THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 14, Issue of April 8, pp. 13241–13249, 2005
Printed in U.S.A.

Cellular Fate of Truncated Slow Skeletal Muscle Troponin T Produced by Glu^{180} Nonsense Mutation in Amish Nemaline Myopathy*

Received for publication, December 6, 2004, and in revised form, January 10, 2005
Published, JBC Papers in Press, January 23, 2005, DOI 10.1074/jbc.M413696200

Xin Wang‡§, Qi-Quan Huang§, Mark T. Breckenridge§, Aihua Chen§, Thomas O. Crawford¶, D. Holmes Morton‖, and Jian-Ping Jin‡§**

From the ‡Section of Molecular Cardiology, Evanston Northwestern Healthcare and Northwestern University Feinberg School of Medicine, Evanston, Illinois 60201, the §Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106, the ¶Departments of Neurology and Pediatrics, Johns Hopkins University, Baltimore, Maryland 21287, and the ‖Clinic for Special Children, Strasburg, Pennsylvania 17579

A nonsense mutation at codon Glu^{180} in exon 11 of slow skeletal muscle troponin T (TnT) gene (*TNNT1*) causes an autosomal-recessive inherited nemaline myopathy. We previously reported the absence of intact or prematurely terminated slow TnT polypeptide in Amish nemaline myopathy (ANM) patient muscle. The present study further investigates the expression and fate of mutant slow TnT in muscle cells. Intact slow TnT mRNA was readily detected in patient muscle, indicating unaffected transcription and RNA splicing. Sequence of the cloned cDNAs revealed the single nucleotide mutation in two alternatively spliced isoforms of slow TnT mRNA. Mutant *TNNT1* cDNA is translationally active in *Escherichia coli* and non-muscle eukaryotic cells, producing the expected truncated slow TnT protein. The mutant mRNA was expressed at significant levels in differentiated C_2C_{12} myotubes, but unlike intact exogenous TnT, truncated slow TnT protein was not detected. Transfective expression in undifferentiated myoblasts produced slow TnT mRNA but not a detectable amount of truncated or intact slow TnT proteins, indicating a muscle cell-specific proteolysis of TnT when it is not integrated into myofilaments. The slow TnT-(1–179) fragment has substantially lower affinity for binding to tropomyosin, in keeping with the loss of one of two tropomyosin-binding sites. Our findings suggest that inefficient incorporation into myofilament is responsible for the instability of mutant slow TnT in ANM muscle. Rapid degradation of the truncated slow TnT protein, rather than instability of the nonsense mRNA, provides the protective mechanism against the potential dominant negative effect of the mutant TnT fragment.

Striated muscle contraction is initiated by a series of Ca^{2+}-induced allosteric changes in troponin and tropomyosin. The conformational changes in these thin filament regulatory proteins allow the interaction of myosin in the thick filament to form a strong cross-bridge with F-actin, activate myosin ATPase, and generate contractile force (1). Troponin is a complex of three component proteins: TnC[1] (the Ca^{2+}-binding subunit), TnI (the inhibitory subunit), and TnT (the tropomyosin-binding subunit) (2–4). In anchoring the troponin complex to tropomyosin and actin, TnT occupies an organizer position and role for this Ca^{2+} signaling machinery (3, 5).

Higher vertebrates have evolved three different types of muscle, and three homologous TnT genes exist that encode isoforms of TnT specific to each type: slow skeletal muscle TnT (*TNNT1*), fast skeletal muscle TnT (*TNNT3*), and cardiac TnT (*TNNT2*) (6–9). In adult animals, these TnT isoforms are specifically expressed within their respective muscle fiber types. Whereas the three TnT isoforms have diverged in structure, each shows evolutionary conservation across the vertebrate phylum (10), suggesting that each isoform possesses a differentiated functional role.

A nonsense mutation at codon Glu^{180} in exon 11 of slow skeletal muscle TnT gene *TNNT1* is found to cause an autosomal-recessive inherited form of nemaline myopathy named Amish nemaline myopathy (ANM) (11). This is the first skeletal muscle troponin disease identified. Whereas most human skeletal muscles contain mixed fast and slow fibers (12) and express both fast and slow TnTs (13), the fact that loss of only one isoform of TnT causes lethal myopathy provides the first evidence for the critical role of the functionally diverged fiber type-specific TnT isoforms. This mutation is predicted to produce a truncated slow TnT protein (slow TnT-(1–179)), but muscle of ANM patients showed no intact or truncated slow TnT (13). This raises a question about the expression of mutant *TNNT1* or fate of transcribed mutant slow TnT gene products in ANM muscle cells.

Imaging and structural studies suggest that TnT adopts an extended conformation (14–17). Studies using proteolytically cleaved or genetically engineered TnT fragments have identified two protein-binding domains of TnT. The C-terminal (T2) region binds to the central region of tropomyosin and interacts with TnI, TnC, and F-actin (18–20). The central region of TnT has another tropomyosin-binding site that binds to the tropomyosin head-to-tail overlap junction in the thin filament (21–23). The crystal structure of partial human cardiac troponin complex including the C-terminal domain of TnT agrees with

* This work was supported in part by National Institutes of Health NIAMS Grant AR-048816 and NICHD Grant HD-044824 (to J.-P. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY762903 and AY762904.

** To whom correspondence should be addressed. Tel.: 847-570-1960; Fax: 847-570-1865; E-mail: jpjin@northwestern.edu.

[1] The abbreviations used are: TnC, troponin C; TnI, troponin I; TnT, troponin T; ANM, Amish nemaline myopathy; CMV, cytomegalovirus; FBS, fetal bovine serum; mAb, monoclonal antibody; MHC, myosin heavy chain; RT, reverse transcription; DMEM, Dulbecco's modified Eagle's medium.

these protein binding data (17). The ANM nonsense mutation in codon Glu^{180} of the slow TnT gene results in a deletion of the C-terminal 83 amino acids of the protein, removing the majority of the T2 domain that contains the TnC, TnI, and C-terminal tropomyosin-binding sites. Nonetheless, the truncated slow TnT-(1–179) fragment retains the central tropomyosin-binding site that participates in the anchoring of troponin complex to the thin filament (18).

Despite encoding a potentially deleterious functional structure, a single copy of the mutant slow TnT allele does not appear to affect ANM carriers (11). This is in marked contrast to a human cardiac TnT truncation mutation, deleting the C-terminal 14 amino acids, which causes a dominantly inherited familial hypertrophic cardiomyopathy (24). In addition to increasing our understanding of the molecular pathology of ANM, investigation of the expression and fate of mutant slow TnT may also provide information about the protective mechanism that effectively eliminates mutant slow TnT from ANM muscle cells.

In the present study, we found that the mutant slow TnT mRNA was readily detected in patient muscle, indicating unaffected transcription and RNA processing. Sequence of the cloned cDNA reveals the single nucleotide mutation in two alternatively spliced slow TnT isoform mRNAs. The mutant cDNA can be expressed in *Escherichia coli* and non-muscle eukaryotic cells to produce truncated slow TnT protein at high levels. Transfective expression of the mutant slow TnT cDNA in C_2C_{12} myotubes produces significant amounts of the mutant mRNA, but no detectable truncated slow TnT. Furthermore, transfective expression in undifferentiated myoblasts do not produce a detectable amount of truncated or intact slow TnT proteins, indicating a muscle cell-specific proteolysis of TnT when it is not integrated into myofilaments. These results demonstrate instability of slow TnT-(1–179) specifically within muscle cells, suggesting that the loss of TnI, TnC, and one tropomyosin-binding site restricts incorporation of mutant slow TnT into the myofilament, which in turn leads to accelerated degradation. Therefore, rapid degradation of TnT-(1–179) protein, rather than loss of the nonsense mRNA, is responsible for the absence of a dominant effect of this mutation.

EXPERIMENTAL PROCEDURES

Cloning of Mutant Human Slow TnT cDNA from ANM Patient Muscle—A frozen diagnostic muscle biopsy obtained from the quadriceps muscle of a 7-week-old ANM patient was used for RNA isolation. This investigation was determined to be exempted research under section IV C criteria by the Johns Hopkins Hospital Institutional Review Board. This patient was previously confirmed for homozygous mutation at codon Glu^{180} in the slow TnT gene (11) and the muscle sample showed no detectable intact or truncated slow TnT protein (13).

