The Characterisation of a novel mRNA TA553, that is up-regulated by passive stretch of skeletal muscle *in-vivo*

Dissertation for BSc (Hons) Basic Medical Sciences with Biochemistry

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Abstract

The factors which link increased functional demand to muscle adaptation are incompletely understood. TA553 is a novel mRNA identified by subtractive hybridization comparing stretched and unstretched skeletal muscle. This project utilised the techniques of Northern hybridization, RT-PCR and RRT-PCR to determine: the true size of the mouse mRNA encoded by TA553, the tissue specificity of expression of TA553 in mice and to compare the expression of TA553 in a range of tissues. Results show that TA553 encodes an mRNA of greater than 9Kb in length and that expression is seen in a range of skeletal muscles and cardiac muscle (heart); very low expression is seen in liver. It is therefore proposed that the protein coded for by TA553 and related homologues plays a role in the phenotypic change induced by stretch in striated muscle.

Acknowledgements

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Introduction

For thousands of years, man has exercised skeletal muscle for needs of survival, aesthetic and athletic purposes. The process of developing skeletal muscle in order that demand will be met either by making the muscle able to provide power or resist fatigue is a facet of human life. However, the mechanisms that link skeletal muscle phenotypic change with increased work are incompletely understood.

It is well accepted that a normal dynamic equilibrium exists in skeletal muscle.

Table 1 : Muscle dynamic equilibrium, supply and demand.

DEMAND	SUPPLY
Increased demand for force	Hypertrophy
Increased demand for fatigue resistance	Class switching to slow muscle
Decreased demand for force	Atrophy
Decreased demand for fatigue resistance	Class switching to fast muscle

The factors above (table 1) interplay with each other to provide subtle responses to the needs of a skeletal muscle allowing a muscle to meet its expected demand.

Unfortunately, the tight regulation of equilibrium may be disturbed leading skeletal muscle to be ‘maladapted’ to its demand. Examples of this include the poor skeletal muscle adaptive response to exercise in the elderly, the muscle atrophy seen in astronauts and atrophy seen in patients with muscular dystrophy.

A significant quantity of research has allowed understanding of the contractile mechanisms of skeletal muscle, however, only recently has research started to uncouple the physiological and molecular basis of the regulation of skeletal muscle phenotype. Additionally, there may also be conservation of pathways within the three types of muscle (skeletal, cardiac and smooth) and that research in the area of skeletal muscle may also have implications for other fields of science and medicine, notably cardiology.

The importance of work in this field is that eventually, the full scheme of events occurring in skeletal muscle adaptation will be uncovered. Hence, therapeutic targets may be explored and

developed as to provide a regimen that can alleviate the effects of various pathologies that may impart an effect on the dynamic equilibrium within skeletal muscle.

In this introduction, the established basic science of skeletal muscle shall be presented, the physiological and molecular factors regulating muscle phenotype shall be discussed and finally the work leading to the start of this project will also be presented.

Skeletal muscle - Basic aspects

Skeletal muscle's main function is locomotion with a subsidiary function of an amino acid store and occupies 40% of body mass. **(Maltin et al, 2001)**.

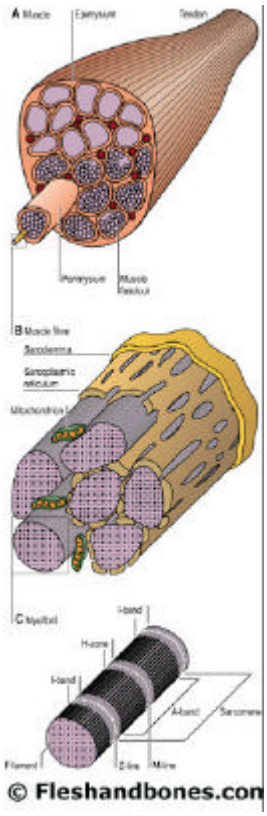


Figure 1 : Gross structure of skeletal muscle. Source www.Fleshandbones.com (Human Physiology by Davies et al (2001) Churchill Livingstone publishers.

Muscle Contraction

Morphologically, the muscle fibre represents the functional unit of skeletal muscle. When examined, one muscle fibre in transverse section is seen to be made up of numerous myofibrils which individually measure approximately 1μm in diameter. In turn, myofibrils comprise protein filaments, which can be divided into sarcomeres.

A sarcomere is made up of thin and thick filaments representing actin and myosin respectively. In the process of muscle contraction, actin filaments are pulled by myosin, the result is that the actin and myosin filaments overlap each other more. This allows contraction of the sarcomere, if a sufficient amount of sarcomeres are recruited, the skeletal muscle will contract **(Huxley, 1974) (Weber and Franzini-Armstrong, 2002)**.

Myosin has three functions **(Stryer, 1988)**.

1. Spontaneous polymer assembly at normal physiological ionic strength and acidity:
2. An ability to hydrolyse ATP to ADP (the presence of actin can increase this activity by 200 times).
3. Binding to the polymerized form of actin.

Myosin is made up of six polypeptide chains, two heavy and four light. Microscopically, a head and rod can be seen. The rod consists of heavy chains arranged as an alpha-helix, which in turn connects to two globular heads. Each head is bound to two different light chains. The head binds ATP and actin **(Stryer, 1988)**.

Actin, the major component of thin filaments. At physiological ionic strength and acidity the actin monomer forms an actin polymer. The synthesis of the actin polymer also requires the presence of ATP. In addition the thin filament also comprises tropomyosin and troponin.

During the process of skeletal muscle contraction, ATP is hydrolysed to ADP and the energy release allows the head region of the myosin molecule to prime allowing binding to the actin molecule. The cyclical nature of these events is referred to as the ‘cross-bridge cycle.’

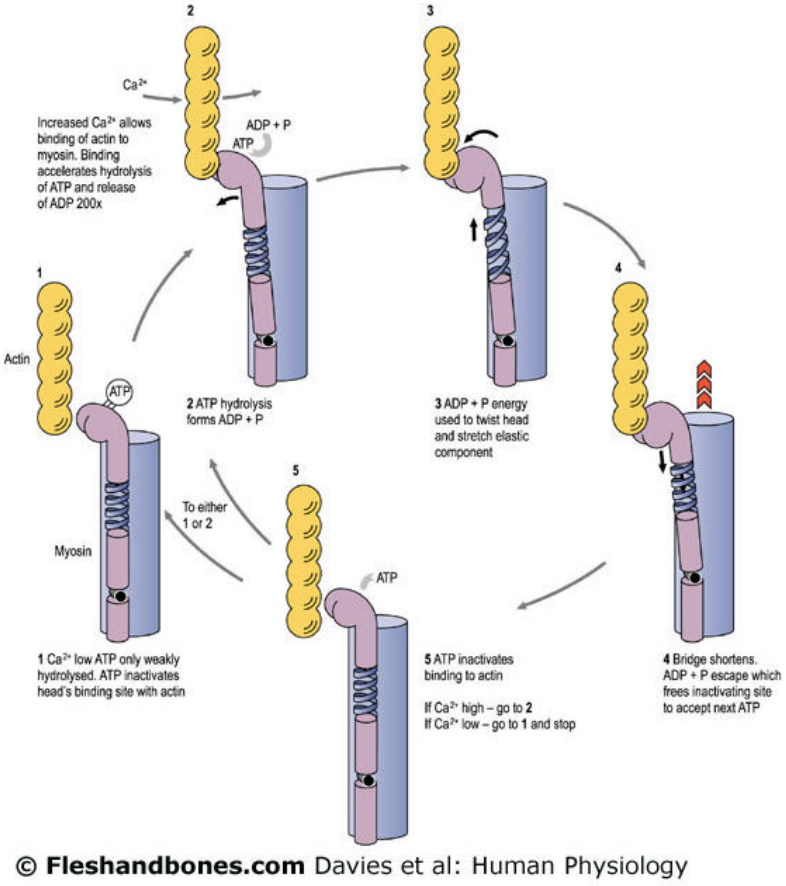


Figure 2: Muscle Contraction. Source: www.Fleshandbones.com Human Physiology by Davies et al (2001) Churchill Livingstone publishers.

The cross bridge cycle comprises four main stages.

1. ATP-binds to myosin.
2. The ATP molecule is hydrolysed to ADP forming a myosin-ADP complex + phosphate molecule.
3. Actin binds to form an actin-myosin-ADP complex.
4. ADP is released leaving an actin-myosin complex.

