THE ISOLATION AND CHARACTERIZATION OF TROPONIN T FROM THE SLOW AND FAST MYOTOMAL MUSCLES OF ATLANTIC SALMON

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by

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Abstract

1. Subunits of troponin (Tn) were isolated and characterized from the fast and slow swimming muscles of Atlantic salmon (*Salmo salar*). Troponin C (TnC) from both slow and fast muscle was determined, by electrophoretic analysis to be present as single and distinct isoforms. Three isoforms of fast troponin T (TnT 1F, 2F and 3F) and two from slow muscle (TnT 1S and 2S) were detected. These proteins were determined to be TnT by the following criteria: copurification with whole Tn, SDS PAGE, amino acid composition, partial protein sequence data, immunoreaction with an anti-TnT antibody and affinity chromatography with immobilized tropomyosin (TM). The TnTs were specific to the corresponding muscle type.

2. All TnTs, with the exception of TnT 1F were N-terminally blocked. Partial protein sequence data was obtained for all of the isoforms. The various isoforms of TnT possess similar amino acid compositions with the exception of their proline contents. The higher molecular weight isoforms (TnT 1F and 1S) contain higher amounts of proline than the lower molecular weight forms (TnT 2F, 3F and 2S). All TnTs contain one tryptophan, with the exception of TnT 1S which contains two. None of the isoforms contain cysteine. TnT 2F and 3F contained phospho-serine while TnT 1F, 1S and 2S did not.

3. A full length TnT clone was isolated from a brown trout (*Salmo trutta*) slow muscle cDNA library and sequenced. The clone is believed to correspond to TnT 1S based on identity (94-112) with protein sequence data derived from a CNBr fragment of TnT 1S and similar amino acid composition data. The

nucleotide sequence encodes 278 amino acids with a predicted molecular mass of 32362 and a negative charge at neutral pH. The N-terminal region is highly acidic (26 acidic residues occur in the first 55 amino acids). In addition, all of the 13 proline residues are located within the first 73 amino acids. The central part of the molecule contains a large number of charged residues, while the C-terminus is positively charged.

4. During exploratory sequencing of the slow cDNA library, a full length clone corresponding to actin was isolated and sequenced. Comparative sequence analyses revealed that this clone encodes a striated muscle class II α-actin. The nucleotide sequence encodes 377 residues with an acidic pI and a predicted molecular mass of 41852.

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Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
CAPS	(3-[cyclohexylamino]-1-propanesulfonic acid)
cDNA	complementary deoxyribonucleic acid
CNBr	cyanogen bromide
DFP	diisopropylfluorophosphate
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
LB	Luria Broth
PMSF	phenylmethylsulfonic acid
PNK	polynucleotide kinase
PVDF	polyvinylidine difluoride
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
TBE	Tris HCl, borate, EDTA
TE	Tris HCl, EDTA
TM	tropomyosin
Tn	troponin

Chapter 1

INTRODUCTION

MOTILITY

Movement is a fundamental characteristic of life. Examples include the mobilization of white blood cells during the inflammatory response, the flagellar driven propulsion of spermatozoa and bacteria, the spreading of fibroblasts across a surface, the migration of chromosomes during cell division and the contraction of muscle (Bagshaw et al., 1993).

The minimum criteria for all motile systems are a cable, such as actin or microtubules, to direct movement and a motor protein to drive the movement. The motor is simply an energy transducer, which converts chemical energy in the form of adenosine triphosphate (ATP) to mechanical energy (Bagshaw, 1993; Bray, 1992). Myosin, dynein and kinesin are examples of motor proteins. In recent years, dramatic advances have been made with kinesin and to a lesser extent dynein. However, muscle contraction involving the interaction of actin filaments and myosin remains the most well characterized motile system at the molecular level, owing to the high abundance of muscle and relatively easy purification of its constituent proteins.

MUSCLE

In vertebrate organisms there are two distinct muscle types; striated (cardiac and skeletal) and smooth. Smooth muscle, under involuntary control is involved with the functioning of internal organs such as stomach,

diaphragm and uterus. Contractions are slow but a great range of control is achieved. Cardiac muscle is under vagal and sympathetic control and can undergo regular, sustained contraction for many decades. Under voluntary control, skeletal muscle has diverse physiological properties; including locomotion, eating, breathing, facial expressions and the maintenance of posture (Bagshaw, 1993).