As described previously (25), total RNA was isolated from ~5 mg of muscle tissue using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two μg of the muscle RNA was used to synthesize cDNA by reverse transcription (RT) using an oligonucleotide primer (5′-T19V-3′, V = A, C, or G) complementary to the poly(A) tail of mRNA in an anchored manner. From the cDNA mixture copied from all poly(A)$^+$ mRNA, cDNA encoding slow skeletal muscle TnT was amplified by PCR using oligonucleotide primers synthesized according to the human slow TnT gene sequence (GenBank™ accession number NT_011109.15, position 27912387.27928764 in the chromosome 19 genomic contig). The forward primer sequence corresponds to a part of exon 2 flanking the translation initiation codon plus an NdeI cloning site and the reverse primer sequence corresponds to a part of exon 14 flanking the translation termination codon with an EcoRI cloning site incorporated. The PCR products were verified by a secondary PCR using a slow TnT-specific internal reverse primer paired with the exon 2 forward primer, cut at the NdeI and EcoRI sites built at the ends of the cDNA, and cloned into TOPO pCR4 vector (Invitrogen). After transformation of host *E. coli* cells, colonies containing the recombinant plasmid were identified by PCR using slow TnT-specific primers and the plasmid DNA was purified for sequencing the cDNA insert by the dideoxy chain termination method at a service facility.

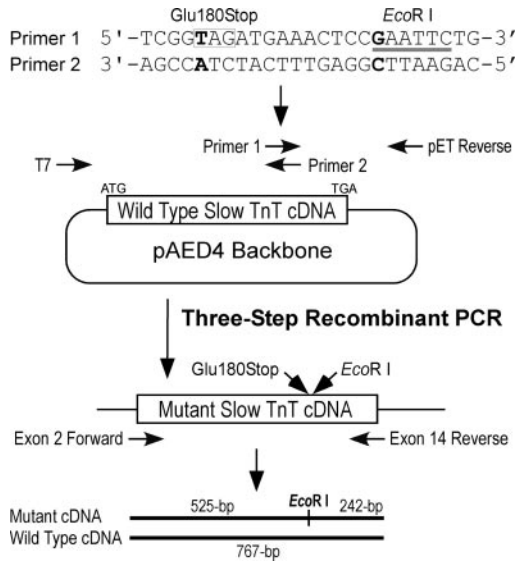

FIG. 1. **Construction of mutant mouse slow TnT cDNA and expression vectors.** A forward and a complementary reverse mutagenesis primers (*Primers 1* and *2*) were synthesized containing the G to T point mutation in codon 180 and a downstream EcoRI site to generate a unique marker for the mutant cDNA. Three-step recombinant PCR mutagenesis was carried out on mouse slow TnT cDNA templates previously cloned in pAED4 plasmid. The two DNA fragments produced by the first step PCR using primers 1 and 2 paired with pET Reverse and T7 primers, respectively, were annealed to form full length mutant cDNA that was further amplified by PCR using T7 and pET Reverse primers for cloning into pAED4 vector. The predicted RT-PCR products from the mutant and wild type slow TnT mRNA using Exon 2 forward and Exon 14 reverse primers are *outlined*. The size of the DNA fragments represents that of the low molecular weight isoform lacking the 33-bp encoded by exon5 (Fig. 3*B*).

Mutagenesis in Mouse Slow TnT cDNA—We have previously cloned full-length mouse cDNA encoding the alternatively spliced high and low molecular weight slow TnT isoforms (10). Using the cDNAs cloned in pAED4 plasmid (26) as template, recombinant PCR was used to create a stop codon at Glu^{180}. A pair of complementary mutagenesis oligonucleotide primers was synthesized to reproduce the G to T mutation found in ANM patients (11). The primer sequences and the mutagenesis strategy are shown in Fig. 1. An EcoRI restriction endonuclease cutting site was constructed in the primer sequences downstream of the mutant stop codon to introduce a unique genotyping marker for the transgene product. The mutagenesis primers were used together with the T7 and pET reverse primers flanking the 5′- and 3′-ends of the mouse slow TnT cDNA in the vector sequence to construct the mutant cDNA by a three-step recombinant PCR procedure. The reconstituted full-length slow TnT cDNA containing the point mutation and EcoRI site was digested with restriction enzymes XbaI and XhoI and ligated into compatibly cut pAED4 vector DNA. After transformation of JM109 *E. coli* cells, colonies containing the recombinant plasmids were identified by PCR using T7 primer in the 5′-flanking region of the vector sequence and a reverse primer specific to the mouse slow TnT cDNA insert.

The PCR-positive cDNA clones were further screened by mini-scale expression in *E. coli*. Briefly, the transformed JM109 *E. coli* colonies were expanded as 0.5 × 2–3-cm smear cultures on LB/ampicillin agar plates (~20 smears per 100-mm plate). Approximately one-third of each smear was transferred using a toothpick to a 0.5-ml microcentrifuge tube containing 20 μl of 1:1 phenol:chloroform and 20 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The tubes were touch spun to get the bacteria into the chemical mixture and then vortexed for 2 s. The tubes were then spun in a microcentrifuge at top speed at room temperature for 2 min. One μl of the aqueous layer that contains nucleic acid extracted from the bacteria was used to transform BL21(DE3)pLysS *E. coli*. Cells from 4 to 6 transformations can be plated in separate areas on an 100-mm LB/ampicillin/chloromphenicol plate for culture at 37 °C overnight. From each transformation, multiple bacterial colonies were collected to start 2-ml cultures in LB/ampicillin/chloromphenicol media containing 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. After being cultured at 37 °C with shaking for 3 h, the induced bacteria were collected by centrifugation, lysed in SDS gel sample buffer, and used for SDS-

PAGE and Western blot analysis as described below. After confirming expression of the anticipated slow TnT fragment, the recombinant plasmid DNA was purified from the original JM109 cultures and the slow TnT cDNA insert was sequenced as above to verify the mutagenesis as well as authenticity.

SDS-PAGE and Western Blotting—Muscle samples or bacterial extracts were homogenized in SDS-PAGE sample buffer containing 2% SDS. After being heated at 80 °C for 5 min and clarified by spinning in a microcentrifuge at top speed and room temperature for 5 min, the total protein extracts were resolved by polyacrylamide gel using the Laemmli buffer system. The resulting gels were stained with Coomassie Blue R-250 to reveal the resolved protein bands. Duplicate gels were electrically transferred to nitrocellulose membranes as previously described (27). After blocking in Tris-buffered saline containing 1% bovine serum albumin, the nitrocellulose membranes were incubated with (*a*) monoclonal antibody (mAb) CT3 recognizing slow and cardiac TnT (28), (*b*) mAb T12 against fast TnT (Ref. 29, a gift from Dr. Jim Lin, University of Iowa), (*c*) rabbit polyclonal antibody raised against fast TnT, or (*d*) mAb TnI-1 against TnI (30). The membranes were then washed with high stringency using Tris-buffered saline containing 0.5% Triton X-100 and 0.05% SDS, incubated with alkaline phosphatase-labeled anti-mouse or anti-rabbit IgG second antibody (Sigma), and washed again. The blots were developed in 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium substrate solution as previously described (27) to reveal TnT or TnI bands.

Expression and Purification of Truncated Human Slow TnT—cDNA encoding mutant human slow TnT was subcloned into the pAED4 prokaryotic expression vector as described for the mutant mouse slow TnT cDNAs. By transformation of BL21(DE3)pLysS *E. coli* cells, large scale protein expression was carried out in 2× TY media containing 100 mg/liter ampicillin and 12.5 mg/liter chloroamphenicol with vigorous shaking at 22 °C. When $A_{600\ nm}$ reached ~0.2, the cultures were induced by 0.4 mM isopropyl 1-thio-β-D-galactopyranoside and further incubated for 6–8 h. The induced bacterial cells were collected by centrifugation and lysed in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 15 mM β-mercaptoethanol by passing a French cell press at 1,000 p.s.i. three times. The cell lysis was fractionated by ammonium sulfate precipitation and the fraction between 30 and 55% saturation at 0 °C was dialyzed against 0.1 mM EDTA containing 6 mM β-mercaptoethanol. Following dialysis, the fraction was brought to 6 M urea, 0.1 mM EDTA, 15 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, clarified by centrifugation, and chromatographed on a DE-52 cellulose anion-exchange column equilibrated in the same buffer. The column was eluted with a linear KCl gradient (0–300 mM) in the same buffer and the protein peaks were analyzed by SDS-PAGE. The fractions containing human slow TnT-(1–179) fragment were dialyzed against 0.1 mM EDTA and concentrated by lyophilization. The truncated slow TnT was further purified by Sephadex G-75 gel filtration chromatography in 6 M urea, 0.5 M KCl, and 10 mM imidazole-HCl, pH 7.0, as described previously (31). Both high and low molecular weight variants of truncated human slow TnT were purified by this procedure.