In effect, the interaction between the myosin head and the actin polymer appears to 'pull' the actin molecules towards each other. Hyper-extension of the molecular structure is prevented by anchoring of one end of the actin molecule to 'Z-lines' which allow the actin molecule to act as a molecular 'bungee' rope. Gross muscle contraction is terminated when the association between actin and myosin is terminated.

Skeletal muscle contraction is dependent on the presence of calcium ions and ATP. In effect, the thick and thin filaments of skeletal muscle slide against each other **(Huxley, 1965)**.

The role of calcium in skeletal muscle contraction is primarily one of regulation and control. Skeletal muscle contraction is primarily initiated by neural mechanims. Neural signals are integrated at the neuromuscular junction where a depolarisation wave is initiated stimulating calcium ion release from the sarcoplasmic reticulum (SR). Calcium ions binds to troponin C which alters the resting troponin structure which allows actin-myosin binding to occur.

Formation of skeletal muscle

Myoblasts (skeletal muscle precursors) develop from multipotent mesodermal precursor cells of the paraxial mesoderm. Myoblasts are cells that are committed to a skeletal muscle fate that have the ability to proliferate and migrate. After migration myoblasts express MRFs (muscle regulatory factors), exit the cell cycle and begin differentiation. **(Yun and Wold, 1996)**.

Myogenic cell fate is specified by signalling factors referred to as MRFs. Myf5 and MyoD are the factors required for the acquisition for myogenic activity. They are all intranuclear transcription factors of the bHLH class and ectopic expression will differentiate a majority of non-myogenic cells to skeletal muscle **(Yun and Wold, 1996)**. The factors Myf5 and MyoD are necessary for the formation of myoblasts. Myogenin in conjuction with either MyoD or MRF4 is involved in myotube terminal differentiation **(Maltin et al, 2001)**.

Mesodermal somite cells are converted into myoblasts and myoblasts fuse together to form myotubes (multinucleated myofibres) **(Slack, 2001)**. Myogenic identity is regulated by a balance of various factors; Noggin, Wnt-1 and SHH (Sonic hedgehog) released from the neural tube and notochord are positive regulators. Wnt-7a form the surface ectoderm acts a positive regulator whereas BMP4 (bone morphogenetic protein) acts as an inhibitory factor **(Maltin et al, 2001)**.

After myoblasts have migrated to their respective anatomical areas, myoblasts can either maintain proliferative activity or enter a terminal differentiation process. The course of the cell's action is dependent on effects on the cell cycle. The presence of FGF (fibroblast growth factor) will keep the cell dividing **(Slack, 2001)** whereas the presence of myogenin will cause the cell to leave the cell cycle and enter terminal differentiation **(Maltin et al, 2001)**. Additionally, satellite cells which are remnants of embryological development in adult life and can proliferate and contribute to muscle repair, terminal differentiated skeletal muscle fibres lack this ability **(Slack, 2001)**.

After the initial formation of a skeletal muscle fibre (primary), secondary myogenesis can take place. Differential gene expression for contractile proteins such as the myosin heavy chain can take place, which correlates, to whether the muscle phenotype is ‘fast’ or ‘slow.’ The regulatory factors underlying this process are not elucidated in great detail at present **(Maltin et al, 2001)**. The role of initial nerve innervation and of calcium dependent calcineurin activation which in turn leads to the activation of gene transcription through NFAT transcription **(Buckingham, 2001)**. The increased cell calcium through nerve activity would cause increases activation of the calcineurin / NFAT pathway and hence genes for a ‘slow’ skeletal muscle phenotype would be expressed **(Maltin et al, 2001) (Buckingham, 2001)**.

Skeletal muscle adaptation to functional demand

In contrast to embryological development of skeletal muscle which requires systemic somatotrophic hormone release, adult skeletal muscle hypertrophy is based on individual stimulation of muscle fibres by mechanical forces which consequently lead to locally acting factors **(Brahm et al, 1997)**.

Skeletal muscle can adapt to functional demand (or lack of) by changing the predominant muscle fibre type present and hence the gross functional properties of the muscle or muscle fibre may undergo muscle hypertrophy (or atrophy). The importance of these adaptive mechanisms is to ensure that future demands on the skeletal muscle may be met with greater efficiency and a reduced risk of damage by overuse of muscle.

Class switching of skeletal muscle

Class switching of skeletal muscle is a useful mechanism by which the predominant fibre types in skeletal muscle may be altered to efficiently meet the demand placed upon the muscle. For, example, it may be hypothesised that a ‘fast’ muscle with glycolytic fibres that is exposed to chronic stretching would respond by altering muscle fibres to ‘slow’ oxidative fibres, the biochemistry of which would be less likely to suffer from fatigue than ‘fast’ skeletal muscle fibres.

Functionally, skeletal muscle can be defined into three subtypes **(Goldspink, 1996)**. Altering specific gene expression may change the phenotype to reflect varying proportions of subtypes.

Table 2: The main fibre types in skeletal muscle (Based on Goldspink, 1996 and Ganong, 1987)

FIBRE TYPE	TYPE I	TYPE IIB	TYPE IIA
Name	Slow oxidative	Fast glycolytic	Fast oxidative + glycolytic
Mysoin ATPase activity	Slow	Fast	Fast
Ca2+ pumping activity of Sarcoplasmic Reticulum	Moderate	High	High
Oxidative capacity[1]	High	Low	High

The composition of fibre types that make up an individual skeletal muscle is dependent on function. For example, type IIB fibres are designed for fine, skilled movements and are seen in the extraocular muscles and some muscles of the hand. Type I fibres are useful more long protracted action such as posture and are seen in the long muscles of the human posterior trunk **(Ganong, 1987)**.

The phenotypic variation in muscle fibre type is due to differential expression of the isoforms for the various contractile proteins. There are over seven genes of the myosin heavy chain in skeletal muscle including embryonic, neonatal, adult fast and adult slow **(Leinwand et al, 1983) (Goldspink, 1996)**. Examples of skeletal myosin heavy chain genes include 2B and 2A. Different genes code for the different myosin heavy chain proteins. The protein products of these genes have different rates of ATP hydrolysis leading to varying speeds of the cross-bridge cycle and hence the velocity of muscle contraction varies.

There are also two cardiac myosin heavy chain genes, α and β which can also be expressed in skeletal muscle. The β gene protein product codes for a very slow form of myosin heavy chain protein and has very slow cross-bridge cycle hence it is found in the type I fibres of the postural soleus muscle. The α gene for myosin heavy chain is also expressed in some head muscles which are embryologically separate from other skeletal muscles **(Goldspink, 1996)**.

The myosin light chain protein isoforms are derived from differential splicing of exons from the same gene. This cut and paste approach allows more than one myosin light chain to be derived

[1] Based on mitochondria content, capilliary density and myoglobin content.

from one gene **(Periasamy et al, 1984)**. The troponin and tropomysoin protein isoforms range from fast to slow skeletal muscle types and also in a subset of cardiac isoforms, which can be derived by alternative splicing methods in-vivo **(Goldspink, 1996)**.

Therefore, skeletal muscle class switching involves highly regulated changes in myosin heavy chain and myosin light chain expression patterns.

The predominant fibre type in a skeletal muscle is believed to be determined embryologically **(Condon et al, 1990)** however, endocrine factors, neural innervation changes or alterations in physical activity may 'override' the basal pattern of skeletal muscle. Overriding the basal genetic pattern is believed to be due to variations in gene transcription for the proteins responsible for skeletal muscle function namely actin, myosin and tropomyosin **(Ganong, 1987)**.

Skeletal muscle hypertrophy

Skeletal muscle hypertrophy in effect results in an increase in contractile tissue, which in turn would allow the skeletal muscle in question to hopefully fulfil the demand.

Surgical increase of workload by removal of a synergist muscle has been shown to increase the mass of tested muscle. Hypertrophic growth is evident by 24 hours and a plateau reached by the fifth day of overload. Systemic thyroid and hypophyseal hormones are redundant in this change **(Goldberg, 1967) (Carson et al, 2002)**.

Skeletal muscle hypertrophy also involves synthesis of proteins that will ameliorate the functional demands on a given skeletal muscle.. This will in turn require amino acid uptake and an increase in cell RNA content **(Carson et al, 2002)**. Treatment of functionally overloaded skeletal muscle with an inhibitor of RNA synthesis from DNA (actinomycin D) prevents skeletal muscle hypertrophy in vivo **(Goldberg and Goodman, 1969)**.

