3\) 25, and glucose 11. The buffer was equilibrated with 95% O\(_2\) and 5% CO\(_2\), with a pH of 7.4, and maintained at 37.4°C. A PE-50 catheter was inserted into the left atrium, advanced into the left ventricle, and forced through the ventricular apex. The proximal end of the catheter remained in the left ventricle, with the distal end connected to a pressure transducer. After the Langendorff mode retrograde perfusion was established, the pulmonary vein was opened and connected to the venous return cannula. Anterograde work-performing perfusion was initiated at a workload of 250 mg Hg ml/min, which was achieved with a venous return of 5 ml/min and aortic pressure of 50 mg Hg. Pressure loading was carried out in work-performing heart preparations. Venous return was kept constant at 5 ml/min, and afterload (mean arterial pressure) was increased to the point where the maximal rate of pressure development (\( +dP/dt \)) was not further elevated. Isoprotene (Sigma) was added to the Krebs-Henseleit solution entering the heart using a microperfusion pump (MasterFlex L/S). The signals were digitized and analyzed by software “Biobench.” Heart rate, left ventricular pressure, and the mean coronary perfusion pressure were continuously monitored. The pressure curve was used to calculate the rate of pressure development (\( +dP/dt \)) and decline (\( -dP/dt \)), time to peak pressure (TPP), and time to half-relaxation (RT\(_{50}\)). TPP and RT\(_{50}\) were normalized with respect to peak left ventricular pressure because they are dependent upon the extent of pressure development. Pressure loading was carried out at a venous return of 5 ml/min and maximum afterload values (mean aortic pressure) that can be generated at this venous return (preload). To test for a trend in the \( +dP/dt \) as an index of cardiac performance over the range of increasing afterloads, mean arterial pressures were gradually increased from 10 to 50 mm Hg with a custom-made micrometer at a constant preload of 5 ml/min. Starling curves were generated by linear regression using the software “Origin” (version 4.0, Microcal™ software). For the regression lines, the average slopes were calculated using only the initial part of the Frank-Starling curve (at cardiac work from 0 to 250 mg Hg/ml/min). Data are presented as means ± S.E., and the statistical differences were estimated by paired and unpaired t test analyses when appropriate.

**Preparation of Skinned Fiber Bundles**—The relationship between tension (stress) and ATPase activity was measured simultaneously, over a range of \( \rho \)Ca (-log of the molar free Ca\(^{2+}\) ) values, in detergent-extracted fiber bundles as described previously (6, 15). The fiber bundles (150–175 \( \mu \)m in diameter and 1.2 mm in length) were dissected from the left ventricular papillary muscle of the mice hearts and skimmed overnight in a relaxing buffer containing (in mM) KCl 150, MOPS 20, EGTA 10, free Mg\(^{2+}\) 1, MgATP\(^{2-}\) 5, creatine phosphate 12, dibutylr-biotol 0.5. The solution also contained 1% Triton X-100, creatine kinase 10 IU/ml, and protease inhibitors (1 \( \mu \)M pepstatin A, 10 \( \mu \)M leupeptin, and 100 \( \mu \)M phenylmethylsulfonyl fluoride). Fiber bundles were mounted at one end to a rod and to a force transducer at the other end. Resting sarcomere length was set at 2.2 \( \mu \)m using a laser diffraction pattern. The transmural area of the fiber bundles was computed using a optical model, and the measurements of fiber width and height were obtained. Tension was expressed as force/cross-sectional area. The ATPase activity of the fiber bundles was measured by a linked enzyme assay coupled to the breakdown of NADH as described previously (6). Tension and ATPase activity were determined over a range of \( \rho \)Ca values. ATPase activity was measured by adding ATP, to the relaxed skinned fibers as described above. Data were linearized using the Hill transformation and the \( \rho \)Ca tension or \( \rho \)Ca ATPase activity relations were fitted to the Hill equation using non-linear regression analysis to derive the \( \rho \)Ca value and Hill coefficient (6).