Construction of Eukaryotic Expression Vectors—The mutant mouse slow TnT cDNA was isolated as an NdeI/XhoI fragment and subcloned into BamHI/XhoI cut pcDNA3.1(+) eukaryotic expression vector (Invitrogen) downstream of the cytomegalovirus (CMV) promoter for expression in non-muscle and undifferentiated muscle cells. Recombinant plasmids with slow TnT cDNA inserted in the sense orientation were selected by PCR using a CMV promoter-specific forward primer and a slow TnT cDNA-specific reverse primer. The positive plasmids were verified by restriction endonuclease mapping.

To construct an expression vector specific to differentiated muscle cells, the mutant mouse slow TnT cDNAs were inserted into the XhoI site of a promoter-less pcDNA3.1(+) plasmid upstream of the SV40 intron sequence. The CMV promoter in the pcDNA3.1(+) had been deleted by NheI and NruI digestion and self-ligation. A 5.5-kb mouse genomic DNA fragment containing the β-myosin heavy chain (β-MHC) promoter (32) (a gift from Dr. Jeffrey Robbins, University of Cincinnati) was then inserted into the NotI site upstream of the mutant mouse slow TnT cDNA. The rational for using β-MHC promoter that is active in differentiated C_2C_{12} myocytes (33) for the expression of exogenous slow TnT cDNA is to allow the transgene to be independent of the endogenous slow TnT gene regulation. The recombinant plasmids were screened by PCR and verified by restriction enzyme mapping.

Transfection of Monolayer Cell Cultures—For use in cell transfection experiments, the recombinant pcDNA3.1(+) plasmid DNA was prepared in large quantities from transformed JM109 *E. coli* using the QIA-Well cartridge (QIAgen) following the manufacturer's instructions.

Transfection of human kidney epithelial cell line 293 (ATCC CRL-1573), mouse fibroblast cell line NIH 3T3 (ATCC CRL-1658), and mouse skeletal myoblast cell line C_2C_{12} (ATCC CRL1772, Ref. 34) was carried out using the Lipofectamine™ transfection reagent (Invitrogen) following the manufacturer's instructions. Two to five μg of the recombinant supercoil plasmid DNA in 10–50 μl of TE buffer was mixed with 10–15 μl of Lipofectamine in DMEM and incubated at room temperature for 20 min. The Lipofectamine-DNA complex was gently mixed with 5 ml of DMEM without fetal bovine serum (FBS) and added to monolayer cell cultures at 50–60% confluent after removing the old medium. After incubation at 37 °C in 5% CO_2 for 6 h, 5 mL DMEM containing 20% FBS was added and the culture was continued for 12 h before change to fresh culture media containing 10% FBS. For transient transfective expressions, the cells were suspended using the Versene solution (0.537 mM EDTA, 136.8 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) (35) and washed three times with phosphate-buffered saline, pH 7.2, for the examination of slow TnT mRNA and protein expression 30 h after transfection. The elimination of trypsin digestion from the collection of cells for SDS gel and Western analysis avoided artificial enzymatic degradation of the cellular proteins.

To establish stable transfection of C_2C_{12} cells, the cells transfected with the G418 resistant recombinant pcDNA3.1 plasmid were cultured in DMEM containing 10% FBS plus G418 (500 μg/ml, ICN Biomedical, Inc.). Results from testing the tolerance of non-transfected C_2C_{12} cells to G418 showed that this cell line is sensitive to G418. In culture media containing 200 μg/ml G418, all cells died after 9 days of culture. The C_2C_{12} cell colonies resistant to G418 were individually picked up from the culture dish by trypsin digestion in small cylinders greased to the dish. The cells were expanded for extracting DNA to verify the transfection by PCR as described above. The C_2C_{12} cell lines stably transfected with the mutant slow TnT cDNA were expanded and stored in DMEM containing 35% FBS and 10% Me_2SO in liquid nitrogen for later characterization.

In Vitro Differentiation of C_2C_{12} Myocytes—C_2C_{12} is an immortalized myoblast cell line derived from adult mouse skeletal muscle (34). The cells were purchased from American Type Culture Collections and cultured in DMEM containing 10% FBS, penicillin (100 μg/ml), and streptomycin (50 μg/ml) at 37 °C in 5% CO_2. The untransfected or transfected C_2C_{12} cells were placed for *in vitro* differentiation in DMEM as above with 2% horse serum instead of FBS. The expression of TnT isoforms was examined by Western blots in both undifferentiated C_2C_{12} myoblasts and differentiated myotubes. As described above, the cells in the culture dish were suspended using the Versene solution and washed three times with phosphate-buffered saline. SDS gel sample buffer was added to dissolve the cells and to extract total cellular protein by vortexing. After heating and centrifugation of the samples, SDS-PAGE and Western blotting were carried out as above.

Restriction Endonuclease Analysis to Identify and Quantify the Mutant Slow TnT mRNA Expressed in C_2C_{12} Cells—As described above, total RNA was extracted from the stably transfected C_2C_{12} cells using the TRIzol reagent and RT-PCR was carried out to amplify the entire coding region of mouse slow TnT mRNA (Fig. 1). To obtain a quantitative comparison between the endogenous and exogenous slow TnT cDNAs, the PCR program was designed based on preliminary experiments for a pre-saturated level of amplification (data not shown). The total PCR product containing both endogenous and exogenous slow TnT cDNAs were digested with EcoRI at the site built in the exogenous cDNA but absent in the endogenous cDNA (Fig. 1). Agarose gel electrophoresis was carried out to determine the restriction pattern, and densitometry analysis of the ethidium bromide-stained bands was used to quantify the relative levels of endogenous and exogenous slow TnT mRNA. The original RNA extract equivalent to 10-fold of the amount used for the RT-PCR was used in control PCR to avoid samples with any cellular DNA contamination.

Expression of Intact Exogenous TnT in C_2C_{12} Cells—We have previously cloned and characterized a 5.4-kb mouse genomic DNA containing the slow skeletal muscle TnT gene promoter (36). To evaluate the compatibility of C_2C_{12} cellular environment in expressing exogenous TnT cDNA, we used the mouse slow TnT promoter to direct an expression of chicken fast skeletal muscle TnT in differentiated C_2C_{12} myotubes. A cDNA encoding chicken breast muscle TnT (37) was constructed downstream of the mouse slow TnT promoter using the pcDNA3.1 backbone as described above. Stably transfected cell lines were established and *in vitro* differentiation was carried out as above. Expression of chicken fast TnT in differentiated C_2C_{12} myotubes was examined by Western blotting using a polyclonal antibody, RATnT, raised against chicken breast muscle TnT (27) and an mAb, 6B8, specific to the chicken TnT isoform expressed (27) together with mAb CT3 control of endogenous cardiac and slow TnT expression.

Protein Binding Assay—To investigate the binding affinity of the ANM truncated slow TnT to tropomyosin, enzyme-linked immunosorbent assay solid phase protein binding experiments (27) were applied to compare binding of the truncated and intact slow TnT to α-tropomyosin. Intact mouse slow skeletal muscle TnT was expressed from cloned cDNA and purified as described previously (28). α-Tropomyosin was purified from rabbit heart as described previously (27). Intact slow TnT, high and low molecular weight truncated slow TnT proteins, were dissolved individually at 5 μg/ml in Buffer A (100 mM KCl, 3 mM $MgCl_2$, 10 mM PIPEs, pH 7.0) and coated onto triplicate wells of microtiter plates by incubation at 4 °C overnight. After washes with Buffer A plus 0.05% Tween 20 (Buffer T) to remove the unbound TnT, the plates were blocked with Buffer T containing 0.1% bovine serum albumin (Buffer B). The plates were then incubated with serial dilutions of rabbit α-tropomyosin in Buffer B at room temperature for 4 h. After washes with Buffer T, the bound tropomyosin was quantified via an anti-tropomyosin mAb CH1 (38), a gift from Dr. Jim Lin, University of Iowa, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin second antibody (Sigma), and H_2O_2/2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) substrate reaction using the enzyme-linked immunosorbent assay procedure described previously (27). The enzyme-linked immunosorbent assay results were recorded by an automated microplate reader (Bio-Rad Benchmark) and the $A_{405\ nm}$ values from the linear range of color development were used to plot the TnT-tropomyosin binding curves. Bovine serum albumin-coated wells were used to produce a negative control curve.

Data Analysis—DNA and protein sequence analysis was carried out using the DNA Star computer programs. Densitometry of SDS-PAGE gels and Western blots was performed on digital images scanned at 600 d.p.i. and analyzed using NIH Image program version 1.61. Statistical analysis was carried out by Student's *t* test.