The remainder of this discussion is restricted to striated muscle only. The characteristics of smooth muscle, although interesting, are outside the scope of this work. Striated muscle fibers are multinucleated cells that range in diameter from 20-100μm and vary in length from less than a millimeter to many centimeters, and in certain instances can span the entire length of the muscle (Bagshaw, 1993; Bray, 1992). Three types of muscle fibers can be distinguished based on a number of parameters including, speed of contraction and method of ATP synthesis. These are: slow oxidative (type I), fast oxidative (type IIa) and fast glycolytic (type IIb) (Table 1). Slow contracting (darker toned) muscle is rich in myoglobin, cytochromes and mitochondria and can therefore carry out oxidative phosphorylation, an aerobic process, occurring in mitochondria, that provides a copious supply of ATP from fatty acid oxidation. This type of muscle is well suited for sustained activity and is more abundant in athletes involved in endurance type exercises. Fast glycolytic fibers (lighter toned) are larger (due to higher amounts of actin and myosin) than slow oxidative fibers and also contain myosin that has a higher Mg^{2+}ATPase activity. Type IIb fibers derive energy from anaerobic breakdown of glycogen. Since the ATP yield is low, these fibers fatigue quickly. By contrast, type IIa fibers have some oxidative capacity and can therefore sustain

Table 1. Differentiating characteristics of slow and fast type muscle fibers. Adapted from Sherwood, 1989.

	Fiber Type		
Characteristic	Slow-oxidative (Type I)	Fast-oxidative (Type IIa)	Fast-glycolytic (Type IIb)
Speed of Contraction	slow	fast	fast
Resistance to Fatigue	high	intermediate	low
Myosin-ATPase activity	low	high	high
Fiber diameter	small	intermediate	large

rapid contractions longer than the fast glycolytic fibers (Sherwood, 1989; Bagshaw, 1993; Syrovy, 1987).

Most mammalian muscle contains a mixture of these three fiber types, the proportions of which depend upon the type of muscle and the physiological demands placed upon it. For example, muscles that control posture such as those of the back and legs must contract for long periods of time therefore are rich in slow oxidative fibers. Conversely, the muscles of the arm that perform rapid movements are well endowed with fast glycolytic fibers (Bagshaw, 1993; Sherwood, 1989).