**RESULTS**

**Generation of Chimeric \( \alpha \)-\( \beta \)-TM Transgenic Mice**—To elucidate the relation between the carboxyl-terminal end amino acids of TM and contractile behavior in striated muscle, we
developed a transgenic mouse model with an overexpressed chimeric α/β-TM protein in the heart. The chimeric TM cDNA was generated by joining the murine α-TM cDNA (containing amino acids 1–257) to the murine β-TM cDNA (containing amino acids 258–284 and the entire 3′-UTR). This TM sequence was ligated at the 5′ end to the murine α-myosin heavy chain (α-MHC) promoter, which confers cardiac-specific expression (Fig. 1). An SV40 polyadenylation and termination cassette is located 3′ of the TM cDNA to ensure correct transcript processing. Founder mice were identified by PCR and confirmed by Southern blot analysis. Six lines of transgenic mice were produced with copy numbers ranging from 2 to 5.

**Chimeric α/β-TM Transcript and Protein Expression in the Transgenic Mice**—We measured TM expression using Northern blot analysis following RNA isolation from hearts of TG and NTG littermates. RNA from each transgenic line was isolated and probed with 32P-radiolabeled 3′-UTR sequences from α-TM and β-TM cDNAs; these nucleotide regions are specific to the particular striated muscle isoform and do not cross-react with other striated muscle TM isoforms (4). As seen in Fig. 2, endogenous α-TM is expressed in all hearts and in the skeletal muscle control. When the β-TM 3′-UTR sequence is used as a probe, the expression of chimeric TM is seen to be restricted to transgenic heart samples; this probe also hybridizes to control skeletal muscle RNA, but it is not detected in NTG cardiac RNA. The expression levels of the endogenous α-TM transcript remain relatively constant in the various transgenic mouse lines. However, the α/β-TM mRNA is expressed more variably, ranging from 93% in line 22 to 300% in line 14 of endogenous α-TM levels. This varied expression of the chimeric mRNA correlates with the copy numbers of the transgene in the different lines. GAPDH expression was ascertained to account for any loading differences among the various samples.

To compare contractile protein profiles from chimeric α/β-TM transgenic mice with controls, we isolated myofibrillar proteins from the hearts of these mice. These proteins were subject to SDS-PAGE gels and stained with Coomassie Blue to visualize protein content (Fig. 3). Results show there are no changes in the expression of cardiac contractile myofilament proteins between NTG and α/β-TM transgenic mice. Also, quantification of total TM protein levels demonstrated no significant differences among the 6 transgenic lines nor between the TG and NTG mice. Under SDS-PAGE conditions, endogenous α-TM and the chimeric α/β-TM isoforms are not separated electrophoretically, and their isoforms cannot be distinguished. To enhance the sensitivity of the SDS-PAGE assay, we conducted a Western blot analysis using a striated muscle TM-specific antibody (Fig. 3B). This antibody recognizes the three striated muscle TM isoforms with the same affinity (data not shown). However, we detected only one band in either the TG or NTG samples. Even though the chimeric α/β-TM has one more negative charge than the wild-type α-TM, there are no apparent differences in protein mobility under these experimental conditions.

There is a discrepancy between observing two TM messages (endogenous α-TM and chimeric α/β-TM), yet only one striated muscle TM protein band is detected. To resolve this issue, we conducted additional analyses to define whether only a single TM protein was being translated or whether two TM proteins were co-migrating in the gel. Previous studies in our laboratory demonstrated that addition of urea to SDS-PAGE gels facilitates separation of TM species, including the α-TM, β-TM, and TPM 3′-UTR, andLOYEE 3′-UTR, or GAPDH. MHC, myosin heavy chain; cTnl, cardiac troponin I; MLC, myosin light chain. B, 25 µg of total cardiac myofibrillar protein from NTG or TG mice (or limb skeletal muscle) run on SDS-PAGE gels and probed with the striated muscle TM-specific antibody, CH1.