In addition to the accumulation of protein content, the satellite cells, remnants of the embryological development of skeletal muscle re-enter the cell cycle and hence proliferate **(Carson et al, 2002)**. Irradiation of skeletal muscle prior to overload prevents satellite cell division and the subsequent skeletal muscle hypertrophy **(Rosenblatt and Parry, 1992)**. However, there is evidence that states

that satellite cells can be redundant in skeletal muscle hypertrophy **(Baar and Esser, 1999)**. The role of satellite cells in skeletal muscle hypertrophy requires further investigation.

In summary skeletal muscle hypertrophy, involves increased expression of the protein machinery pre-requisite for satisfactory skeletal muscle contraction and this involves the addition of sarcomeres in parallel to increase contractile strength.

Signal transduction between mechanical stimuli and skeletal muscle adaptation

Identified factors in muscle adaptation pathways

Microarray analysis compared expression between normal and gastrocnemius-ablated rats (hence functional overload). Results show that IGF-1 mRNA expression was increased when comparing functionally overloaded skeletal muscle to normal muscle **(Carson et al, 2002)**. These results indicate that if IGF-1 is expressed in greater quantities resulting from skeletal muscle functional overload, the hypothesis that IGF-1 is involved in muscle hypertrophy may be proposed.

In fact, the local synthesis, release and action of IGF-1 have been implicated in various aspects of skeletal muscle development in adults **(Florini et al, 1991)**. Viral introduction of IGF-1 genes into mice skeletal muscle shows individual local muscle skeletal muscle hypertrophy and reduced atrophy associated with ageing **(Barton-Davis et al, 1998)**. Exercised muscle also appears to play a role in maintaining circulation IGF-1 concentration **(Brahm et al, 1997)**.

In addition to the liver form of IGF-1 recent data points to the existence of an IGF-1 isoform that is synthesised in exercising skeletal muscle, undetectable in un-exercised skeletal muscle and has a local action as opposed to a systemic action. This isoform is designated MGF (mechano growth factor) **(Goldspink, 2000) (McKoy et al, 1999)**. The structure of MGF bestows smaller size and a reduced half-life when compared to liver IGF-1 **(Yang et al, 1996)**. Insulin and dexamethasone are also seen to show similar effects to IGF-1 **(Semsarian et al, 1999)**.

The ability of IGF-1 to mobilise intracellular calcium is important. Intracellular calcium and calmodulin are pre-requisite for the activation of calcineurin (a phosphatase enzyme) **(Semsarian et al, 1999)**. Transcription factors such as GATA-2 and NF-ATc1 are transcription factors believed to lie downstream of calcineurin in the skeletal muscle hypertrophy pathway. IGF-1 or activated calcineurin increases GATA-2 expression, GATA-2 localises in the nucleus and

cooperates with isoforms of NF-Atc1 to allow specific gene transcription relevant to muscle adaptation **(Musaro et al, 1999)**.

Surgical overload (removal of gastrocnemius and soleus) of ‘fast’ mouse plantaris muscle was performed to induce hypertrophy, addition of a calcineurin inhibitor such as cyclosporin A was shown to inhibit skeletal muscle hypertrophy **(Dunn et al, 1999)**. This result shows calcineurin signal transduction is implicated in skeletal muscle hypertrophy and that it may be implicated in class switching of skeletal muscle fibre type from fast to slow forms. Functional overload of ‘fast’ skeletal muscle such as plantaris would be expected to switch its dominant fibre type to a slower forms **(Dunn et al, 1999) (Goldspink, 1996)**.

Prostaglandin F2α that acts by a G-protein receptor has also been implicated in the pathways for skeletal muscle hypertrophy by use of in-vitro methods **(Vandenburgh et al, 1995)**. Interleukin-6 (IL-6) expression is increased 15-fold in rat soleus after 3 days of rat soleus work overload **(Carson et al, 2002)**. IL-6 is believed to act via a JAK-STAT pathway **(Narazaki et al, 1994)**. However the role of locally produced IL-6 in skeletal muscle has yet to be determined **(Pederson et al, 2001)**. IL-6 overexpression can lead to skeletal muscle atrophy that can be blocked by IL-6 antibodies **(Tsujinaka et al, 1996)**. The role of IL-6 in inducing VEGF (vascular endothelial growth factor) production and hence neo-vascularisation and the importance that IL-6 plays in glucose and fatty acid metabolism may also play a part **(Pederson et al, 2002)**.

In summary, several factors that are implicated in muscle hypertrophy have been identified such as IGF-1, IL-6 and PGF-2α and some of the pathways that link these factors to nuclear transcription have also been identified (figure 3). However, at present, the full understanding of how the pathways allowing skeletal muscle phenotypic change have yet to be fully uncoupled in terms of regulation and interaction.

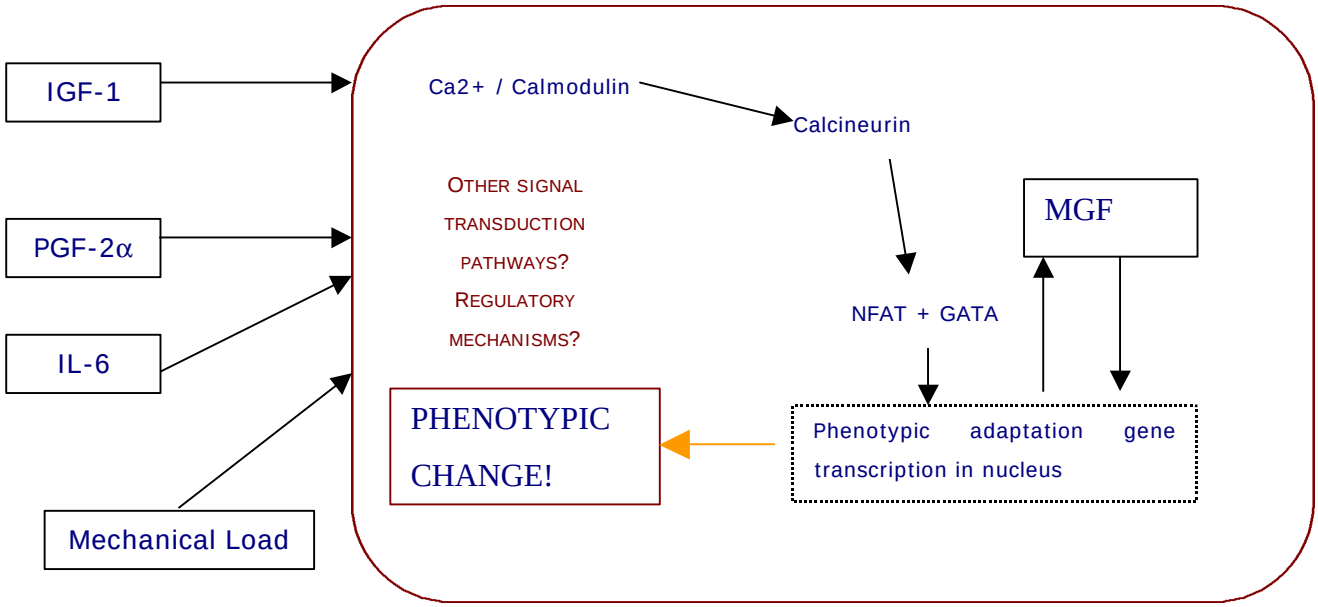


Figure 3: Some pathways that contribute to skeletal muscle hypertrophy.

Work leading directly to this project

A subtractive hybridization technique was used by Dr. G. Coulton and colleagues in order to identify candidate regulators of skeletal muscle hypertrophy **(Kemp et al, 2001) (Sadusky et al, 2001) (Kemp et al, 2000).** This technique allowed comparison between gene expression in un-stretched (control) and stretched Extensor digitorum longus (EDL) muscle from C57BL/10 mice (age and sex matched).

The EDL muscle was passively stretched for seven days and then subtractive hybridization was performed at this point. The reason for choosing seven days of stretch was to maximise the presence of genes involved with hypertrophy and to avoid genes involved with damage repair which can peak relatively early after the stretch. These include c-fos and c-jun and genes for proteosomes and ubiquitin (which remove cellular detritus) **(Osbaldeston et al, 1995)**.

At the present time, 18 known genes and 13 novel mRNA sequences have been identified that are consistently up-regulated by passive stretch in vivo. It is proposed that since these genes have increased expression resulting from prolonged passive stretch, that they may play an important role of the phenotypic adaptation of skeletal muscle to demand.