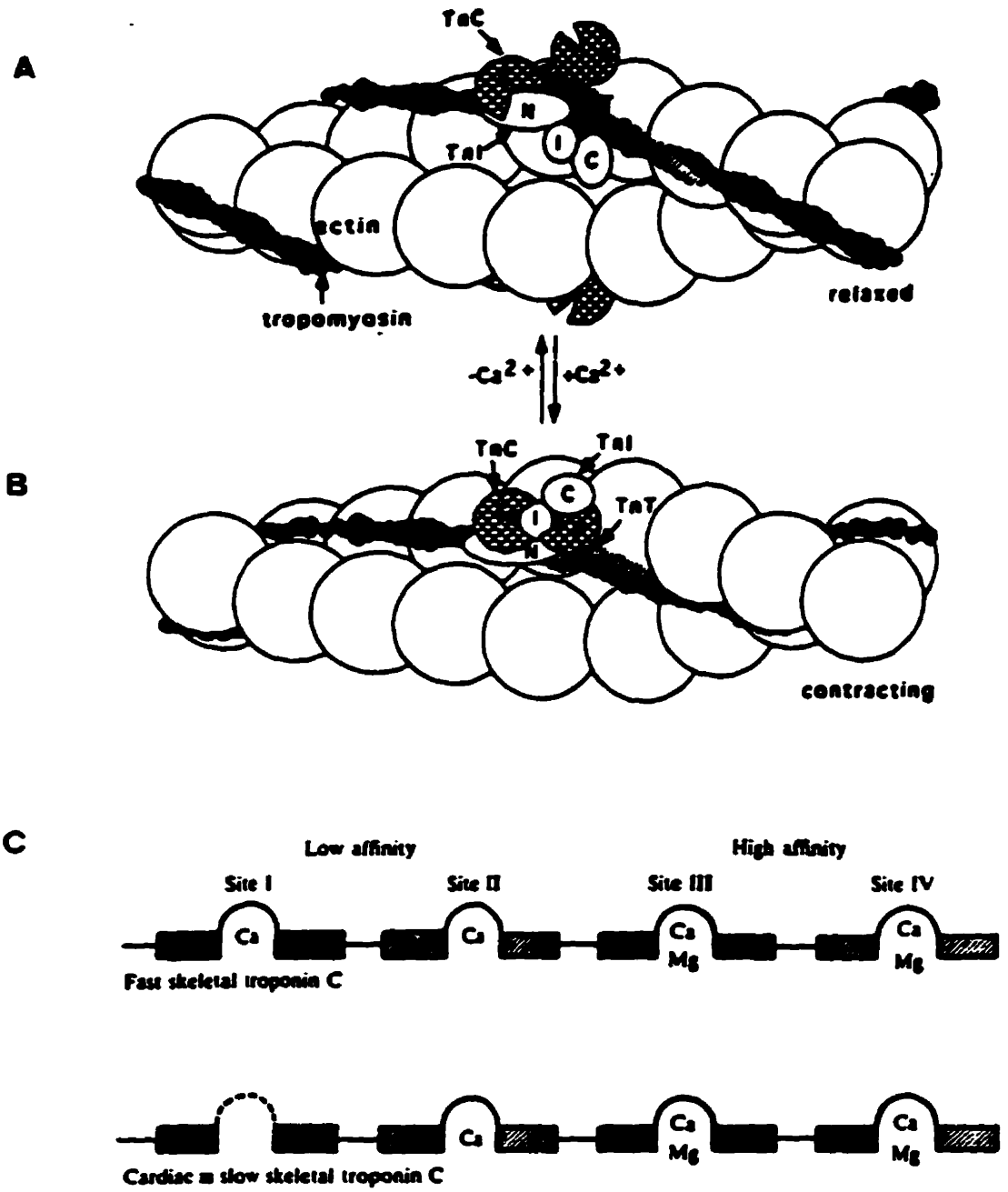
Each muscle fiber contains longitudinal elements called myofibrils. Electron microscopy of skeletal and cardiac muscle sections reveal an ordered, repeating pattern so that the muscle appears striated. The sarcomere, the repeating unit of the myofibril, consists of two types of interdigitating filaments; thick and thin. The thick filaments are composed of myosin. The thin filament (Fig.1) is a complex of five proteins: actin, TM, TnT (tropomyosin binding subunit), TnI (inhibitory component) and TnC (Ca^{2+} binding protein) (reviewed by: Huxley, 1972; Bagshaw, 1993; Chalovich, 1993; Leavis and Gergely, 1984; Adelstein and Eisenberg, 1980).

MYOFIBRILLAR PROTEINS

Myosin

Striated muscle myosin is a large (M_r = 500kDa), acidic, hexameric complex of two heavy chains and four light chains. At the C-terminus, the heavy chains self associate to form a long α-helical coiled coil. The N-

Fig. 1. Schematic representation of the thin filament in the absence (A) of calcium and in the presence (B) of calcium. In both diagrams, the high affinity sites of TnC are filled with Mg^{2+}. In (A) the low affinity sites of TnC are devoid of Ca^{2+} and only the C-terminal domain of TnC interacts with TnI. This leaves both the C-terminal as well as the inhibitory region of TnI free to interact with actin and TM. The result is inhibition of actin and myosin interaction. When Ca^{2+} binds to the Ca^{2+} specific sites of TnC (B), there is increased affinity for the inhibitory and C-terminal regions of TnI, resulting in changes in the interaction of TnI with TM and actin. TM moves to a new position (deeper into the actin groove) allowing actin and myosin to interact productively and contraction to occur. This model is based on studies of mammalian muscle proteins and was taken from a review article by Farah and Reinach, (1995). (C) The calcium binding domains of troponin C from striated muscle. The shaded boxes represent α-helices, while the semi circles denote Ca^{2+} binding loops. Fast muscle TnC contains four sites capable of binding Ca^{2+}, while cardiac/slow TnC only has three functional sites. Taken from Perry, (1985).


A
TnC
N
I
C
TnI
actin
tropomyosin
relaxed
+Ca2+
-Ca2+
B
TnC
TnI
C
I
TnT
N
contracting
C
Low affinity
High affinity
Site I
Site II
Site III
Site IV
Ca
Ca
Ca
Mg
Ca
Mg
Fast skeletal troponin C
Ca
Ca
Mg
Ca
Mg
Cardiac ≡ slow skeletal troponin C

terminal portion of each heavy chain forms a globular head. Two nonidentical pairs of light chains are associated with each head. These are termed regulatory and essential light chains (~20kDa each) (Bray, 1992; Bagshaw, 1993). The role of each of these components is not fully understood, however they may function to stabilize the heavy chain (Bagshaw, 1993).