**Fig. 2. RNA expression in transgenic mice.** 10 µg of total RNA (pooled from 5 mice from each line) was electrophoresed, transferred to nitrocellulose membrane, and probed with 32P-radiolabeled β-TM 3′-UTR, α-TM 3′-UTR, or GAPDH. SKM, skeletal muscle.

**Fig. 3. Contractile protein expression in chimera α/β-TM transgenic mice.** A, 25 µg of total cardiac myofibrillar protein from NTG or TG mice or limb skeletal muscle (SKM) was isolated and purified, electrophoresed on SDS-PAGE gels, and stained with Coomassie Blue dye. cTnl, cardiac troponin I; MLC, myosin light chain. B, 25 µg of total cardiac myofibrillar protein from NTG or TG mice (or limb skeletal muscle) run on SDS-PAGE gels and probed with the striated muscle TM-specific antibody, CH1.

To ascertain the distribution of α-TM and chimeric α/β-TM dimeric species in control and transgenic mice, myofibrillar...
Expression of Chimeric α-/β-Tropomyosin

proteins from NTG and TG hearts were subject to two-dimensional gel electrophoresis, 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 (14) was employed to resolve the different TM dimers and determine their subunit composition (see “Experimental Procedures”). Under reduced-reduced conditions, heart myofibrillar protein samples from NTG mice show a single area, labeled a, that is reactive with the TM antibody (Fig. 4). In agreement with previous studies on TM composition in skeletal and cardiac musculature, the results show the α-TM isomorph is predominant in the heart. The results from Fig. 2 show that in the TG mice, both α- and chimeric α-/β-TM transcripts are generated in the hearts. When the TM protein subunit composition of TG hearts is examined by two-dimensional gel electrophoresis, results show that the TM species resolve into α- and α-/β-subunits in TG samples under reduced-reduced conditions (Fig. 4). A PhosphorImager quantitative analysis shows that 51 ± 2.8% of the total cardiac TM content is the α-/β-TM chimeric protein in the TG hearts. Also the total TM protein levels are equivalent between TG and NTG hearts, which is in agreement with the results obtained from the Coomassie Blue staining analysis. These results demonstrate that both α-TM and chimeric α-/β-TM are present in the TG cardiac myofibrils.

To negate the possibility that an unexpected mutation or deletion of the α-/β-TM DNA sequences occurred during transgenesis, we conducted PCR analyses on TG mouse tail DNA. By using oligonucleotides corresponding to the α-MHC and β-TM 3′-UTR sequences as 5′ and 3′ primers, a PCR fragment was generated, amplified, cloned, and sequenced. Results show there are no mutations or deletions in the α-/β-TM nucleotide sequence (or the corresponding amino acid sequence) in the transgene construct.

Histological and Physiological Studies—None of the founder mice or their progeny demonstrates any gross phenotypic alterations or reduced viability. Over 15 transgenic mice from 3 different lines and ranging in age from 2 months to 11 months were examined for morphological changes. Hematoxylin and eosin-stained heart sections show no signs of cardiac hypertrophy, fibrosis, thrombi, necrosis, or any other pathological condition in any of the TG lines examined (data not shown). Also, no differences in percent heart/body weight ratios from the transgenic to the NTG mice are observed. Thus, incorporation of the chimeric α-/β-TM protein into the cardiac myofibrils does not lead to morphological or pathological alterations in the sarcomere.

We pursued several lines of experiments to discover the physiological significance of the 27 carboxyl-terminal amino acids of striated muscle TM in the regulation of cardiac muscle contractility. Transgenic animals from line 14 were studied with different approaches, including the isolated-work performing heart. Mice from TG lines 25 and 31 were also examined to confirm the results. Previous studies have shown that overexpression of wild-type α-TM in transgenic mice does not lead to alterations in morphology or physiological performance of the heart (6, 7); overexpression of striated muscle β-TM does lead to decreases in the rate of relaxation and an increase in the half-time of relaxation (1).