This methodical manner of investigating gene expression has produced reports on the following three novel genes that are responsive to passive muscle stretch in vivo; *Ankrd2* **(Kemp et al, 2000)**, *Smpx* **(Kemp et al, 2001)** and *Serhl* **(Sadusky et al, 2001)**.

Stretch responsive protein mRNA TA553

Accession number: AJ250188

Nucleotide Database http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide

TA553 is a novel mRNA that is significantly up-regulated after seven days of passive stretch of EDL muscle in vivo **(Unpublished personal communication – Dr. Gary Coulton)**. The main aim of this project was to characterise this novel mRNA sequence in mice.

The sequence derived from the subtractive hybridization experiment was shown to be 1042 base pairs (bp) long. A BLAST search[2] showed 100% homology to an expressed sequence tag (EST) which was expressed in the tongue of an adult male mouse. The RIKEN consortium sequenced this EST which is 1325bp long and its accession number is AK010194. The RIKEN sequence overlaps TA553 at both the 5' and 3' ends.

The RIKEN sequence was subsequently put into a BLAST search linking it with the human genome. A single BLAST hit was seen on chromosome 5 of the human genome. A homologous EST transcript was noted that had high similarity to the RIKEN sequence. This cDNA is CMYA5 (Cardiomyopathy associated gene 5).

Software amino acid translation (www.expasy.org.ch) showed CMYA5 contained an open reading frame of 915 amino acids. Protein motif analysis (SMART) showed the presence of coiled-coil, fibronectin-3-like and SPRY domains. The smaller mouse TA553 also contained the SPRY domain. At this stage it was unsure whether CMYA5 constituted the entire coding sequence either in the 5' or 3' regions.

[2] http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

The increased expression of TA553 in the negative hybridization study suggests that TA553 may play a role in the regulation of skeletal muscle phenotype. It also raised the possibility of the human homologue playing an important role in heart muscle and the pathogenesis of cardiomyopathy.

Project Hypothesis

The protein coded by partially TA553 plays a role in the regulation of stretch induced phenotypic change in skeletal muscle.

Specific Project Aims

1. Determine the size of mouse mRNA partially encoded by TA553 (AJ250188) and the RIKEN sequence (AK010194).
2. Determine the pattern of tissue specificity of this mRNA in mice.
3. To compare quantitatively, the expression of TA553 in a range of tissues.

Methods and Materials

This project will use the techniques of Northern hybridization, RT-PCR (reverse-transcriptase polymerase chain reaction) and RRT-PCR (ratiometric reverse-transcriptase polymerase chain reaction) to investigate the role of TA553.

General Methods

Animal tissue collection

For this study, the tissues of C57/BL mice were dissected; this species is a standard normal laboratory strain. Rearing and dissection of mice was carried out at SGHMS in the Biological Research Facility. Tissues were removed at autopsy using Schedule 1 accredited procedures. The aim of dissection was to provide samples from various contractile tissues (i.e. skeletal, smooth and cardiac muscle) and non-contractile tissues. After dissection, the collected tissue samples were immediately placed in Eppendorf tubes and plunge frozen in liquid nitrogen until processing to extract RNA.

Maintaining RNA integrity

Ribonuclease (RNase) contamination of samples was prevented by maintaining a sterile environment at all times when working with RNA. This entails:

1. Wearing gloves at all times when working with samples.
2. The designation of a particular part of the laboratory for RNA specific work.
3. Distilled water treated with 0.1% Diethyl Pyrocarbonate (DEPC) and autoclaved overnight and only this water was used in the experiments.
4. Disposable sterile plasticware was used when possible and non-disposable glassware was baked at 200^{o}C overnight.
5. When running electrophoresis with RNA samples, the electrophoresis tanks were treated with 3% hydrogen peroxide solution for 15 minutes and rinsed with RNase free water,

Extraction of RNA from tissue samples

There are four distinct steps in the extraction of RNA from tissue samples: For this procedure, an SV RNA extraction kit made by Promega was used.

1. Cell lysis
2. Inhibit RNases
3. Removal of protein from the sample

4. Recovery of intact RNA

Tissue samples were homogenised in tubes containing RNase denaturing solution (RDS) and lysing matrix in a 'Fast-prep' machine on speed setting six. The samples were homogenised in ten second cycles until no solid matter could be visualised by eye. In-between cycles, samples were kept cool in ice to prevent RNA degradation.

RNA was treated using a modified version of the Promega SV RNA extraction protocol (see appendix). After this protocol the extracted pellet of RNA was resuspended in solution. For the Northern analysis, the pellet was dissolved in formamide and for RT-PCR (and cDNA synthesis), the pellet was dissolved in water. It is believed that formamide would affect the Taq polymerase enzyme and general reaction kinetics.

Northern Analysis

For the creation of a Northern blot, a NorthernMax ™-Gly kit produced by Ambion was used.

Gel electrophoresis.

5µm of each RNA sample was diluted with 1ml of Rnase free water and the absorbance was measured at 260 nm and 280 nm. This data was then used to load the gel with 40µg of RNA per lane (This work was kindly carried out by Dr. Godfrina McKoy).

40µg of extracted total RNA from required samples was combined with 15µm of Glyoxal Load Dye. The samples were incubated to denature for 30 minutes at 50°C in a water bath and briefly spun. The gel was run on a 1.0% agarose gel at 150V for 2.5 hours.

RNA transfer to membrane

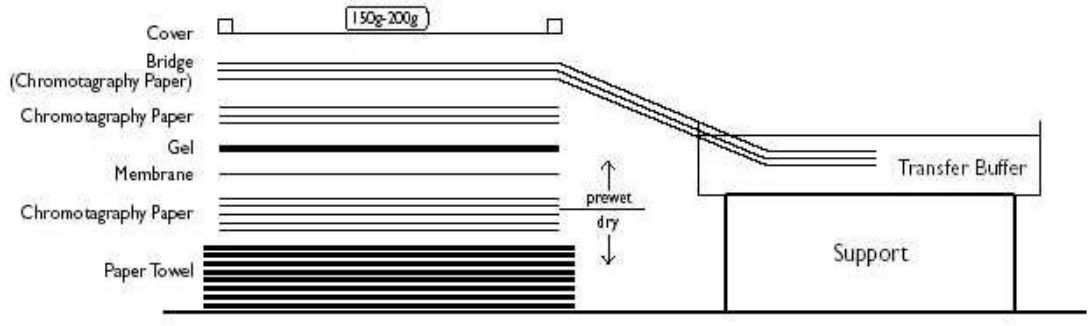


Figure 4: Transfer assembly, from Ambion Northern hybridization protocol.

An apparatus was assembled as shown in the above diagram (figure 4). This allows the transfer of RNA from the agarose gel to a positively charged nylon membrane.

Once set up, the apparatus was left to allow downward migration of the size-fractionated RNA onto the nylon membrane for 15-20 minutes per mm of gel thickness. The transfer apparatus was disassembled. The RNA on the membrane was cross-linked by baking for 80^{O}C for 15 minutes. At this point, the membrane was illuminated briefly with ultraviolet light to visualise the ribosomal RNA bands and to mark the position of lanes for future reference.

Prehybridization and hybridization

Using 10ml ULTRAhyb hybridization buffer per $100cm^2$ of membrane, the membrane was pre-hybridized at 45^{o}C for 2 hours in a hybridization tube placed in a roller oven. After the process of pre-hybridization, the oligonucleotide probes were added. The probes used in this experiment were biotin labelled single stranded DNA probes (designed and created at SGHMS). Once the probe was added to the hybridization tube, the tube was placed in the roller oven and hybridized overnight at 45^{o}C.

In this experiment, two oligonucleotide probes were created and were labelled with biotin at the 5' end (This work was kindly carried out by Dr. Vincent Ang). They were mixed prior to hybidization.

Table 3: Oligonucleotide probes for Northern analysis

TA553/622UTR (Untranslated region)	5'TGTGGGAGACTATCTAAATAATGCACATTGCCTGAGAGACAGAATGATAGGTCTGACCCCCAACACTTGCTCATATTCTGATAAACTCTGAATGCCCAGG 3'
TA553/459CD (Coding region)	5'TCTGGCTGGACGCTCGGTTGCGTGCACCTCACTCACAATGCCACTGTAGAAAAATGTGTATCTCTGTGGCTCAGAGCAGTGCATGTACCATGAGGTCTCG 3'

Washing and exposure to photographic film

For the subsequent washing steps, 20ml per $100cm^2$ of membrane was used.