RESULTS

The Mutant Slow TnT mRNA Is Present at Significant Amounts in ANM Patient Muscle—Because the limited quantity of diagnostic biopsy muscle samples precludes the use of Northern blot to detect and quantify the amount of slow TnT mRNA, we applied RT-PCR to examine the mRNA with the opportunity to clone cDNA from the mutant mRNA. Slow TnT cDNA was obtained from patient RNA on the first trial of RT-PCR using standard conditions. The easy detection of slow TnT mRNA suggests that the slow TnT mRNA was present at significant amounts in the muscle of the homozygous ANM patient. Expression of cDNA clones in *E. coli* yields protein products with the predicted sizes that are recognized by the anti-slow TnT mAb CT3 but not the anti-fast TnT mAb T12 (Fig. 2). The results verified the authenticity of the cloned cDNA and demonstrate that the CT3 epitope is preserved in the truncated slow TnT, confirming that the negative CT3 Western blots of ANM patient muscle biopsy samples indicates the absence of truncated slow TnT (13). Sequences of the ANM slow TnT cDNAs showed for the first time at the mRNA level the presence of the G to T mutation, at nucleotide 538 from the translation initiation codon, which converted codon Glu^{180} into a stop codon (Fig. 3*A*).

The Two Normally Occurring Alternatively Spliced Variants of Slow TnT mRNA Are Both Present in the ANM Muscle—Like the cardiac and fast skeletal muscle TnT genes, the slow skeletal muscle TnT gene contains alternatively spliced exons in the 5′-variable region (6). Alternative splicing of exon 5 produces a high molecular weight and a low molecular weight isoform of human slow TnT (39, 40). Sequences of multiple original slow TnT cDNAs cloned by RT-PCR from the ANM muscle revealed two alternative splicing variants including or excluding exon 5 (Fig. 3*B*). Both variants were cloned at significant frequencies. The results indicate that the Glu^{180} nonsense mutation in exon 11 did not affect the alternative splicing of exon 5 in the 5′-region. Sequences of the alternatively spliced high and low molecular weight ANM slow TnT mutants have been submitted to the GenBank™/EBI Data Bank with accession numbers AY762903 and AY762904, respectively.

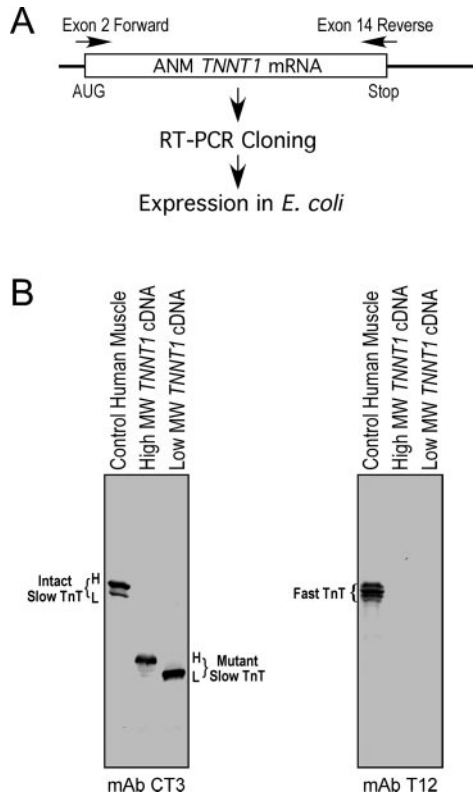

FIG. 2. **Cloning of mutant *TNNT1* cDNA from ANM muscle.** *A*, the coding region of slow TnT cDNA was cloned from ANM muscle mRNA by RT-PCR using the exon 2 forward and exon 14 reverse primers and subcloned into pAED4 vector for expression in *E. coli*. *B*, Western blots of total protein extracts from transformed *E. coli* cultures and control human skeletal muscle using mAb CT3 against slow TnT and mAb T12 against fast TnT verified authenticity of the ANM slow TnT cDNA. The results also demonstrate that both the high (*H*) and low (*L*) molecular weight ANM slow TnT fragments retain the CT3 epitope.

The Mutant Slow TnT mRNA Can Be Effectively Translated in E. coli—To investigate whether the mutant slow TnT mRNA is translatable, we first examined its capacity as a translation template in *E. coli*. The SDS gel and Western blots in Fig. 4*A* show that the high and low molecular weight variants of the mutant human slow TnT and the engineered mouse counterparts were expressed in *E. coli* at significant levels. The apparent molecular weight of the human and mouse slow TnT fragments produced in *E. coli* as reflected by SDS gel mobility is in agreement with that predicted from the cDNA sequences (Table I). Both the high and low molecular weight protein products were confirmed as slow TnT by their recognition by mAb CT3 (Fig. 4*A*). The successful expression of truncated slow TnT demonstrates that the mutant mRNA is normally translatable until the nonsense mutation-generated stop codon (Fig. 3*A*).

The effective expression of mutant cDNA in *E. coli* produced sufficient truncated slow TnT for functional analysis. Fig. 4*B* demonstrates the purity of the high and low molecular weight variants of the truncated human slow TnT produced. The high and low molecular weight slow TnT fragments behaved similarly in all steps of the purification procedure, consistent with their similar physical properties as predicted from their amino acid composition (Table I). Nonetheless, the high molecular weight slow TnT fragment was eluted from the DE52 anion exchange column at a slightly higher KCl concentration than that for the low molecular weight variant, in agreement with their charge difference because of the additional acidic residues encoded by the exon 5 sequence (Fig. 3*B*, Table I).

Expression of Endogenous TnT in C_2C_{12} Cells—We used C_2C_{12} mouse myocytes to examine the expression of mutant

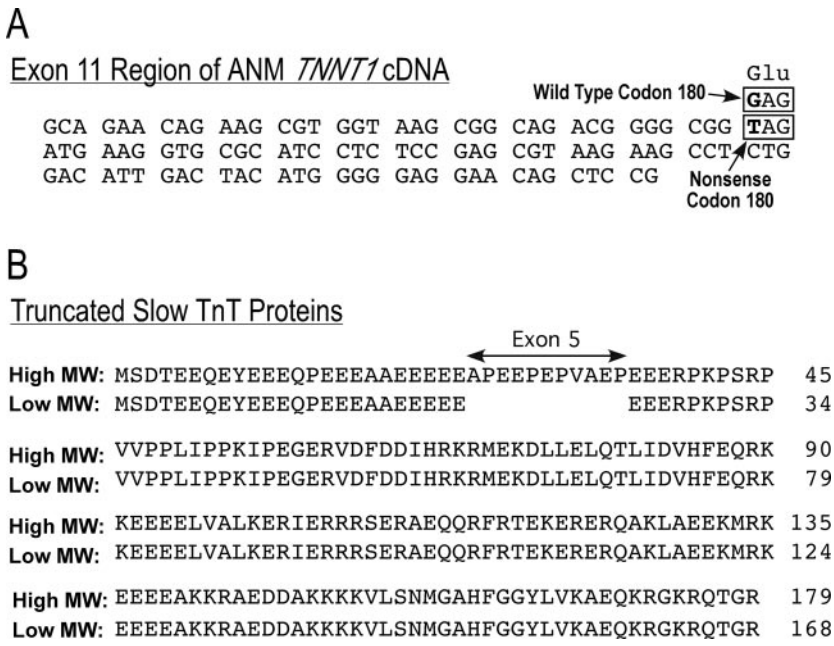

FIG. 3. **Glu[180] nonsense mutation in two alternatively spliced variants of slow TnT mRNA cloned from ANM muscle.** *A*, exon 11 sequence of slow skeletal muscle TnT cDNA cloned from the mRNA of a homozygous ANM patient muscle demonstrates the G to T nucleotide substitution at position 538 from the translation initiation codon, converting codon Glu[180] into a premature stop codon. *B*, amino acid sequence alignment of the two classes of truncated slow TnT predicted from the ANM cDNA sequences demonstrates alternative splicing of exon 5 to produce the high and low molecular weight protein variants (Table I).

