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

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 differentiating C_{2}C_{12} myotubes, but unlike intact exogenous TnT, truncated slow TnT protein was not detected. Transfection experiment 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 (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 phyllum (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 GenBankTM/EBI Data Bank with accession number(s) AY762903 and AY762904.

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these protein binding data (17). The ANM nonsense mutation in codon Glu180 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 C2C12 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 specimen 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 Glu180 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 the plasmid DNA was purified for sequencing the cDNA insert by the manufacturer’s protocol. Two transformations of JM109 E. coli were 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. 3B).

dideoxy chain termination method at a service facility.

**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 Glu180. 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 two 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 Xhol 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 BL21DE3pLyS S E. coli. Cells from 4 to 6 transformations can be plated in separate areas on an 100-mm LB/ampicillin/chloramphenicol plate for culture at 37 °C overnight. From each transformation, multiple bacterial colonies were collected to start 2-ml cultures in LB/ampicillin/chloramphenicol 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 Comassie 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 (29), (c) rabbit polyclonal antibody raised against fast TnT, or (d) mAb Tn-I against Tn(I) (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-indolyphosphate/nitro blue tetrazolium substrate solution as previously described (26) to reveal TnT or TnI bands.

**Expression and Purification of Truncated Human Slow TnT—cDNA encoding mutant human slow TnT was subcloned into the pAE4 prokaryotic expression vector as described for the mutant mouse slow TnT cDNAs. By transformation of BL21(DE3)pLyS E. coli cells, large scale protein expression was carried out in 2 x 10^6 T-cell media containing 100 mg/liter ampicillin and 12.5 mg/liter chloramphenicol with vigorous shaking at 22 °C. When A600nm 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 lysate was fractionated by ammonium sulfate precipitation and 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% 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 separation on a HiLoad 16/60 Superdex 200 column (Pharmacia). The purified proteins were verified by restriction endonuclease mapping analysis.

**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 (In vitro) 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 analysis.

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 NdeI 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 C2C12 myocytes (33) for the expression of exogenous slow TnT cDNA was that the expression of the transgenic 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 fibroblasts cell line NIH 3T3 (ATCC CRL-1658), and mouse skeletal myoblast cell line C2C12 (ATCC CRL1772, Ref. 34) was carried out using the Lipofectamine™ transfection reagent (Invitrogen) following the manufacturer’s instructions. Two to three days of the recombinant supercoiled 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% CO2 for 6, 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 transfection experiments, the cells were suspended using the Versene solution (0.537 mM EDTA, 136.8 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4, 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 C2C12 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 C2C12 cells revealed that this cell line is sensitive to G418 and transfected cells were resistant to G418. The C2C12 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 C2C12 cell lines stably transfected with the mutant slow TnT cDNA were expanded and stored in DMEM containing 200 μg/ml G418, all cells died after 9 days of culture. The C2C12 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 C2C12 cell lines stably transfected with the mutant slow TnT cDNA were expanded and stored in DMEM containing 25% FBS and 10% MeSO in liquid nitrogen for later characterization.

**In Vitro Differentiation of C2C12 Myocytes—**C2C12 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% CO2. The untransfected or mock-transfected C2C12 cells were cultured in DMEM containing 200 μg/ml G418 as above with 2% horse serum instead of FBS. The expression of TnT isoforms was examined by Western blots in both undifferentiated C2C12 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 boiling. The extracted total cellular protein was separated by SDS-PAGE. The separated protein bands were visualized by Coomassie Blue R-250 to reveal the resolved protein bands.
Truncated Slow Troponin T in Nemaline Myopathy

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 Glu180 into a stop codon (Fig. 3A).

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. 3B). Both variants were cloned at significant frequencies. The results indicate that the Glu180 nonsense mutation in exon 11 did not affect the alternative splicing of exon 5 in the 5′-regions. Sequences of the alternatively spliced high and low molecular weight ANM slow TnT mutants have been submitted to the GenBankTM/EBI Data Bank with accession numbers AY762903 and AY762904, respectively.

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. 4A 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. 4A). The successful expression of truncated slow TnT demonstrates that the mutant mRNA is normally translatable until the nonsense mutation-generated stop codon (Fig. 3A).

The effective expression of mutant cDNA in E. coli produced sufficient truncated slow TnT for functional analysis. Fig. 4B 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. 3B, Table I).