1. Low stringency washing: Using the Low Stringency Wash Solution #1 supplied with the Northern-Max™ -Gly kit, the membrane was washed with agitation once for 10 minutes.

2. High stringency washing: One, 2-minute wash with Low Stringency Wash Solution #1 at 45^{o}C with agitation was performed.

These steps demarcate the termination of the northern hybridization protocol. The following steps for exposure to film are taken from the BrightStar™ BioDetect ™, nonisotopic detection kit produced by Ambion (Table) 4, the instructions were as dictated by the manufacturer's instructions.

Table 4: Steps for exposing nylon membrane to film. The volumes are based on the 100cm^2 area of the membrane.

STEP	TIME (MINUTES)	AGENT	VOLUME (ML)
1.	2 * 5	1X Wash buffer	50
2.	2 * 5	Blocking buffer	25
3.	30	Blocking buffer	50
4.	30	Diluted Strep-Alkaline phosphatase (Strep-AP)	20ml blocking buffer + 1µl Strep-AP
5.	15	Blocking buffer	25
6.	2 * 15	1X wash buffer	50
7.	2 * 2	1X Assay buffer	25
8.	5	CDP-Star incubation	5

After the completion of the steps described, excess fluid was removed from the nylon film. The film was covered in plastic and exposed to film after two hours.

Reverse transcriptase - Polymerase chain reaction (RT-PCR)

For cDNA synthesis, see appendix.

After cDNA synthesis. The polymerase chain reaction (PCR) can be initiated as if working with standard DNA. In this project three primer pairs (F: Forward and R: Reverse) were used (Table 5), they were designed to produce distinct bands on agarose gel electrophoresis.

Table 5: Primer design for RT-PCR

PRIMER	FORWARD	REVERSE	EXPECTED AMPLIFIED LENGTH (BASE PAIRS)
GAPDH[3]	5'-GGACCAGGTTGTCTCCTGCGAC-3'	5'-GAGGTCCACCACCCTGTTGCTG-3'	500
Beta Myosin heavy chain	5'-GCCAACACCAACCTGTCCAAGTTC-3'	5'-TGCAAAGGCTCCAGGTCTGAGGGC -3'	200
TA553	5'-GTGGACAGCTGCTCTTCATCGTGAG-3'	5'-CCTCCACTCACACACTGCTCCATG-3'	300

The GAPDH primer set was used because GAPDH acts a 'housekeeping' gene (i.e it changes very little in expression in muscle) **(Kuo et al, 1999)**. The beta-myosin heavy chain primer set was act as a validating control in order. Beta-myosin heavy chain expression was studied in order to act as a validating control, since beta-myosin heavy chains make up part of a phenotypically slow muscle, it would be expected that the most amplification (hence band intensity) would be seen in slow or postural muscles. If this pattern can be replicated, extra validity would be added to any 'new' results generated.

Beta-myosin heavy chain accession number: AY056464
GAPDH accession number: NM_008084

The PCR reaction mix (Table 6) is 100μm in volume and cycling is as described in Table 7. All negative controls were set up normally except for a lack of template cDNA, the shortfall in reaction volume was made up with an extra 1μm of water.

[3] Glyceraldehyde-3-phosphate dehydrogenase.

Table 6: PCR reaction mixture

COMPONENT	QUANTITY (µl)
Template cDNA	1
Water	85
Primer (Forward and reverse)	1
Taq polymerase enzyme	2
dNTPs	2
PCR reaction buffer	10

Table 7: Thermal cycling protocol for PCR. 'N' is substituted for the desired value of cycles.

STAGE	TEMPERATURE (^{O}C)	TIME (MINUTES:SECONDS)
Initial denaturing	95.0	7:00
Denaturing	**94.0**	**0:50**
Annealing	**63.0**	**0:45**
Extension	**68.0**	**1:00**
Final termination	72.0	7:00

Repeat 'N' times.

When the PCR reaction programme has been completed, the reaction tubes are removed from the thermal cycler and can be stored at 4^{o}C. 20µm of PCR product and 10µm of loading dye can be run on a 1.2% agarose electrophoresis gel with a 1Kb DNA ladder, and after sufficient running a photograph of the gel in ultraviolet light can be taken.

Ratiometric RT-PCR (RRT-PCR)

25 cycles of RT-PCR were completed as described previously. The primers used were GAPDH F and R, the subsequent gel was loaded with 20µm of PCR DNA product and 10µm of loading dye. The resulting GAPDH bands were scanned in to provide a relative measure of each band's intensity using Image/J http://rsb.info.nih.gov/imageJ produced by the National Institute of Health, United States. This arbitrary intensity level which is supposed to reflect DNA concentration was converted into loading volumes to ensure all lanes with GAPDH were at the same intensity, hence the same concentration.

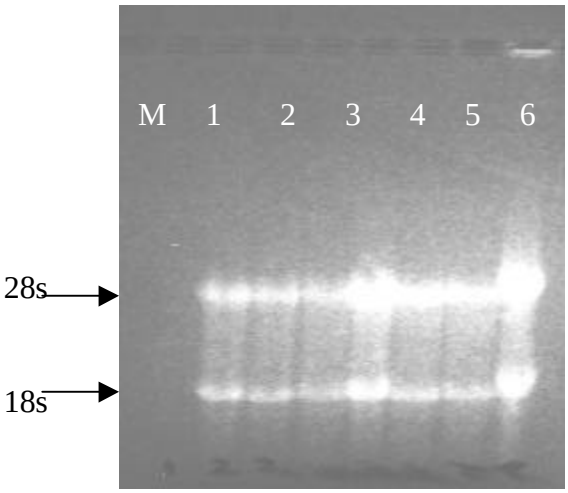
A second PCR reaction at 25 cycles was performed, this utilised primers GAPDH F / R (1µm) and TA553 F / R (1um) and to maintain the total reaction volume at 100µm, the water was reduced to 84µm from 85µm as seen in Table 6. PCR product was loaded into a gel with the volumes stipulated by the analysis described previously and run at 150V until satisfactory migration had occurred.

The expression of GAPDH in this project was normalised in the manner described because previous evidence suggests that GAPDH is a gene expressed at constant levels throughout all cells **(Kuo et al, 1999)**. Differential volume loading of PCR product to allow constant GAPDH bands will allow valid comparisons of TA553 gene expression throughout a variety of cell types.

Results

Northern Analysis

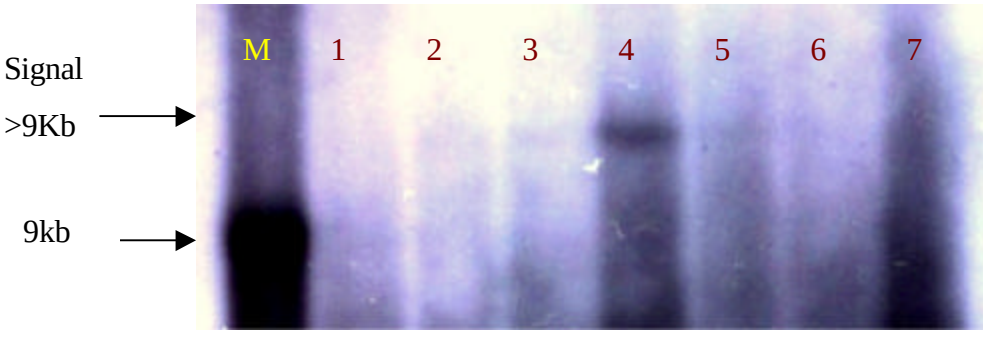
The principle underlying the use of a Northern blot in this project was to identify the full size of the sequence partially encoded in the mouse by TA553 and the Riken cDNA sequence and to hopefully show a pattern of expression throughout various tissues.



Lanes: M=Marker (Not visible here)
1=Liver
2=Lumbar paraspinal muscle
3=Diaphragm
4=Gastocnemius muscle
5=Heart (Cardiac muscle)
6=Tongue
7=Gut

Figure 5: Denaturing 1.0 % agarose gel electrophoresis of RNA samples prior to hybridization.

Initially, an agarose gel electrophoresis (figure 5) was performed to determine the integrity of purified RNA and as the first step in the Northern blot technique. After the completion of the Northern blot preparation, imaging of the film produced was done. This gel shows that RNA was present since the 28s and 18s rRNA bands can be seen and there is little smearing inferiorly to the 18s ribosomal RNA band suggesting the extraction technique did not allow much RNA degradation. This gel was subsequently used for the transfer procedure.