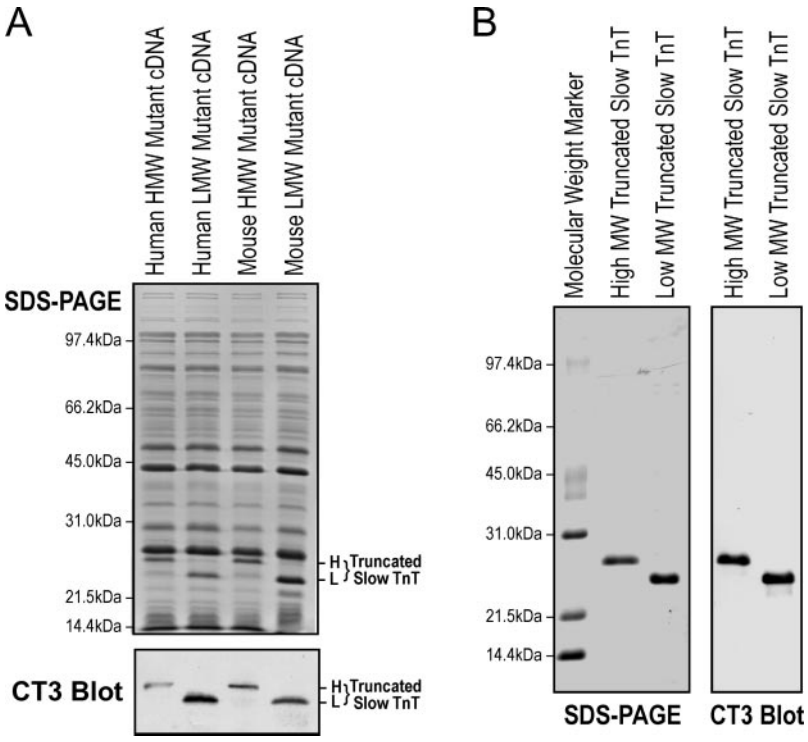

FIG. 4. **Expression of truncated slow TnT in *E. coli* and purified proteins.** *A*, SDS-PAGE using a 14% gel with a acrylamide:bisacrylamide ratio of 180:1 and Western blots using mAb CT3 demonstrated the expression of high (*H*) and low (*L*) molecular weight truncated human and mouse slow TnT proteins in *E. coli*. *B*, the SDS-PAGE gel and CT3 Western blot show the purified high and low molecular weight truncated human slow TnTs.

slow TnT mRNA and truncated protein in skeletal muscle cells. Although the C_2C_{12} cell line has been widely used in muscle studies and is known to form differentiated myotubes in culture with abundant myofilaments and muscle-specific protein expression (33, 34), the expression of TnT isoforms in C_2C_{12} cells has not been characterized. The Western blots in Fig. 5 demonstrate that very little TnT was expressed in confluent C_2C_{12} myoblasts before switching to 2% horse serum media. With differentiation, C_2C_{12} myocyte-myotubes express cardiac, slow, and fast TnT isoforms. Cardiac TnT is expressed earliest during *in vitro* differentiation and slow TnT expression follows. Fast TnT expression reaches its highest level after longer duration in the differentiation media at a time when the expression levels of cardiac and slow TnT decline. The early expression of cardiac TnT during C_2C_{12} cell differentiation mimics developmental regulation in which cardiac TnT is expressed in embryonic skeletal muscle (13, 41). A similar pattern is seen in the transition of TnI isoforms (Fig. 5). The expression of endogenous slow TnT in C_2C_{12} cells justifies its use as an experimental system to study the expression and fate of the mutant slow TnT mRNA and protein.

Multiple alternatively spliced fast TnT protein bands were detected at various levels in differentiated C_2C_{12} cells. However, only adult cardiac TnT and high molecular weight slow TnT were detected throughout the course of *in vitro* differentiation (Fig. 5). In addition to reflecting the adult muscle origin of the C_2C_{12} cell line (34), this observation suggests that alternative splicing of the transcripts of the three homologous TnT genes is differentially regulated in C_2C_{12} cells. Whereas the regulatory mechanism remains to be investigated, this feature does not preclude the use of the C_2C_{12} cell line as a muscle cellular environment to study the fate of mutant slow TnT

TABLE I

Physical properties of the truncated human and mouse slow TnT

The molecular weight (M_r) and isoelectric point of the slow TnT proteins were calculated from amino acid sequences using the DNA Star computer program. The data for intact human (39, 40) and mouse (10) slow TnT isoforms were deduced from previously published sequences.

	Molecular weight		Isoelectric point	
	High M_r	Low M_r	High M_r	Low M_r
Human intact	31,241.2	30,095.0	5.67	6.33
Human truncated	21,325.7	20,179.5	4.97	5.18
Mouse intact	31,214.2	30,008.9	5.94	6.58
Mouse truncated	21,227.7	20,022.4	5.05	5.23

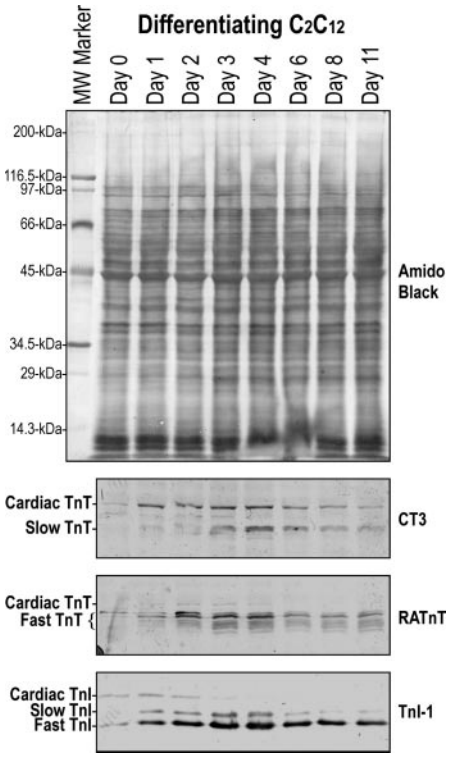

FIG. 5. **Expression of TnT and TnI isoforms in C_2C_{12} cells during *in vitro* differentiation.** Confluent C_2C_{12} cells cultured in DMEM containing 2% horse serum were harvested on various days of differentiation. Total cellular protein extracted (shown in the Amido Black-stained nitrocellulose membrane, as expected, the high molecular weight proteins were transferred less effectively) was examined by Western blotting to monitor the expression of TnT isoforms. Cardiac and slow TnT expression was examined using mAb CT3, fast TnT was examined using polyclonal antibody RATnT, and TnI was examined using mAb TnI-1. The results show that cardiac TnT was expressed in C_2C_{12} cells during the earlier phase of *in vitro* differentiation. The expression of slow TnT followed and fast TnT was expressed latest with alternatively spliced isoforms. A similar transition pattern was seen for TnI isoforms.

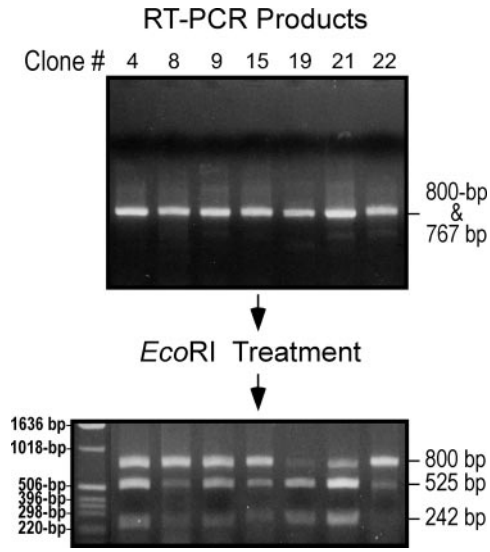

FIG. 6. **Expression of mutant slow TnT mRNA in transfected C_2C_{12} cells.** The expression of mutant low molecular weight slow TnT isoform mRNA in stably transfected C_2C_{12} cell lines after 5 days of *in vitro* differentiation was examined by RT-PCR and 1.2% agarose gel electrophoresis. The *upper panel* shows the total slow TnT cDNA (endogenous and exogenous) amplified. The endogenous slow TnT expressed in C_2C_{12} cells is mainly the high molecular weight isoform (Fig. 5). The 800-bp and 767-bp high and low molecular weight cDNAs ran together in the 1.2% gel. The total cDNA was digested with EcoRI that specifically cleaves the exogenous 767-bp mutant but not the wild type endogenous cDNA (Fig. 1). The *lower panel* agarose gel demonstrates that the mutant slow TnT cDNA was detected in the transfected cells at various levels as compared with that of endogenous wild type cDNA. Normalized by the fragment sizes, densitometry analysis was performed to quantify the exogenous *versus* endogenous slow TnT mRNA in each sample.

mRNA and protein, because overexpression of mutant slow TnT cDNA does not involve RNA splicing.

Transfective Expression of Mutant TNNT1 cDNA in C_2C_{12} Myotubes Produces Mutant Slow TnT mRNA but No Detectable Truncated Protein—We established multiple stably transfected C_2C_{12} cell lines to test the expression of mutant slow TnT cDNA. PCR on DNA extracted from the cells confirmed the presence of the β-MHC promoter-directed slow TnT transgene (data not shown). RT-PCR using the Exon 2 forward and Exon 14 reverse primer pair that is common to the wild type and mutant mRNAs (Fig. 1) with non-saturating cycling numbers readily detected slow TnT cDNA from total RNA extracted from transfected C_2C_{12} cells that had undergone 5 days of differentiation in culture. Digesting the PCR-amplified slow TnT cDNA with EcoRI produced fragments specific to the exogenous mutant slow TnT mRNA as well as uncut cDNA from endogenous slow TnT mRNA (Fig. 6). The results show that expression levels of mutant mRNA can reach 30–80% that of the total slow TnT mRNA (exogenous plus endogenous).