The tail region has a repeating pattern of nonpolar and polar amino acids. Hydrophobic amino acids occur every alternate third and fourth residue, thereby creating a nonpolar strip running the length of the helix that promotes self association between adjacent heavy chains. The myosin tail is responsible for thick filament aggregation, creating a bipolar cable that has myosin heads protruding from both ends.

All myosins possess actin activated Mg^{2+}ATPase activity. Located within each head is the site of ATP hydrolysis as well as the actin binding site. The crystal structure of the myosin head revealed the presence of two clefts, an ATP binding pocket and a crevice that runs from directly beneath the ATP binding site almost to the back of the head. The second cleft may play a role in information relay between the actin and ATP binding sites which were shown to be approximately 4nm apart (Rayment et al., 1993).

The interaction of thick and thin filaments occurs through the binding of myosin heads to actin. It is this interaction, or crossbridge, coupled to the hydrolysis of ATP that generates force production and movement.

Actin

A monomer of globular (G) actin, is an acidic, single polypeptide chain of M_r 42kDa. Two globular domains separated by a narrow cleft are apparent

in the crystal structure (Kabsch et al., 1990; Holmes et al., 1990). Each actin has associated with it a nucleotide (ATP or ADP) and a divalent cation (Ca^{2+} or Mg^{2+}) that bind the cleft region. The smaller domain contains both the N- and C-termini of the protein as well as the myosin binding site.

Actin is one of the most abundant and highly conserved proteins in eukaryotic cells (Sheterline and Sparrow, 1994; Syrovy, 1987). The high degree of conservation of actin is a reflection of an extensive quaternary structure. Each monomer has binding sites on its surface that allow association with at least three other actin molecules. This leads to self polymerization, thereby forming a continuous filament. The first 25-30 amino acids of actin contain a high proportion of acidic residues that facilitate binding to myosin. Actin also interacts extensively with TM along the entire length of the thin filament, with a single TM molecule making contact with at least 7 actin monomers. In addition, the inhibitory component of the troponin complex (Tn I) also binds to actin (Huxley, 1972; Bagshaw, 1993; Leavis and Gergely, 1984).

In view of its conserved nature, it is surprising that as many as six variants of actin are synthesized in higher mammals. These are classified (α,β and γ) based on their isoelectric points and exhibit a tissue specific distribution (Bray, 1992; Herman, 1993; Sheterline and Sparrow, 1994). Very few differences exist between some of these isoforms (4 substitutions out of 375 amino acids in skeletal versus cardiac muscle actins). The sequence variations do not seem to translate to significant functional differences (no change in myosin Mg^{2+}ATPase activity in assays that included either cardiac or skeletal muscle actin) (Mossakowska and Strzelecka-Golaszewska, 1985). However, there may be subtle differences in actin mediated activity that go

undetected due to limitations in the techniques that are presently available. The sequence and functional similarity of actin isoforms from mammalian muscles suggests that the role of actin in the contractile cycle is similar in all muscles.

Polymerized actin has a distinct polarity as revealed by the characteristic arrangement of myosin heads which can form on its surface in the absence of ATP. Under the electron microscope, the lateral projections of myosin appear as arrowheads thus distinguishing the two filament ends as either pointed or barbed (Bray, 1992; Sheterline and Sparrow, 1994). The pointed end is directed toward the centre of the sarcomere. This is significant because myosin is allowed to move in the pointed to barbed direction only. The result is that myosin heads 'pull' thin filaments on opposite sides of the sarcomere toward one another upon contraction (Bray, 1992).

Tropomyosin

Tropomyosin was first isolated in 1946 (Bailey, 1946; 1948) and exists as an acidic, coiled coil of two α-helical chains aligned in parallel and in register with a M_r of 66kDa. In the presence of reducing agents and high concentrations of denaturants, such as urea, TM is dissociated into two chains of 33kDa (Smillie, 1979). Tropomyosin contains two heptad repeat sequences thereby forming a continuous nonpolar strip which runs the length of the molecule and promotes self association. This arrangement is further stabilized by the interaction of charged groups located next to hydrophobic amino acids on both α-helices. All muscle TMs have 284 amino acids and are acetylated at the N-terminus (Smillie, 1979).