Lanes: M=Marker
1=Liver
2=Lumbar paraspinal muscle
3=Diaphragm
4=Gastocnemius muscle
5=Heart (Cardiac muscle)
6=Tongue
7=Gut

Figure 6: Northern blot with probes for TA553.

The Northern blot shows a signal at greater than 9Kb, which is at a greater length than expected, hence the markers used did not go up to the length of the sequence. Liver definitely shows no signal. Gut is inconclusive due to excess interference. Tongue appears to have a very faint band which may not be apparent on the picture. Definite bands are seen in the lumbar paraspinal muscle, diaphragm, gastrocnemius and heart.

RT-PCR

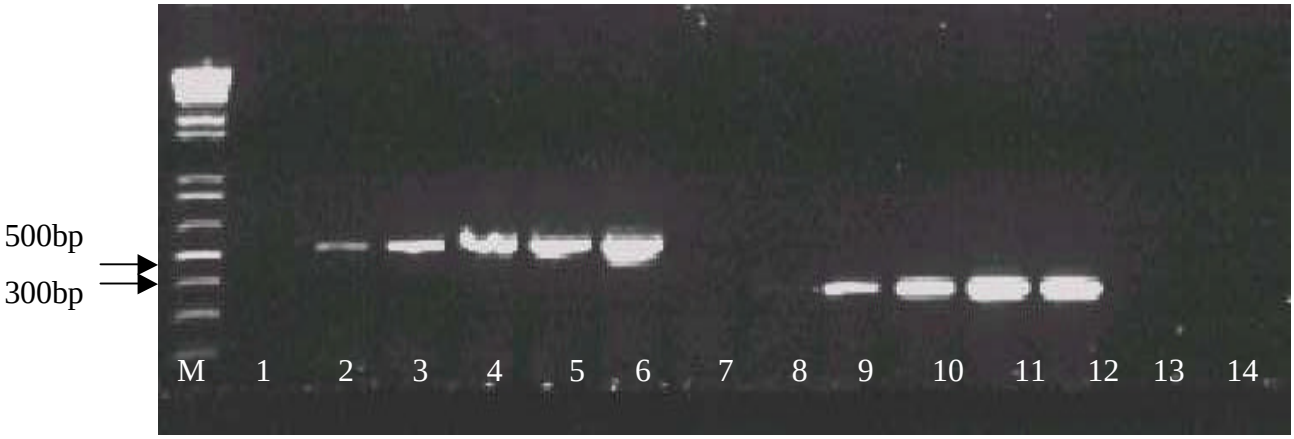


Figure 7: RT-PCR GAPDH and TA553 mRNA at varying cycle number (N) to identify the log phase of amplification. The sample was a mix of psoas muscle of C57/BL and leg from the KY mouse species.

Lanes 1-6 = GAPDH: 10,15,20,25,30,40 cycles.
Lanes 7-12 = TA553: 10,15,20,25,30,40 cycles.
Lane 13 = GAPDH neg. control. Lane 14 = TA553 neg. control.

An initial experiment was set up to determine the log phase of PCR amplification for GAPDH and TA553 products. This was to ensure a number of cycles where products would show visible bands before the plateau phase of amplification had been reached. Runs were made at 10, 15, 20, 25, 30 and 40 cycles in the thermal cycler. Based on these results it was determined that to form products that are within the log phase of PCR amplification, 25 cycles is the optimum value. When required to produce saturation of PCR product, 40 cycles of amplification was used.

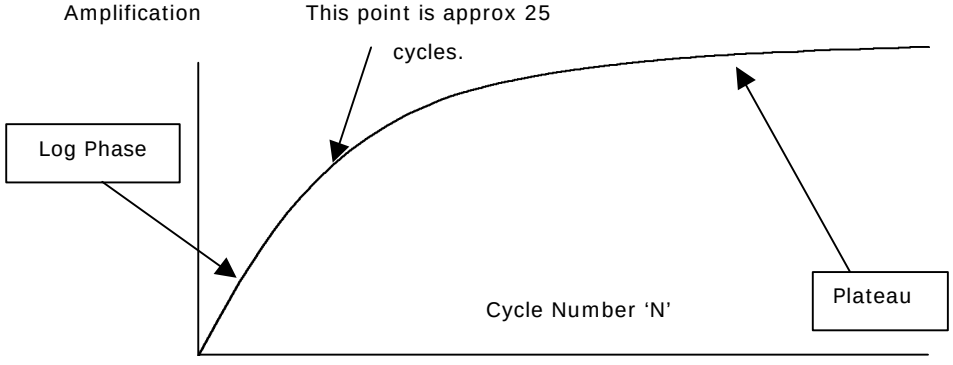
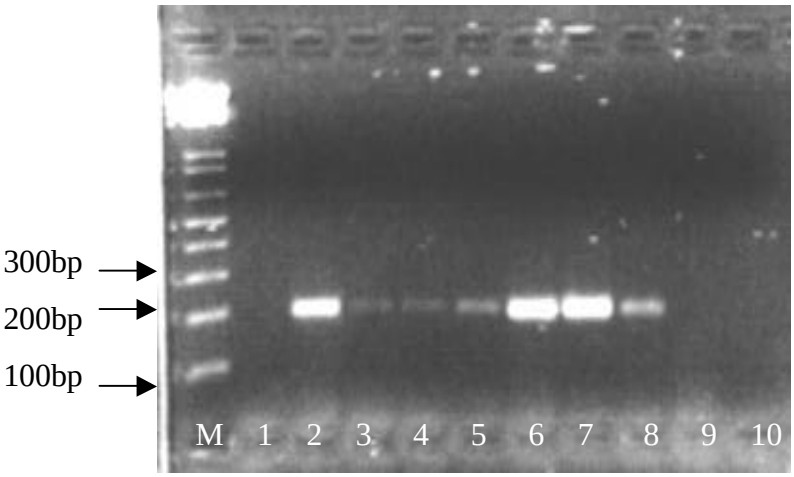


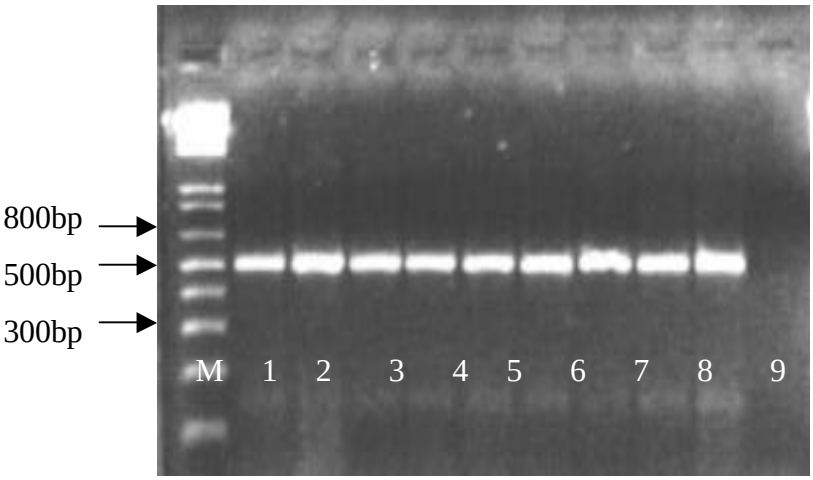
Figure 7b: Graphical representation of PCR product amplfication.



Lanes: M=Marker
1=Tongue
2=Lumbar paraspinal muscle
3=Tibialis anterior muscle
4=Extensor digitorum longus muscle 5=Gastrocnemius muscle
6=Diaphragm
7=Soleus muscle
8=Heart
9=Liver
10=Negative control

25 cycles of RT-PCR as described in methods.