An isolated antegrade perfused heart preparation was employed to carry out a functional analysis of the chimeric α-/β-TM hearts. This model allows a comparison of myocardial contractile parameters under identical base line conditions (50 mm Hg mean aortic pressure, venous return 5 ml/min, intrinsic heart rate of 320–400 beats/min, and cardiac minute work of 250 mm Hg ml/min), and without the complicating variables imposed by reflex changes in the intact animal. Under conditions of identical load, the TG hearts produce systolic pressures that are significantly lower than the control hearts (Table I). The maximal rate of pressure development for contraction (+dP/dt) is significantly decreased: 4504 ± 421 mm Hg/s in the controls versus 3137 ± 221 mm Hg/s (p < 0.05) in the TGs, along with an increased time to peak pressure (0.38 ± 0.026 ms/mm Hg versus 0.55 ± 0.039 ms/mm Hg for NTG versus TG, respectively) (Table I and Fig. 5). Coupled with the decreased contractility, the maximal rate of pressure decline (−dP/dt) is also decreased from 3688 ± 297 mm Hg/s in wild-type to 2768 ± 238 mm Hg/s in the TG heart. Additionally, there is an increase in the end diastolic pressure and in the heart rate in the chimeric α-/β-TM hearts, coupled with an increased basal coronary flow (3.14 ± 0.35 ml/min for NTG versus 4.34 ± 0.20 ml/min for TG). Other parameters of cardiac function (time to peak pressure – TPP and the half-time of relaxation – RT1/2) were derived from intraventricular pressure tracings. TPP is defined as the time between the beginning of systole and the peak development of pressure. RT1/2 is the time from the peak intraventricular pressure to the point of 50% ventricular relaxation. Both parameters were normalized with respect to peak intraventricular pressure, because they are dependent upon the extent of pressure development. As seen in Fig. 5 and Table I, the TPP of the chimeric mice is significantly longer than that of control mice, whereas the RT1/2 is similar between the two genotypes.

Response to Change in Workload—The Starling law reflects the increase in cardiac performance in response to increased intraventricular pressure or volume load. As the cardiac muscle is stretched, cardiac myofibrils respond with increased force generation. To determine to what extent the TG and NTG hearts could be loaded with increasing workload, cardiac minute work was varied from 50 mm Hg ml/min to the maximal level of mean aortic pressure (afterload) that can be generated at a given venous return (preload) of 5 ml/min. The hearts from both NTG and TG groups were sensitive to increases in afterload. However, at a constant preload of 5 ml/min, TG hearts generate mean arterial pressure (afterload) up to 55 mm Hg, whereas NTG hearts produce afterloads up to 70–80 mm Hg. The plots of cardiac work versus the rates of pressure development (+dP/dt) are shown in Fig. 6. TG hearts demonstrate an overall reduction in +dP/dt over a range of cardiac work that can be maintained by both experimental groups, indicating a higher intrinsic contractile state in the NTG hearts. The slope

Fig. 4. Two-dimensional electrophoretic analysis with Western blot analysis of NTG and TG myofibrillar proteins. Myofibrillar TM subunit composition was analyzed by reduced-reduced two-dimensional gel electrophoresis as described under “Experimental Procedures.” The positions of the α and α-/β-chimeric TM subunits are marked.
of the initial part of the Frank-Starling left ventricular functional curves (range 0 to 250 mm Hg ml/min) calculated by linear regression analysis are not statistically different (5.89 ± 0.88 for NTG versus 4.75 ± 0.29 for TG).

Response to β-Adrenergic Stimulation—Transgenic hearts display positive inotropic and chronotropic responses to β-adrenergic receptor stimulation; however, diminished baseline +dP/dt values are only partially restored after infusion of the β-adrenergic agonist, isoproterenol (Fig. 7). In contrast, lusitropic indices (+dP/dt mm Hg/s) and parameters of cardiac dynamics (TPP and RT\(_{1/2}\)) are restored to control levels, indicating that depressed relaxation can be overcome by cAMP-dependent protein kinase-dependent phosphorylation, which increases the rate of calcium cycling and calcium re-uptake by the sarcoplasmic reticulum.