Like actin, TM exists in muscle in a filamentous form. Molecules of TM interact in a head to tail fashion via an 8-9 residue overlap between the N-termini of one molecule and the C-termini of another (McLachlan and Stewart, 1975). This interaction is stabilized, in part, by an electrostatic attraction between the two ends and is crucial for normal thin filament function. The requirements for effective polymerization include an intact N- and C-terminus (Johnson and Smillie, 1977; Mak and Smillie, 1981; Heeley et al., 1987) as well as an acetylated N-terminus (Hitchcock-DeGregori and Heald, 1987).

Distinct forms of TM are found in nonmuscle, skeletal and smooth muscle cells. Although these forms are similar, variations exist in important domains that give rise to structural and functional differences (Lees-Miller and Helfman, 1991). This diversity arises from a combination of alternative promoters and differential splicing of multiple genes (Lees-Miller and Helfman, 1991). Through these mechanisms, the rat α-gene, for example, not only encodes striated muscle α-TM but also smooth muscle TM (Ruiz-Opazo and Nadal-Ginard, 1987; Lees-Miller and Helfman, 1991) and low molecular weight TM isoforms consisting of 248 amino acids that are expressed in fibroblast and brain (Lees-Miller and Helfman, 1991). The complexity of this system will likely increase as more TM genes are characterized. Additional diversity can be generated by phosphorylation at serine position 283 (Mak et al., 1978). The extent of phosphorylation may play a role in modulating the regulatory properties of TM (Heeley et al., 1989; Heeley, 1994).

The role of TM in muscle remained unclear until the 1960's, when Ebashi showed that TM was associated with troponin in regulating the

actomyosin ATPase activity in striated muscle (Ebashi and Ebashi, 1964). Tropomyosin inhibits the myosin Mg^{2+} ATPase activity of reconstituted systems composed of purified actin and myosin present in molar ratios of 1:1 or less (Heeley et al., 1989). Tropomyosin is an essential component in regulating the Ca^{2+} sensitivity (concentration of Ca^{2+} required for 50% maximal activation) of the Mg^{2+}ATPase activity of actomyosin in the presence of troponin. When the stoichiometry of the thin filament is considered the importance of TM becomes clear. By interacting with both TnT of the Tn complex and actin, TM serves as a relay, conveying conformational information from one Tn to seven actins.

Troponin C

Troponin C, an acidic, calcium binding protein isolated in the 1960s (Hartshorne and Mueller, 1968; Schaub and Perry, 1969 is the smallest component of the Tn complex, with a molecular weight of approximately 18kDa. The TnC crystal structure predicts a dumb-bell shaped molecule with two globular domains (N- and C-terminal) separated by a flexible linker helix of 8-9 turns (Herzberg and James, 1988). Located within each domain are two helix-loop-helix motifs. In fast skeletal muscle, TnC contains four loops (numbered I to IV according to their order in the primary sequence) each capable of binding a divalent cation (Fig. 1c). The N-terminal domain contains Ca^{2+} binding loops I and II, which are low affinity binding sites (Leavis et al., 1978) specific for Ca^{2+}. The high affinity Ca^{2+}-Mg^{2+} binding sites III and IV are located within the C-terminal domain and possess a higher affinity for divalent metal ions. Under physiological conditions these sites

are thought to be permanently filled with Mg^{2+} (Potter and Gergely, 1975; Leavis et al., 1978). The Ca^{2+} induced contraction of skeletal muscle is due to the binding of Ca^{2+} to sites I and II (Johnson et al., 1978), while sites III and IV are believed to be involved in maintaining stability of the TnI-TnC complex (Zot and Potter, 1982, Negele et al., 1992).

Troponin C exists as two isoforms in mammals and birds, one is found in fast skeletal muscle, while the other is common to both cardiac and slow muscle (Wilkinson, 1980). These arise from two distinct genes. The major difference between cardiac/slow TnC and fast TnC is that Ca^{2+} binding loop I of cardiac/slow TnC is mutated so that it no longer binds Ca^{2+} ions (Collins et al., 1977). At least three amino acids responsible for Ca^{2+} coordination are different in cardiac/slow TnC, these include Asp 27 (rabbit fast TnC) for Val (rabbit cardiac TnC); Asp 29 for Ala and Asp 33 for Cys. Thus, cardiac/slow TnC only possesses one trigger site (site II) (Sweeney et al., 1990). Although cardiac and slow TnC have the same primary sequence, the Ca^{2+} binding properties of the respective Tn complexes are not identical. This is because the Ca^{2+} binding properties are modified by the nonidentical isoforms of TnI present in slow and cardiac muscle (Perry, 1985).