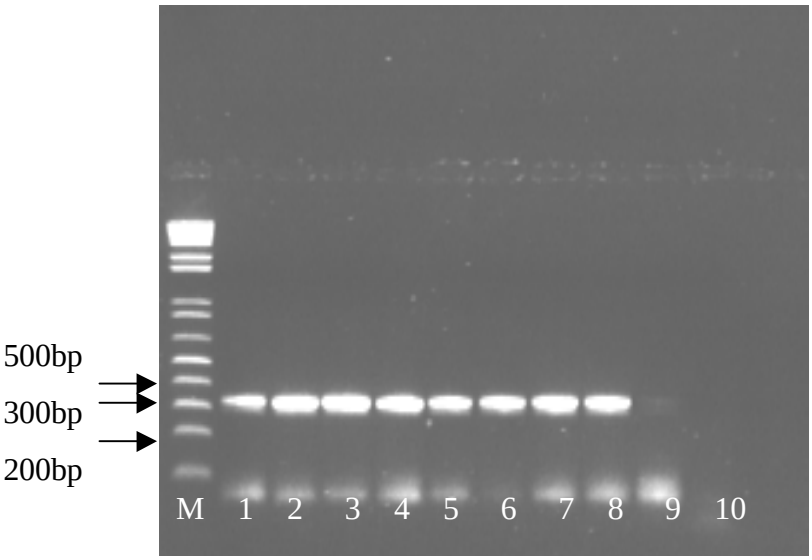
Figure 8: RT-PCR for Beta-Myosin heavy chain mRNA.



Lanes: M=Marker
1=Tongue
2=Lumbar paraspinal muscle
3=Tibialis anterior muscle
4=Extensor digitorum longus muscle 5=Gastrocnemius muscle
6=Diaphragm
7=Soleus muscle
8=Heart
9=Liver
10=Negative control

25 cycles of RT-PCR as described in methods.

Figure 9: RT-PCR GAPDH mRNA.



Lanes: M=Marker
1=Tongue
2=Lumbar paraspinal muscle
3=Tibialis anterior muscle
4=Extensor digitorum longus muscle 5=Gastrocnemius muscle
6=Diaphragm
7=Soleus muscle
8=Heart
9=Liver
10=Negative control

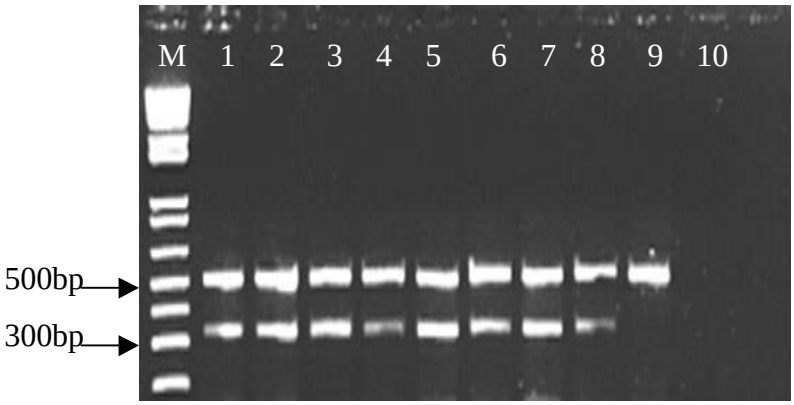
40 cycles of RT-PCR as described in methods.

Figure 10: RT-PCR TA553 mRNA. (Note – Faint band at bottom is a primer dimer)

Apart from showing established expression specificity, the GAPDH and beta-myosin heavy chain initial PCR experiments were designed to show that the PCR process was successfully completed. .The predicted PCR product bands (Table 3) for GAPDH, TA553 and beta-myosin heavy chain mRNA were visualised on agarose gel electrophoresis on their respective agarose gels for various samples (Figures 8, 9, 10). The GAPDH band is seen at 500bp, the beta-myosin heavy chain band is seen at 200bp and TA553 is seen at 300bp.

The TA553 (Figure 10) sample was run at 40 cycles in the thermal cycler because this would ensure that the amplification of product would be maximal, this was to see if any samples expressed TA553. All samples showed presence of TA553 amplification product; however, despite reaching the plateau stage of the PCR amplification curve the liver lane only showed a low intensity band that may not be apparent on the figure.

RRT-PCR



Lanes: M=Marker
1=Tongue
2=Lumbar paraspinal muscle
3=Tibialis anterior muscle
4=Extensor digitorum longus muscle
5=Gastrocnemius muscle
6=Diaphragm
7=Soleus muscle
8=Heart
9=Liver
10=Negative control

25 cycles of RT-PCR as described in methods.

Figure 11: RRT-PCR TA553 (bottom) compared to GAPDH (top) expression.

A standard 1.2% agarose gel loaded with equal amounts of PCR product was run and photographed. This photograph was analysed with ImageJ. The analysis provided the values of PCR product loading into the gel for GAPDH and TA553. This allowed running of a gel following RT-PCR with primers for GAPDH and TA553 (Figure 11).

This gel allows comparison of TA553 levels in a range of tissue samples. The band intensity proceeds from highest to lowest in the following order (intensity of the TA553 band) however, the resulting band intensities are hard to separate by eye.

Table 8: Relative expression of TA553 mRNA based on RRT-PCR based on an arbitrary visual examination by eye of the gel photograph.

Higher intensity	Lumbar paraspinal muscle, Gastrocnemius muscle, Tongue, Soleus muscle, Tibialis anterior muscle.
Lower intensity	Extensor digitorum longus muscle, Diaphragm, Heart (Cardiac muscle).
No band detected	Liver, Negative control.

All skeletal muscles surveyed showed a band at the region of TA553; however, there were differences in expression levels. Another finding was that there was expression of TA553 mRNA within samples from the heart that can be assumed to originate from cardiac muscle. Despite showing a band after 40 cycles of RT-PCR, the TA553 band was not seen in the liver lane after only 25 cycles are RT-PCR. Experimental contamination of the gut sample meant that it could not be used for RT-PCR. In addition the gut lane on the Northern blot was obscured by non-specific hybridisation and a signal could not be seen. The result of this is that in the reported experiments here, TA553 expression in smooth muscle (gut) could not be investigated.

Discussion

The hypothesis presented stated that the novel mRNA transcript TA553 was involved in the phenotypic adaptation of skeletal muscle to perceived demand.

This project is based on the previous unpublished work that has shown that passive stretch 'in-vivo' increases the expression of TA553. The techniques used in this project namely Northern hybridization analysis, RT-PCR and RRT-PCR were used to determine the normal pattern of TA553 mRNA expression in the mouse. The results shown here suggest that within skeletal and cardiac muscle in the mouse, the expression of TA553 is high.

Analysis of results

The Northern blot shows a signal band at a length greater than 9Kb in length, which is present in the skeletal muscles surveyed and cardiac muscle. The signal cannot be seen in the band for liver. It was predicted that the signal would show up at about 3.40Kb (since CMYA5 is 3.40kb). The Northern blot in this project was obscured by non-specific hybridization and presence of 28s and 18s ribosomal RNA bands. The presence of 28s and 18s ribosomal bands may be removed by performing a Northern blot with just poly-adenylated RNA.

After the completion of project work, another Northern blot was completed by Dr. Godfrina McKoy which repeated the presence of the band at greater than 9Kb in length.

When RT-PCR was carried out on various samples at 40 cycles with TA553 primers. All contractile tissues (skeletal and cardiac) showed high band intensity. The sample from liver showed a dim band on gel electrophoresis inferring a very small amount of expression. A possibility of the vascular supply within liver with related smooth muscle may explain this comparatively small expression of TA553. The source of TA553 may be from experimental contamination; however, when RRT-PCR was run at 25 cycles, no product for TA553 was seen. The expression of TA553 may arise within hepatocytes, either randomly or because the protein product may serve a purpose. Definitive evidence of hepatic expression of TA553 may be sought by separate processing of hepatocytes and vascular smooth muscle from the liver.

User error in the processing of small intestinal samples meant that the expression patterns of TA553 in smooth muscle could not be investigated at this time. Despite certain functional

differences in smooth muscle compared to striated muscle, there may be conserved functions between certain muscle types and the protein encoded by TA553 may play a function. Hence, investigation into the expression of TA553 within smooth muscle would be a prudent measure. In addition, this may resolve the question raised by the expression of TA553 within samples derived from liver after 40 cycles of RT-PCR.

The experiment with beta-myosin heavy chain primers was run in an attempt to validate the TA553 findings shown here. Since the beta-myosin heavy chain is normally expressed in 'slow' muscles due to slow cross-bridge cycle formation, it would be expected to be seen in muscles that are involved in postural maintenance or muscles that require fatigue resistance. In small mammals β-myosin is predominant in the developing myocardium whereas in adult small mammals, β-myosin is superseded by an increased presence of α-myosin **(Goldspink, 1996)**. The results for RT-PCR show β-myosin heavy chain expression is at its highest in muscles that need to be fatigue resistant such as soleus, diaphragm and lumbar paraspinal, with some expression in the heart. Low or non-existent expression levels were seen in muscles that are phenotypically fast such as tongue, tibialis anterior, extensor digitorum longus and gastrocnemius.