Ca\(^{2+}\) Force Measurements in Skinned Fiber Bundles—To examine the correlation between physiological results from the whole heart and chimera α-β-TM TG expression at the sarcomere level, a series of experiments was conducted using detergent-extracted (skinned) fiber bundles. In the first set of experiments, we compared the pCa tension relations obtained from NTG and TG hearts. As illustrated in Fig. 8A, pCa\(_{50}\) (−log of free [Ca\(^{2+}\)] required for half-maximum activation) is significantly different (p < 0.05) between NTG (pCa\(_{50}\) 5.84 ± 0.03) and TG (pCa\(_{50}\) 5.55 ± 0.03) fiber bundles (n = 9). These data demonstrate a significant decrease in Ca\(^{2+}\) sensitivity of TG fiber bundles containing chimeric TM protein. Maximum tension (34 ± 1.9 mN/mm\(^2\)) and the Hill n values (3.5 ± 0.7) of the fiber bundles containing chimeric TM were also significantly (p < 0.05) lower than the maximum tension (45 ± 3.6 mN/mm\(^2\)) and the Hill n value (4.3 ± 2.0) of control fiber bundles. As shown in Fig. 8B, ATPase activity of fiber bundles from TG hearts is less sensitive to Ca\(^{2+}\) (pCa\(_{50}\) 5.88 ± 0.03) than the NTG controls (pCa\(_{50}\) 5.61 ± 0.04). There is, however, no significant difference between the maximum ATPase activity of TG fiber bundles (362 ± 47 pmol/μl/s) and NTG fiber bundles (439 ± 31 pmol/μl/s) or in the Hill n values (TG 4.5 ± 2.2; NTG 3.71 ± 0.99). Interestingly, there are no significant differences in the tension cost as illustrated in relations between tension and ATPase activity shown in Fig. 8C.

### Table I

| Parameters                              | NTG n = 7 | TG n = 6 | p value |
|-----------------------------------------|-----------|----------|---------|
| Systolic pressure, mm Hg                | 111.8 ± 5.1 | 93.0 ± 6.1* | 0.044   |
| Diastolic pressure, mm Hg               | −12.9 ± 2.7  | −12.3 ± 2.8 | 0.98    |
| End diastolic pressure                   | 6.27 ± 1.99   | 14.96 ± 1.58* | 0.006   |
| Maximal rate of pressure development (+dP/dt) mm Hg/s | 4504 ± 421  | 3137 ± 221* | 0.016   |
| Maximal rate of relaxation (−dP/dt) mm Hg/s | 3668 ± 297   | 2768 ± 238* | 0.036   |
| Heart rate, beats/min                   | 351 ± 6.7    | 395 ± 19*   | 0.044   |
| Time to peak pressure (TPP), ms/mm Hg   | 0.38 ± 0.026  | 0.55 ± 0.039* | 0.004   |
| Half-time to relaxation (RT\(_{1/2}\)), ms/mm Hg | 0.53 ± 0.031  | 0.61 ± 0.049 | 0.202   |
| Coronary flow, ml/min                   | 3.14 ± 0.35   | 4.34 ± 0.20* | 0.012   |

* p < 0.05 NTG versus TG.