Calcium binding to TnC induces a conformational change that has been detected by fluorescence, thiol reactivity, circular dichroism (CD) (see Leavis and Gergely, 1984 for review) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (Head and Perry, 1974). The change detected by some of these methods was initially predicted to arise from an increase in α-helical content (from 30% to 50%). Recent studies employing high resolution nuclear magnetic resonance (NMR) have disputed this long

held postulate (Gagné et al.,1994). There may be a relative movement of two α-helices resulting in a more compact structure and increased exposure of a hydrophobic pocket in the N-terminal domain that enhances interaction with TnI and neutralizes its inhibitory actions (Herzberg et al., 1986). Troponin C may relieve TnI induced inhibition by weakening the interaction between TnI and actin-TM (Farah and Reinach, 1995).

Troponin I

Troponin I is a basic protein with a molecular weight of approximately 21kDa. Troponin I binds actin, TnC, TnT and TM and is a potent inhibitor of the actomyosin ATPase (Huxley, 1972; Leavis and Gergely, 1984). The inhibitory region was originally defined by Syska et al. (1976) to be contained within residues 96-116 (rabbit skeletal). In experiments with synthetic peptides, residues 104-115 were found to represent the minimum sequence necessary for inhibition (Talbot and Hodges, 1981). However, recent studies have shown that more of the C-terminal region is required (Farah et.al., 1994).

Fast, slow and cardiac muscle each contain a unique isoform of TnI. Sequence data have demonstrated that although considerable conservation exists in the inhibitory domain, these isoforms arise from distinct genes (Wilkinson and Grand, 1978; Murphy et.al., 1991)

Troponin T

Troponin T is the focus of this thesis. It is the largest (~30-33kDa) subunit of the troponin complex and contains binding sites for TM (Mak and Smillie, 1981; Pearlstone and Smillie, 1977; 1981; White et al. 1987), TnC

(Ohtuski, 1975; Pearlstone and Smillie, 1978) and TnI (Ohtuski, 1975; Pearlstone and Smillie, 1980) (Fig. 2). Several lines of evidence indicate that TnT is asymetric. These include X-ray crystallography (White et al., 1987) and NMR (Brisson et al., 1986). The sequence of TnT is characterized by an unusual arrangement of charged residues. Whereas the N-terminal region is composed of a high percentage of acidic amino acids, the C-terminal region is predominantly basic. Although α-helical in some of its structure (Pearlstone and Smillie, 1977), TnT contains a high proportion of proline residues at the NH_2-terminus, which may inhibit formation of helical segments in this domain. In addition, the high proportion of charged residues throughout the molecule is predicted to form an open structure, free to participate in many protein-protein interactions (Leavis and Gergely, 1984).

Troponin T binds TM at two sites (Mak and Smillie, 1981; McLachlan and Stewart, 1976; Pearlstone and Smillie, 1977; 1981; White et al., 1987). The N-terminus of TnT (1-70) extends over a significant portion of the thin filament and binds to the C-terminus of TM, spanning the overlap region and interacting with the extreme N-terminus of adjacent TM molecules (Mak and Smillie, 1981). This Ca^{2+} insensitive association plays a role in anchoring the Tn complex to the thin filament during Ca^{2+} activation. The central region (71-151) and C-terminal domain of TnT (159-227, rabbit TnT amino acid numbering scheme) contain a helical segment which may stabilize interaction with TM by the formation of a triple helix (Pearlstone and Smillie, 1977). Troponin I and TnC also bind near the C-terminal domain (Ohtuski, 1975), rendering this site Ca^{2+} sensitive.