Despite the presence of mutant slow TnT mRNA at levels comparable with or higher than that of endogenous slow TnT in the transfected C_2C_{12} cells, no truncated slow TnT protein could be detected in the total protein extracts (Fig. 7*A*). Because the mutant mRNA is translatable as shown in *E. coli* expression (Fig. 4*A*), the results suggest that no significant amount of truncated slow TnT was accumulated in the myocytes.

It is notable that, although truncated slow TnT protein was not found, overexpression of this exogenous mutant slow TnT mRNA diminishes the level of endogenous slow TnT. Shown in Fig. 7*B*, an inverted correlation was found between the levels of exogenous slow TnT mRNA (relative to total slow TnT mRNA) and endogenous slow TnT protein (relative to the level of cardiac TnT that is used as a differentiation state control). This observation suggests that overexpression of slow TnT mRNA may have a negative feedback effect on the level of endogenous slow TnT gene expression. Expression of exogenous slow TnT mRNA in the present study was directed by β-MHC promoter and, therefore, not subject to this feedback regulation.

Intact Exogenous TnT Can Be Expressed in Differentiated but Not Undifferentiated C_2C_{12} Cells and ANM Truncated Slow TnT Can Be Produced in Non-muscle Cells—To verify the capacity of C_2C_{12} cells for expressing exogenous TnT, we established stable transfective expression of a cDNA encoding chicken fast skeletal muscle TnT under control of the cloned mouse slow TnT gene promoter. The Western blots in Fig. 8*A*

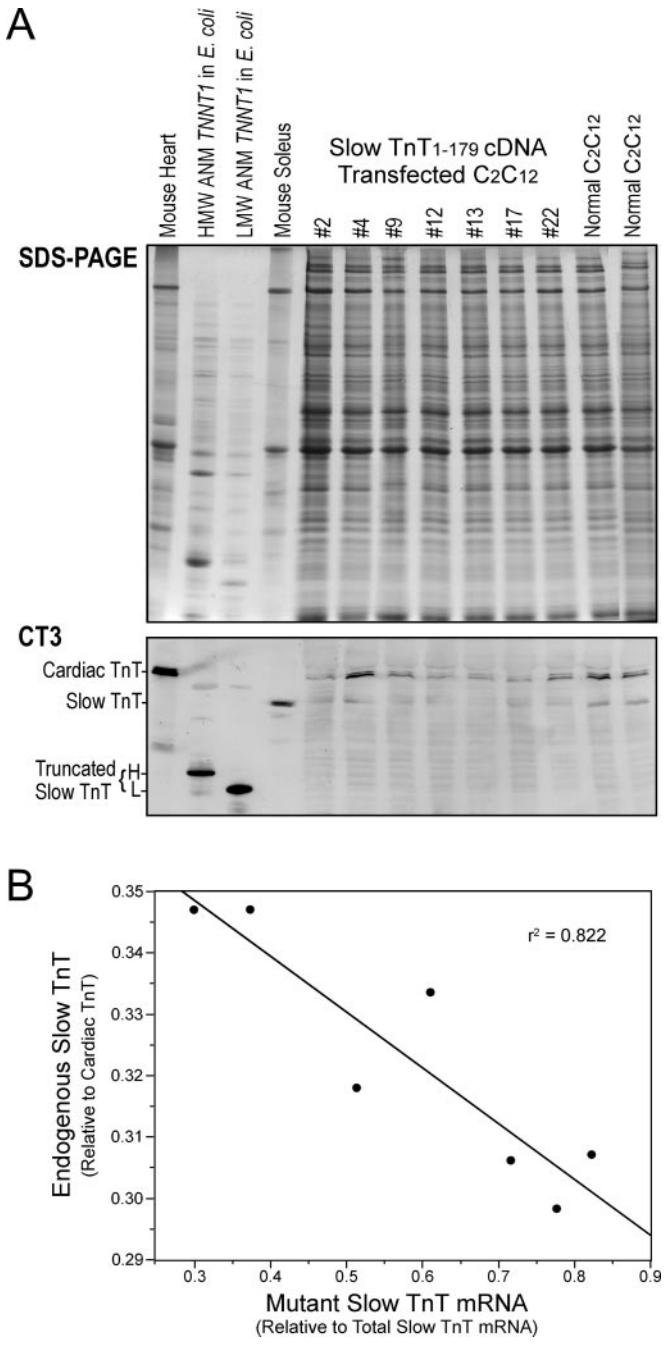

FIG. 7. **Overexpression of mutant slow TnT mRNA in C_2C_{12} cells did not produce detectable truncated slow TnT but reduced the level of endogenous slow TnT.** *A*, Western blots using mAb CT3 showed that no truncated slow TnT protein was detectable in the stably transfected C_2C_{12} myotubes despite the significant amounts of mutant slow TnT mRNA (Fig. 6*B*). High (*H*) and low (*L*) molecular weight truncated slow TnT proteins expressed in *E. coli* were used as control. *B*, the correlation between the level of mutant slow TnT mRNA relative to the total slow TnT mRNA (Fig. 6) and the level of endogenous wild type slow TnT protein normalized with cardiac TnT expressed in each transfected C_2C_{12} cell line (*panel A*) was examined. The result shows an inverted correlation, suggesting an inhibition of endogenous slow TnT expression by overexpression of mutant slow TnT mRNA ($p < 0.05$).

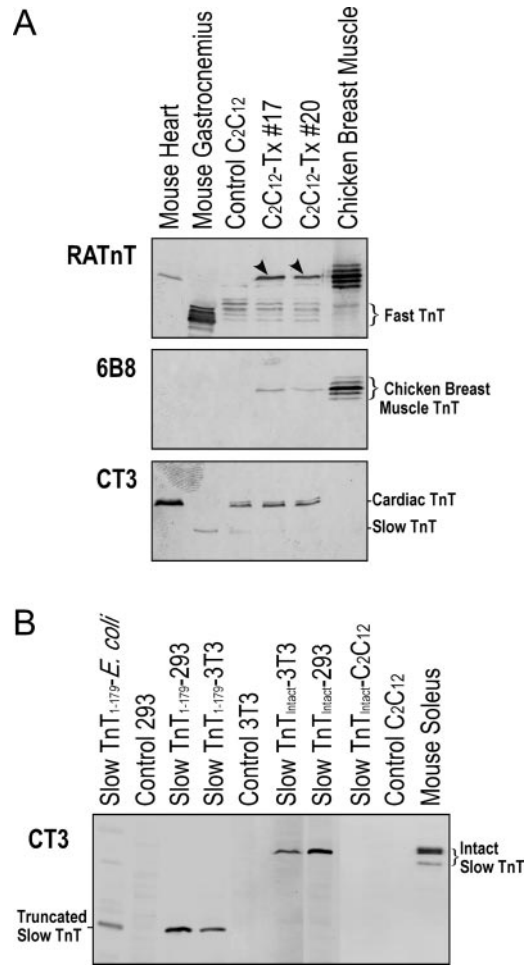

FIG. 8. **Transfective expression of intact exogenous TnT in C_2C_{12} cells and expression of truncated and intact slow TnT in non-muscle cells.** *A*, two stably transfected C_2C_{12} lines were examined along with controls to demonstrate the expression of chicken breast muscle TnT in differentiated C_2C_{12} cells under the direction of the mouse slow TnT promoter. The Western blot using a polyclonal anti-TnT antibody RATnT shows significant amounts of chicken TnT expressed in C_2C_{12} mouse myotubes. The feasibility of expressing intact exogenous TnT in C_2C_{12} myotubes was confirmed by Western blot using mAb 6B8 specific to the chicken TnT isoform. The less intense Western staining of 6B8 *versus* that of the polyclonal RATnT reflects the nature of single *versus* multiple epitope binding. *B*, CT3 Western blots show that transient transfection of the CMV promoter-directed expression vectors produced significant amounts of the ANM truncated slow TnT (*Slow* TnT_{1-179}) in 293 human epithelial cells and 3T3 mouse fibroblasts. Like that in differentiated C_2C_{12} myotubes (Fig. 7*A*), transfective expression using CMV promoter failed to produce truncated slow TnT protein in undifferentiated C_2C_{12} myoblasts. Parallel transient transfections produced significant levels of intact slow TnT in 293 and 3T3 cells but not undifferentiated C_2C_{12} cells, suggesting a muscle cell-specific protein degradation. In contrast to the low level TnT expression in confluent C_2C_{12} cells at the beginning of differentiation (Fig. 5), undifferentiated C_2C_{12} showed no endogenous TnT.

showed high level expression of chicken TnT in differentiated C_2C_{12} myotubes. The successful expression of avian TnT in mouse myocytes demonstrates the feasibility of expressing intact exogenous TnT in differentiated C_2C_{12} cells.