† p < 0.01 NTG versus TG.
DISCUSSION

The results of experiments reported here provide new and important insights into structure-function relationships of the 27 carboxyl-terminal amino acids of TM and their influence on sarcomeric performance. TM has three striated muscle isoforms: α-TM, β-TM, and TPM 3 (16, 17). We have in previous publications (1–3) addressed the functional significance of these isoforms with respect to sarcomeric performance and cardiac function. The carboxyl-terminal region of TM is known to be essential for thin filament structure and function. Its presence is crucial for the affinity of TM to actin and the ability of actin to polymerize. Deletion of this region also impairs the cooperative interaction that occurs among overlapping TM molecules (11, 12). These terminal amino acids anchor the troponin complex to TM through the binding of troponin T. Recent studies (18) show that this region splays out from a tightly packed coiled-coil α-helical structure, perhaps to facilitate troponin T binding and TM cooperativity. Other work suggests that amino acids 251–284 of α-TM may form other structures; residues 253–269 are a canonical coiled-coil; residues 270–279 form a parallel linear α-helices; and the last five residues are non-helical and flexible. Interestingly, the carboxyl-terminal region of the TM striated muscle isoforms displays significant diversity, perhaps contributing to functional differences in sarcomeric function. The work presented here directly addresses whether the amino acid differences between α-TM and β-TM in the carboxyl-terminal region cause alterations in physiological

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Fig. 7. **Isoproterenol dose-response curves in TG and NTG mouse hearts.** Mouse hearts from TG (n = 5) and NTG controls (n = 5) at 3 months of age were subjected to isolated heart analyses with increasing concentrations of isoproterenol (10⁻¹⁰–10⁻⁷). * and **, significant difference (p < 0.05 and p < 0.001, respectively) within the TG group, isoproterenol-treated versus no treatment; † and ‡, significant difference (p < 0.05 and p < 0.001, respectively) within the NTG group: isoproterenol-treated versus no treatment, # significant difference (p < 0.05) between NTG and TG groups.

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*N. Greenfield and S. Hitchcock-DeGregori, personal communication.*
Fig. 8. Ca\(^{2+}\) force relations of skinned fiber bundle preparations from NTG and chimeric α/β-TM TG mouse hearts. A. pCa force measurements. The −log of free [Ca\(^{2+}\)] \(\text{pCa}_{50}\) in the NTG fibers (open circles) was 5.84 ± 0.03; in the chimeric α/β-TM TG fiber bundle preparations (closed circles), the \(\text{pCa}_{50}\) was 5.55 ± 0.03 (significant at \(p < 0.05\)); \(n = 9\). B, pCa-ATPase measurements. ATPase production was measured with a −log of free [Ca\(^{2+}\)] in the NTG fibers (open circles) and TG fiber bundles (closed circles). The \(\text{pCa}_{50}\) for TG fiber bundles is 5.88 ± 0.03, and for the NTG fiber bundles, the value is 5.61 ± 0.04 (\(p < 0.05\)). There is no significant difference between maximum ATPase activities (TG 362 ± performance of the cardiac myofilaments and the whole heart.

What are the differences between α-TM and β-TM that lie within the 27 carboxyl-terminal amino acids that could account for the alterations in cardiac function and thin filament calcium sensitivity? The physiological effects on myofilament function appear certain to involve the flexibility of TM as well as its interactions with troponin T, actin, and with contiguous TMs along the thin filament. Differences in amino acid charge and the size of the side groups between the carboxyl-terminal amino acids of α- and β-TM may account for the functional effects observed with the isoform-specific domain switching. There are 5 amino acid differences between the terminal 27 amino acids of α-TM and β-TM of which 2 are highly conservative (L260V; I284L). Of the remaining 3 amino acid differences (L265M, H276N, and M281I), all have substantial differences in the size of the side group, and 1 contains a change in charge (H276N). This change in charge results in the β-TM having one more (−) charge than α-TM in this region. Our hypothesis is that these size differences and the charge change affect the binding of TNT to TM, altering the transition from myofilament “on” to “off” states as well as off to on states. Cooperativity may also be affected by these different amino acid changes; however, the “splayed” structure of the carboxyl terminus with the β-TM amino acids appears to be conserved (18).