Troponin T can be phosphorylated at serine position 1 in the native

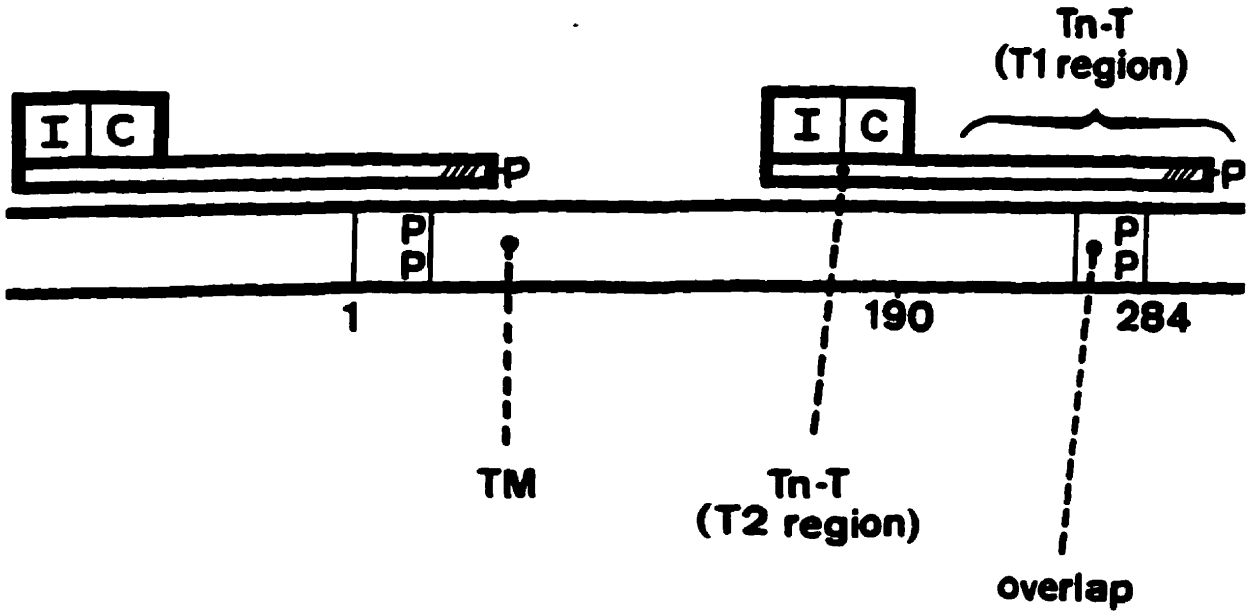


Fig. 2. Schematic representation of the sites of interaction of TM and the Tn complex. The abbreviations are: P, phosphorylation; I, TnI; C, TnC. The numbers correspond to amino acid residues of TM. The elongated N-terminal segment of TnT (T1- corresponding to residues 1-158 of rabbit TnT) extends along the overlap region of TM, while the remainder of the molecule (T2, residues 158-159 of rabbit TnT), along with TnI and TnC is situated on the N-terminal side of cysteine 190 of TM. The hypervariable region of TnT is denoted by (////). Adapted from Heeley, 1994.

troponin complex (Pearlstone et al., 1977, Moir et al., 1977); however, no physiological role for this post translational modification has been found (Heeley, 1994)

Extensive heterogeneity has been observed at the N-terminus of TnT. In connection with this, multiple isoforms have been reported in chicken (Wilkinson et al., 1984), bovine heart muscle (Gusev et al., 1983), rat (Medford et al., 1984; Brietbart et al., 1985), rabbit (Briggs et al., 1984; 1987), human adult and fetal cardiac tissue (Townsend et al., 1995) and human slow muscle (Gahlmann et al., 1987; Samson et al., 1994). Most of the biochemical experimentation has been carried out with TnTs isolated from fast muscle. By comparison cardiac TnT has been studied less and virtually nothing is known about slow TnT. It is known that isoforms of TnT are generated from the tissue specific and developmentally regulated alternative splicing of individual genes (Wilkinson et al., 1984; Breitbart et al., 1985). The exon pattern of the cardiac and fast TnT genes have been delineated (Breitbart et al., 1985). Both have 18 nonidentical exons which mostly occur in the extreme N-terminal region, (9 in the first 43 amino acids (fast) and in the first 82 amino acids (cardiac)). Recently, an additional exon coding for fetal TnT, located between exons 8 and 9 (adult rabbit) has been identified and found to be developmentally regulated (Briggs and Schachat, 1993, Briggs et al., 1994). Combinatorial splicing of five 5' miniexons (4,5,6,7 and 8) occurs at the N-terminus potentially generating 32 fast TnT variants. Only two isoforms (α or β) are generated at the C-terminus by mutually exclusive splicing of exons 16 (α) or 17 (β) of fast TnT. The result is a splicing system capable of generating 64 isoforms (Medford et al. 1984, Putney et al., 1983). The expression of the C-