To verify that the ANM mutant slow TnT mRNA can be translated in eukaryotic cells, Fig. 8*B* shows that transient transfection of the CMV promoter-directed expression construct produced significant amounts of the ANM truncated slow TnT in 293 epithelial cells and 3T3 fibroblasts. These results indicate that the mutant slow TnT mRNA is translatable in eukaryotic cells and the truncated slow TnT protein can be accumulated to a significant level in non-muscle cells.

The results in Fig. 8*B* further show that CMV promoter-directed transient transfection can express intact slow TnT in 293 and 3T3 cells but not in undifferentiated pre-confluent C_2C_{12} myoblasts. RT-PCR on DNase-treated RNA extracted from the transient transfected C_2C_{12} cells confirmed the expression of mutant slow TnT mRNA (data not shown). No

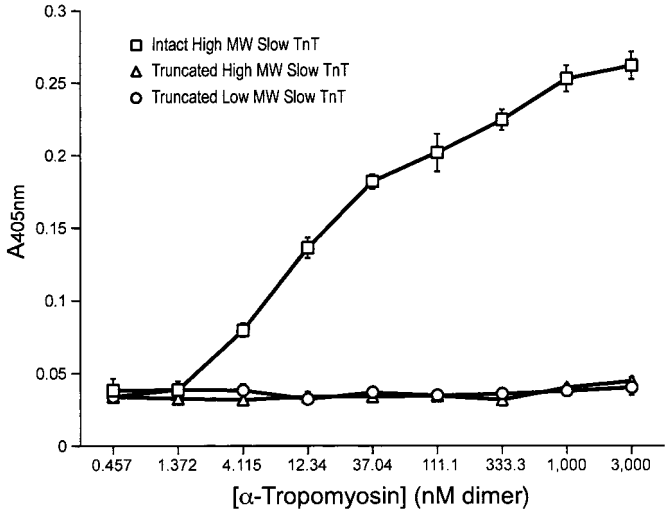

FIG. 9. **Low binding affinity of the truncated slow TnT to tropomyosin.** Solid phase protein binding assays showed that the binding of truncated slow TnT to rabbit α-tropomyosin was undetectable by the high strangency binding assay, in contrast to the saturable binding curve of intact slow TnT. The high and low molecular weight (*MW*) variants showed similar low binding affinity.

endogenous TnT was detected in undifferentiated C_2C_{12} cells (Fig. 8*B*), indicating the absence of myofilaments. The results demonstrate a muscle cell-specific rapid degradation of intact as well as truncated slow TnT proteins in the absence of myofilaments. Because intact exogenous TnT is stable in differentiated C_2C_{12} cells that contain myofilaments (Fig. 8*A*), formation of troponin complex and/or association with the thin filaments may be required for TnT protein to be stable in muscle cells.

Loss of High Affinity Binding of Truncated Slow TnT to Tropomyosin—The truncated slow TnT produced by the ANM Glu^{180} nonsense mutation lacks the C-terminal TnI-, TnC-, and tropomyosin-binding sites (13) and is unable to form troponin complex. However, it retains the central tropomyosin-binding site (42) and can potentially interact with tropomyosin or compete with intact TnT, theoretically producing a dominant negative effect in ANM carriers. The results in Fig. 9 show that binding of the truncated slow TnT to tropomyosin is almost non-detectable in the solid phase protein binding assay, whereas a saturable binding was found between intact slow TnT and tropomyosin. There was no difference between high and low molecular weight truncated variants. Because the solid phase enzyme-linked immunosorbent assay protein binding experiments are done with repeated washing, these results only reflect the loss of high affinity binding (31), although low affinity binding between truncated slow TnT and tropomyosin is expected (42).

DISCUSSION

Slow TnT Gene Transcription and RNA Processing Are Preserved in ANM Muscle—The ANM *TNNT1* mutation was first discovered through genetic linkage analysis and genomic DNA sequencing (11). The present study provides data to show the normal expression of the mutant transcript in ANM muscle. Thus neither nonsense mutation-mediated mRNA decay (43), nor mutation-induced aberration of splicing can explain the absence of truncated slow TnT in ANM muscle. Moreover, the mutation does not alter the native pattern (6) of alternative splicing of the RNA transcript of the slow TnT gene, further demonstrating the absence of mutational effects upon splicing. The apparently normal transcription and RNA processing as well as the abundance of the mutant slow TnT mRNA in ANM muscle provides a potential therapeutic target, using somatic therapies to correct the slow TnT protein deficiency. This finding suggests that reagents that promote translational readthrough of aberrant nonsense mutations, such as the aminoglycoside antibiotic gentamycin (44), may be of potential benefit as a specific treatment. Further testing of this hypothesis would be necessary before contemplating human trials.

Two-site Binding to Tropomyosin Is Essential to the Incorporation of TnT into Myofilament—Predicted from previous studies on various TnT fragments (21, 42), the ANM truncated slow TnT still contains a tropomyosin-binding site and may compete with intact TnT in the ANM muscle cell. This raised the intriguing question as to why ANM heterozygotes do not show a muscle phenotype. The comparison of binding affinity of intact and truncated slow TnT to tropomyosin demonstrates that slow TnT-(1–179) does not bind tropomyosin strongly (Fig. 9). This result supports a hypothesis that the two-site binding to tropomyosin is necessary to anchor TnT and the rest of the troponin complex to the thin filament.

The tropomyosin-binding site in the C-terminal domain of TnT, lost in the truncated slow TnT-(1–179) fragment, is not thought to be of high affinity (21). Therefore, the high affinity binding of TnT to tropomyosin must be a consequence of both sites acting together. This requirement in forming a stable thin filament structure suggests further that the two-site connection between TnT and tropomyosin is constant during muscle activation and relaxation cycles, supporting an elongated conformation of TnT in the thin filament assembly (14, 15). This two-site anchoring requirement may also be a structural base for the observation that the strong binding between TnT and tropomyosin is a corporative process in the absence of F-actin (45).

Rapid Degradation of Unincorporated TnT in Myocytes Is Responsible for the Absence of Truncated Slow TnT Protein in ANM Muscle and Prevents Dominant Negative Effect—To understand the missing link between abundant mutant mRNA and lack of truncated slow TnT protein in ANM muscle cells, we first tested whether the mutant mRNA can be translated into protein. Expression of mutant human *TNNT1* cDNA in *E. coli* and non-muscle eukaryotic cells yields easily detectable levels of the truncated slow TnT protein, proving the stability and capacity for active translation of the mRNA. However, expression of mutant slow TnT mRNA in C_2C_{12} myocytes failed to produce a detectable amount of the truncated slow TnT protein. These results suggest that muscle cell-specific protein degradation is responsible for the absence of truncated slow TnT protein in ANM muscle.

Skeletal muscle protein is a major source of the energy supplied to the organism during the catabolic state (46). Muscle cells may thus have a high intrinsic proteolytic activity. Nonetheless, transfective expression could produce significant amounts of intact exogenous TnT in differentiated but not undifferentiated C_2C_{12} cells (Fig. 8). The data that successful accumulation of TnT in muscle cells requires integrity of TnT as well as the presence of myofilaments suggests that the inability of slow TnT-(1–179) to form troponin complex and/or strongly bind tropomyosin may be the reason for its sensitivity to degradation. Therefore, the intrinsic high proteolytic activity of muscle cells may be specifically directed to non-incorporated myofilament proteins.

Striated muscle contraction is based on the highly organized sarcomere structure. A high constitutive proteolytic activity against unincorporated sarcomeric proteins may represent a general mechanism of surveillance by which the highly organized, multimeric myofilament structure is protected against a potential "weakest link" produced by incorporation of a dam-

aged sarcomeric protein. Damaged or improperly processed proteins that are not incorporated into or dissociated from the myofilaments will be degraded rapidly. Although the single site binding between the truncated slow TnT and tropomyosin is much weaker than that of intact slow TnT (Fig. 9), even a low affinity interaction with tropomyosin may affect physiological function of the muscle if a high level of truncated slow TnT is accumulated in muscle cells. A previous report has shown that the slow TnT-(1–179) fragment does not bind TnC or TnI and cannot reconstitute troponin complex. When used to reconstitute myofilaments, the truncated slow TnT caused severe loss of Ca^{2+} regulation of actomyosin ATPase (47). These authors also showed that when present at a high concentration, the slow TnT-(1–179) fragment could displace endogenous TnT in myofibril *in vitro*. Therefore, the muscle cell-specific degradation of truncated slow TnT converts a potentially dominant mutation (which is a characteristic of most sarcomeric structural protein mutations) to a recessive mutation.