Another distinct possibility for the physiological differences in the function of the whole heart and its myofilaments displayed by the TG mice is the intrinsic nature of the chimeric α/β-TM protein. TNT binds to TM in two distinct regions as follows: a weak binding around amino acids 175–190, and a strong binding between amino acids 258 and 284 (13, 19). These two TM regions may require a specific amino acid structure to synergistically regulate TNT binding and its own TM movement during the contractile and relaxation phases. Through the substitution of β-TM amino acids 258–284 for the corresponding α-TM sequence (which is only one of the domains), we may have disrupted the ability of TNT to properly regulate TM movement during thin filament activation. Work by Thomas and Smillie (20) suggests that binding of TNT to β-TM is weaker than binding to α-TM at the carboxyl terminus, which may be offset by a stronger binding of β-TM to TNT in the 175–190 region. We hypothesize that substitution of a weaker 258–284 β-TM sequence into the α-TM backbone, as performed in the α/β-TM TG mice, may have significantly decreased the ability of TNT to transduce the Ca\(^{2+}\) binding signal and to regulate TM movement on the thin filament because the two TM regions (175–190 and 258–284) can no longer function as a synergistic unit.

Surprisingly, the physiological phenotype found in the chimeric TM TG mice is more dramatic than expected. Overexpression of the entire β-TM molecule in the murine heart results in perturbations in the rate of relaxation and its RT\(_{50}\), but there are no effects on rates of contraction. In these studies, the level of β-TM protein expression (−55%) was comparable with the levels of the chimera protein expressed (−51%) in the current work. Also, there is an increase in calcium sensitivity displayed by the β-TM overexpression myofilaments as demonstrated by a leftward shift in the pCa force measurement curve (5). This effect is opposite that which is observed when the chimeric α/β-TM protein is incorporated into the myofilibr. However, it should be noted 47 versus NTG 439 ± 31 pmol/μl/s. C, stress-ATPase measurements in α/β-TM TG and NTG myofilaments. ATPase production was plotted as a function of stress in NTG (open circles) and TG (closed circles) myofilaments. There is no significant difference between NTG and TG values.
that high expression of $\beta$-TM in the heart (75% $\beta$-TM and 25% $\alpha$-TM) results in significant defects in both contractile and relaxation performance, culminating in lethality (2). The more dramatic alterations in sarcomeric function when the chimeric $\alpha$-$\beta$-TM protein is expressed can be exemplified by the decreases in $+\frac{dP}{dt}$ and $-\frac{dP}{dt}$, coupled with decreases in Ca$^{2+}$ sensitivity. These changes are opposed to the milder phenotype observed when the entire $\beta$-TM protein substitutes for the $\alpha$-TM, and supports the hypothesis that the TM molecule functions optimally as an isoform unit, rather than when specific domains are exchanged between TM isoforms.

In summary, our data show that compared with non-transgensics, hearts of mice expressing the chimeric $\alpha$-$\beta$-TM protein demonstrate decreased contractile and relaxation parameters, decreased myofilament Ca$^{2+}$ sensitivity, and decreased ATPase activity. The unexpected physiological differences associated with this hybrid protein may be due to the unique properties of this molecule. It appears that changing the structure and charge of the 27 terminal amino acids from $\alpha$-TM to $\beta$-TM decreases the transition rate from the on state to the off state of the myofilament structure, leading to decreased basal myocardial function of the chimeric $\alpha$-$\beta$-TM TG mice. As seen with $\alpha$-TM point mutations associated with familial hypertrophic cardiomyopathy and dilated cardiomyopathy, it is possible that a few amino acid changes among the TM isoforms can drastically alter various functions (i.e. cooperativity, blockage of myosin interactions, tropomyosin dimer formation, head-to-tail interactions, and interactions with actin and troponin T). An important finding of this investigation is that the carboxyl-terminal end of $\beta$-TM can impart significant changes in TM structure that alter its function and those of interacting neighbors along the thin filament. Our result also indicates that charge changes alone between $\alpha$- and $\beta$-TM isoforms cannot account for the functional differences in cardiac performance and myofilament sensitivity to calcium.
Functional Importance of the Carboxyl-terminal Region of Striated Muscle Tropomyosin
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