Expression of Endogenous TnT in C2C12 Cells—We used C2C12 mouse myocytes to examine the expression of mutant.
slow TnT mRNA and truncated protein in skeletal muscle cells. Although the C_{2}C_{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_{2}C_{12} cells has not been characterized. The Western blots in Fig. 5 demonstrate that very little TnT was expressed in confluent C_{2}C_{12} myoblasts before switching to 2% horse serum media. With differentiation, C_{2}C_{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_{2}C_{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 TnT isoforms (Fig. 5). The expression of endogenous slow TnT in C_{2}C_{12} cells justifies its use as an experimental system to study the expression and fate of the mutant slow TnT mRNA and protein.

![Truncated Slow TnT Proteins](image)

**A** Exon 11 Region of ANM TnT cDNA

| Codon 186 | Glu |
|-----------|-----|
| GCA       | GAG |
| GAC       | TAG |
| ATG       | AAG |
| ATG       | GGT |
| ATC       | ATG |
| TGG       | ATT |
| GAC       | TCT |
| GAG       | CG  |

**B** Truncated Slow TnT Proteins

| MW  | High MW | Low MW  |
|-----|---------|---------|
| 45  | MSDLTEQEEEEQPEEAEEEAAPEPEPVEAEPEEREPRKPSRP | EEERPKP  |
| 34  | MSDLTEQEEEEQPEEAEEEAAPEPEPVEAEPEEREPRKPSRP | EEERPKP  |
| 79  | VVPPILPKIPEGVDFDDHRRMEKDLLELQTIDVHFQKRK | 79      |
| 135 | VVPPILPKIPEGVDFDDHRRMEKDLLELQTIDVHFQKRK | 135     |
| 124 | KEEEELVALEKERRLREEmEDRQFRRTEKEREQRAKLLAEKR | 124     |
| 179 | EEEEAKRAEDDAKKKVLDSNMGHFGGVLKAEQKRQKRTGR | 179     |
| 168 | EEEEAKRAEDDAKKKVLDSNMGHFGGVLKAEQKRQKRTGR | 168     |

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

Transfective Expression of Mutant TNNT1 cDNA in C2C12 Myotubes Produces Mutant Slow TnT mRNA but No Detectable Truncated Protein—We established multiple stably transfected C2C12 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 C2C12 cells that had undergone 5 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 TnT that is used as a differentiation state control). This observation suggests that overexpression of slow TnT mRNA diminishes the level of endogenous slow TnT. Shown in Fig. 7, an inverted correlation was found between the levels of endogenous slow TnT protein (relative to the level of cardiac TnT) 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 C2C12 Cells and ANM Truncated Slow TnT Can Be Produced in Non-muscle Cells—To verify the capacity of C2C12 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. 8A

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**TABLE 1**

| Molecular weight | Isoelectric point |
|------------------|------------------|
|                  | High $M_c$ | Low $M_c$ | High $M_e$ | Low $M_e$ |
| 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 C2C12 cells during in vitro differentiation. Confluent C2C12 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 C2C12 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 C2C12 cells. The expression of mutant low molecular weight slow TnT isoform mRNA in stably transfected C2C12 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 C2C12 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.
showed high level expression of chicken TnT in differentiated C2C12 cells. The successful expression of avian TnT in mouse myocytes demonstrates the feasibility of expressing intact exogenous TnT in differentiated C2C12 cells.

To verify that the ANM mutant slow TnT mRNA can be translated in eukaryotic cells, Fig. 8B shows that transient transfection of the CMV promoter-directed expression construct produced significant amounts of the ANM truncated slow TnT mRNA (Slow TnT1-179) in 293 human epithelial cells and 3T3 mouse fibroblasts. Parallel transient transfections produced significant levels of intact slow TnT in 293 and 3T3 cells but not in undifferentiated C2C12 cells, suggesting a muscle cell-specific protein degradation. In contrast to the low level TnT expression in confluent C2C12 cells at the beginning of differentiation (Fig. 5), undifferentiated C2C12 showed no endogenous TnT.

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. 8B further show that CMV promoter-directed transient transfection can express intact slow TnT in 293 and 3T3 cells but not in undifferentiated pre-confluent C2C12 myoblasts. RT-PCR on DNase-treated RNA extracted from the transient transfected C2C12 cells confirmed the expression of mutant slow TnT mRNA (data not shown). No
skeletal muscle provides a potential therapeutic target, using somatic therapies to correct the slow TnT protein deficiency. This finding suggests that reagents that promote translational read-through 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 tropinin 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 corporate 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 C2C12 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 C2C12 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-
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