Adult Skeletal Muscle-derived Stem Cells May Retain a Potential to Express Embryonic Genes—It is known that cardiac TnT is expressed in embryonic skeletal muscle cells (41). The presence of cardiac TnT as well as embryonic isoforms of fast skeletal muscle TnT in fetal and neonatal ANM patient muscle is proposed as the molecular basis for the fact that ANM patients have normal muscle function at birth but develop features of myopathy during infancy, correlating with postnatal down-regulation of the fetal TnT isoforms (13). The potential compensation of fetal TnT to the lack of slow TnT in ANM neonates suggests a therapeutic target. Although the C_2C_{12} myoblast cell line was established from adult mouse skeletal muscle (34), the expression of cardiac TnT at significant levels in the early state of *in vitro* differentiation (Fig. 5) indicates that the developmental pattern of skeletal muscle gene regulation can be recapitulated in certain settings. The potential for adult skeletal muscle-derived stem cells to re-express cardiac TnT suggests another possible means to compensate for the absence of functional slow TnT (13). It is also interesting to note that multiple alternatively spliced fast TnT protein bands were detected at various levels in differentiating C_2C_{12} cells. Because the high molecular weight fast TnTs represent acidic embryonic isoforms (25) that may be able to compensate for the loss of slow TnT in embryonic ANM muscle (13), this finding suggests that promoting embryonic alternative splicing pathways for the fast TnT mRNA may also provide compensation to the lack of slow TnT in ANM muscle. Investigating the mechanisms may identify approaches to promote the expression of cardiac TnT and/or embryonic fast TnT in ANM patient muscle as a treatment of this lethal disease.

Acknowledgments—We thank Stephen Chong for technical assistance, Dr. Jim Lin for the T12 and CH1 mAbs, and Dr. Jeffrey Robbins for the β-MHC promoter.

REFERENCES

1. Gordon, A. M., Homsher, E., and Regnier, M. (2000) *Physiol. Rev.* **80,** 853–924
2. Leavis, P. C., and Gergely, J. (1984) *CRC Crit. Rev. Biochem.* **16,** 235–305
3. Tobacman, L. S. (1996) *Annu. Rev. Physiol.* **58,** 447–481
4. Lehrer, S. S., and Geeves, M. A. (1998) *J. Mol. Biol.* **227,** 1081–1089
5. Perry, S. V. (1998) *J. Muscle Res. Cell Motil.* **19,** 575–602
6. Huang, Q.-Q., Chen, A., and Jin, J.-P. (1999) *Gene* (*Amst.*) **229,** 1–10
7. Barton, P. J., Cullen, M. E., Townsend, P. J., Brand, N. J., Mullen, A. J., Norman, D. A., Bhavsar, P. K., and Yacoub, M. H. (1999) *Genomics* **57,** 102–109
8. Breitbart, R. E., and Nadal-Ginard, B. (1986) *J. Mol. Biol.* **188,** 313–324
9. Jin, J.-P., Huang, Q.-Q., Yeh, H.-I, and Lin, J. J.-C. (1992) *J. Mol. Biol.* **227,** 1269–1276
10. Jin, J.-P., Chen, A., and Huang, Q.-Q. (1998) *Gene* (*Amst.*) **214,** 121–129
11. Johnston, J. J., Kelley, R.I., Crawford, T. O., Morton, D. H., Agarwal, R., Koch, T., Schaffer, A. A., Francomano, C. A., and Biesecker, L. G. (2000) *Am. J. Hum. Genet.* **67,** 814–821
12. Baldwin, K. M., and Haddad, F. (2001) *J. Appl. Physiol.* **90,** 345–357
13. Jin, J.-P., Brotto, M. A., Hossain, M. M., Huang, Q. Q., Brotto, L. S., Nosek, T. M., Morton, D. H., and Crawford, T. O. (2003) *J. Biol. Chem.* **278,** 26159–26165
14. Cabral-Lilly, D., Tobacman, L. S., Mehegan, J. P., and Cohen, C. (1997) *Biophys. J.* **73,** 1763–1770
15. Wendt, T., Guenebaut, V., and Leonard, K. R. (1997) *J. Struct. Biol.* **118,** 1–8
16. Jin, J.-P., and Root, D. D. (2000) *Biochemistry* **39,** 11702–11713
17. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) *Nature* **424,** 35–41
18. Mak, A. S., and Smillie, L. B. (1981) *J. Mol. Biol.* **149,** 541–550
19. Pearlstone, J. R., and Smillie, L. B. (1982) *J. Biol. Chem.* **257,** 10587–10592
20. Heeley, D. H., and Smillie, L. B. (1988) *Biochemistry* **27,** 8227–8232
21. Heeley, D. H., Golosinska, K., and Smillie, L. B. (1987) *J. Biol. Chem.* **262,** 9971–9978
22. Ishii, Y., and Lehrer, S. S. (1991) *J. Biol. Chem.* **266,** 6894–6903
23. Schaertl, S., Lehman, S. S., and Geeves, M. A. (1995) *Biochemistry* **34,** 15890–15894
24. Tardiff, J. C., Factor, S. M., Tompkins, B. D., Hewett, T. E., Palmer, B. M., Moore, R. L., Schwartz, S., Robbins, J., and Leinwand, L. A. (1998) *J. Clin. Investig.* **101,** 2800–2811
25. Wang, J., and Jin, J.-P. (1997) *Gene* (*Amst.*) **193,** 105–114
26. Jin, J.-P. (1995) *J. Biol. Chem.* **270,** 6908–6916
27. Wang, J., and Jin, J.-P. (1998) *Biochemistry* **37,** 14519–14528
28. Jin, J.-P., Chen, A., Ogut, O., and Huang, Q.-Q. (2000) *Am. J. Physiol.* **279,** C1067–C1077
29. Lin, J. J.-C., Feramisco, J. R., Blose, S. H., and Matsumura, F. (1984) in *Monoclonal Antibodies and Functional Cell Lines* (Kennett, R. H., Bechtol, K. B., and McKearn, T. J., eds) pp. 119–151, Plenum Publishing Co., New York
30. Jin, J.-P., Yang, F.-W., Yu, Z.-B., Ruse, C. I., Bond, M., and Chen, A. (2001) *Biochemistry* **40,** 2623–2631
31. Biesiadecki, B. J., and Jin, J.-P. (2002) *J. Biol. Chem.* **277,** 18459–18468
32. Rindt, H., Gulick, J., Knotts, S., Neumann, J., and Robbins, J. (1993) *J. Biol. Chem.* **268,** 5332–5338
33. Miller, J. B. (1990) *J. Cell Biol.* **111,** 1149–1159
34. Yaffe, D., and Saxel, O. (1977) *Nature* **270,** 725–727
35. Hossain, M. M., Hwang, D.-Y., Huang, Q.-Q., Sasaki, Y., and Jin, J.-P. (2003) *Am. J. Physiol.* **284,** C156–C167
36. Huang, Q.-Q., and Jin, J.-P. (1999) *J. Mol. Evol.* **49,** 780–788
37. Ogut, O., and Jin, J.-P. (1998) *J. Biol. Chem.* **273,** 27858–27866
38. Lin, J. J.-C., Chou, C.-S., and Lin, J. L.-C. (1985) *Hybridoma* **4,** 223–242
39. Gahlmann, R., Troutt, A. B., Wade, R. P., Gunning, P., and Kedes, L. (1987) *J. Biol. Chem.* **262,** 16122–16126
40. Samson, F., Mesnard, L., Mihovilovic, M., Potter, T.G., Mercadier, J.-J., Roses, A. D., and Gilbert, J. R. (1994) *Biochem. Biophys. Res. Comm.* **199,** 841–847
41. Jin, J.-P. (1996) *Biochem. Biophys. Res. Commun.* **225,** 883–889
42. Pearlstone, J. R., and Smillie, L. B. (1977) *Can. J. Biochem.* **55,** 1032–1038
43. Maquat, L. E. (1995) *RNA* (*N. Y.*)**1,** 453–465
44. Barton-Davis, E. R., Cordier, L., Shoturma, D. I., Leland, S. E., and Sweeney, H. L. (1999) *J. Clin. Investig.* **104,** 375–381
45. Ogut, O., and Jin, J.-P. (2000) *J. Biol. Chem.* **275,** 26089–26095
46. Garrow, J. S. (1982) *Proc. Nutr. Soc.* **41,** 373–379
47. Gomes, A., Miller, T.E., Jaquez, O., Ampie, L., and Potter, J. D. (2003) *Biophys. J.* **84,** 319a