The RRT-PCR shows some variation in expression; however, it is believed that the resolution could be increased to provide more sensitivity. Nevertheless a clear difference in TA553 expression could be seen. Having shown that Beta-myosin heavy chain expression clearly varies in a range of tissue. A comparison between expression of TA553 and Beta-myosin heavy chain expression in various tissues should be compared, this may answer the question whether TA553 is restricted to a specific skeletal muscle fibre type or if ubiquitous skeletal muscle expression is required. Is there a role for TA553 in non-skeletal muscle cells? Our evidence here says probably not but a further investigation in non-skeletal muscle types is required.

Data that links TA553 to a role in the heart is very interesting as a role in cardiomyopathy as suggested by the link to CMYA5 is seen. Our evidence shows that there is some expression of TA553 in the heart. Further functional studies may reveal more of the nature of TA553 in a cardiological perspective.

Both PCR and Northern analysis show that similar pattern of tissue specificity in the fact that expression tends to be high in skeletal and cardiac muscle. The expression of liver TA553 is comparatively very low.

Since ethidium bromide staining of DNA is non-specific, care must be taken when amplifying using PCR to ensure that the correct sequence is amplified, for example, user error in combining mixtures or allowing DNA contamination may give rise to misleading results on gel electrophoresis. With the Northern analysis corroborating findings from RRT-PCR and RT-PCR, a genomic Southern blot can be performed to provide further verification of results.

The method used for quantitative analysis of TA553 mRNA expression was RT-PCR. However, this technique assumes that the PCR reaction is exponential in nature. Factors introduced to the reaction that can affect the exponential nature may affect the result. For example, the use of two sets of primers in RRT-PCR may have an affect on the kinetics of the reaction. Additionally, differences relating from the origin of samples may also have an effect on reaction kinetics **(Rapley, 2000)**.

Three other methods of using RT-PCR to quantify mRNA are available: The first method involves radiolabelling of either primers or nucleotides and detection following PCR product purification **(Rapley, 2000)**. This technique could not be used due to safety implications related to radioactivity. The second method would be use of the TaqMan assay **(Okimoto and Dodgson, 1996)**. This technique involves labelling a oligonucleotide probe with a quencher and fluorescent reporter. The quencher and marker are separated by the 5’ exonuclease activity of Taq polymerase which releases fluorescence in proportion to the amount of starting molecules which can be monitored in real time by a suitable thermal cycling machine **(Rapley, 2000)**. Lack of the necessary equipment prevented this technique from being used.

An alternate method.for quantitative analysis using RT-PCR would be to run samples of constant RNA mass in a reaction tube with primers for GAPDH and TA553 using only samples from one mice. The results would be run on an a gel, the bands would be scanned in and analysed to find a ratio of TA553 expression to GAPDH expression. This would provide a more quantitative analysis; however, to be rigorous would need a large number of repeated experiments for the necessary statistical analysis. Constraints of time meant this method could not be used.

TA553 expression has been seen in all striated muscle surveyed (cardiac and skeletal) and is notably absent from liver (the only non-contractile tissue surveyed). The true size is estimated to be greater than 9kb and not smaller as expected. This knowledge is in addition to previous unpublished work that shows TA553 expression is increased by passive stretch of mouse skeletal muscle in-vivo.

Future lines of investigation

Investigating the role of TA553.

Based on the unexpected result that the true size of TA553 is over 9Kb in size, the immediate priority is to find the exact size and full sequence of the RNA partially coded by TA553.

When the full coding sequence has been identified, various experiments can be carried out that can fully elucidate the role of the protein coded for by TA553 in stretch induced phenotypic change in skeletal muscle. The findings that TA553 expression occurs in the heart may have implications for cardiology research. The following questions need to be answered to fully understand the role of TA553.

1. What is the functional relevance of the coded domains?
2. How does the protein interact with other proteins and what is its cell localisation?
3. Does in-vitro expression of the protein in skeletal muscle cell lines allow phenotypic change?
4. If the action of the protein is inhibited, are the phenotypic changes associated with stretch absent?
5. What mechanisms act to regulate the action of the protein? This question has been partially answered in the fact that stretch can induce increased expression of TA553.
6. Are any aspects of the protein altered in pathological situations affecting the heart or skeletal muscle?
7. Do drugs affect the protein?
8. What is the time pattern of mRNA / protein expression during muscle response to stretch?
9. Does the protein play a role in embryonic development of skeletal or cardiac muscle?

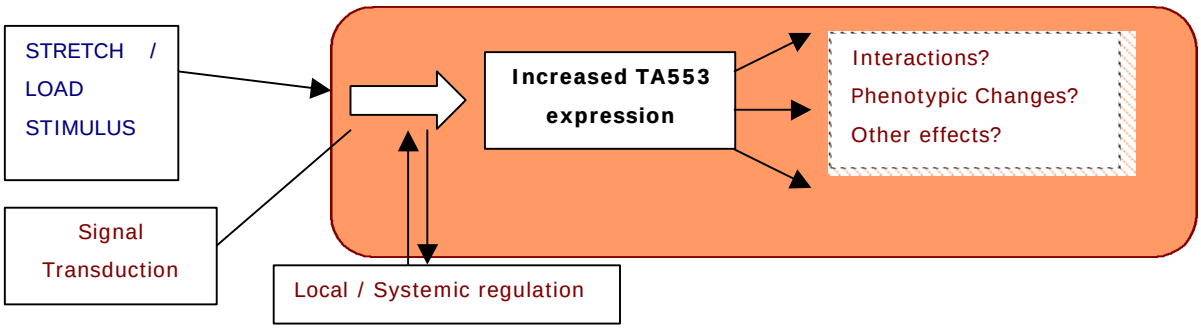


Figure 12: What lies next in TA553 research?

Summary

The experiments described in this project show that TA553 is expressed in all skeletal muscles surveyed. There is also high expression in cardiac muscle, liver shows comparatively very little expression. It has also been shown that the full length of the mRNA sequence TA553 is greater than 9Kb. Hence, once full investigation of the role of TA553 is complete. The full clarification of the pathways involved in skeletal muscle phenotypic change will be closer to completion.

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Appendix

Additional methods

RNA extraction: Variation on the standard protocol.

The Promega protocol was followed except for the initial steps. The tissue samples were homogenised with a FastPrep machine with RDS.

A standard RDS comprises 25g guanidinium thiocyanate, 29.3ml Rnase free water, 1.76ml of 0.75M sodium citrate and 2.64 20% sarcosyl. 1ml Sodium acetate, 10ml of phenol and 2ml chloroform were then added to the RDS and the resultant solution was then to homogenise tissue samples.

After the completion of the homogenisation of tissue, the Promega SV kit manufacturer's protocol was followed to extract total RNA from tissue samples.

Agarose gel electrophoresis

For RNA gels, 1.0% of Rnase free agarose by mass (supplied with Promega SV Kit) was used in solution with 1X Rnase free gel running buffer (diluted from 10X with Rnase free water).

For DNA gels, 1.2% agarose by mass was used and diluted with 1X standard gel running buffer and 1μm of ethidium bromide was added.

The agarose solution was heated in a microwave until any solid matter was dissolved. The gel was poured into an electrophoresis tray and combs were placed into the gel when slightly cooled. The gel was then allowed to cool until it had set, the combs were removed and the gel was loaded with the relevant sample.

When the gel was loaded, the voltage was set to 150V and the gel was run until visual confirmation of satisfactory migration was made.

Synthesis of cDNA (Reverse Transcription)

RNA is denatured at 95^{o}C for 5 minutes and the following reaction mixture is assembled on ice in the order stated in the table. Prior to the addition of RNA template, vortex the mixture briefly and

leave for 2 minutes at room temperature. After template addition, vortex and spin the mixture for 1 minute.

Table 9: Reaction mixture for cDNA synthesis.

COMPONENT	QUANTITY
Primer	1µl
Reaction buffer	5µl
0.1M DTT	2µl
dNTP mix	2µl
Rnasin	1µl
RNA + water	5µl
Reverse transcriptase	2µl

Once the mixture is assembled, vortex gently and incubate at 37^{o}C overnight. The resulting cDNA product can be kept safely at -20^{o}C until required for PCR reactions.