terminal isoforms is developmentally regulated, β occurs in fetal and newborn muscle while α is found in adult (Medford et al., 1984; Breitbart et al., 1985). The slow TnT gene has not been characterized as extensively as the fast and cardiac TnT genes. In human, three distinct slow skeletal muscle TnT isoforms (Gahlmann et al., 1987; Samson et al., 1994) arise from alternative splicing of a single slow TnT gene localized on chromosome 19 (Samson et al., 1990). These sequences are highly similar and differ only by the variable inclusion of an N-terminal 33 nucleotide insertion or a C-terminal 48 nucleotide sequence. The position of alternatively spliced exons known to be expressed in fast, slow and cardiac TnT is illustrated in Fig. 3 (Schiaffino and Reggiani, 1996). Studies of exon splicing patterns of the TnT gene family have revealed a number of interesting findings. The first is that the splicing pattern for each of these three genes is unique. Secondly, TnT isoforms often differ only by the variable inclusion of a very small sequence usually from 3-10 amino acids (Breitbart et al., 1985). The final observation is that not all genetically possible forms are realized at the protein level (Briggs et al., 1987; 1990). For example, 64 distinct variants of rat fast skeletal muscle TnT may be derived from a single gene (Breitbart et al., 1985); however, only 13 have been detected (Sabry and Dhoot, 1991). Troponin T, particularly cardiac specific isoforms, have been implicated in disease states. Mutations in the human cardiac TnT gene have been implicated in familial hypertrophic cardiomyopathy (Thierfelder et al., 1994). Furthermore, many physicians are now assaying for cardiac TnT as a marker in heart failure. The extent of damage is related to the serum levels of cardiac TnT. Such changes are monitored after suspected myocardial infarction and during heart surgery

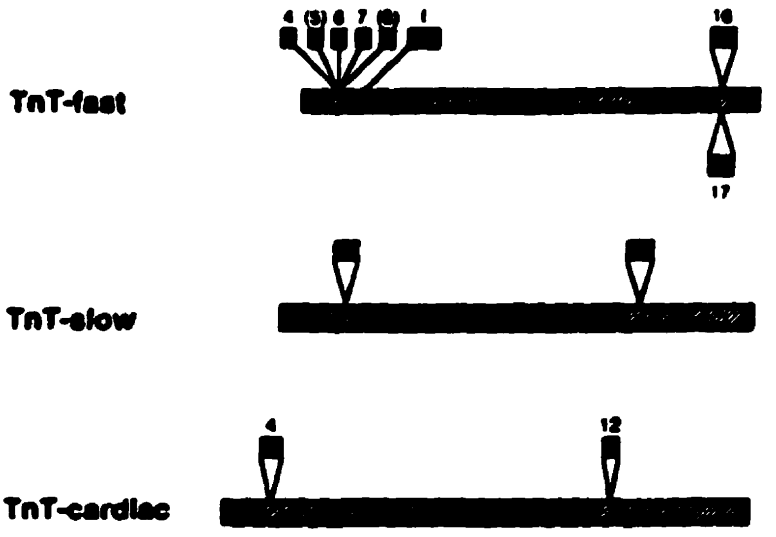


Fig. 3. Schematic representation of the alternative exon patterns of TnT genes. The locations as well as the exon number (when known) are provided. This diagram is taken from Schiaffino and Reggiani (1996) and is based on nucleotide sequence data of rat and rabbit fast TnT (Breitbart et.al., 1985; Briggs and Schachat, 1989; 1993), human slow TnT (Samson et.al., 1994; Gahlmann et.al., 1987), and rat, rabbit and human cardiac TnT (Anderson et.al., 1995; Greig et.al., 1994; Jin et.al., 1992; Mesnard et.al., 1995).

(Wu and Lane, 1995; Voss et al., 1995).

Muscle Contraction: An Overview

Illustrated in Fig. 4 is a schematic of the contractile cycle. In voluntary muscle, the stimulus to move is conveyed in a process known as excitation contraction coupling. An action potential travelling along a nerve reaches the end of the axon and triggers the release of a neurotransmitter such as acetylcholine. The muscle cell membrane becomes depolarized once acetylcholine diffuses to the muscle cell and interacts with its receptor on the cell surface. The excitation continues to move along the muscle cell membrane into invaginations known as transverse tubules (T-tubules), whereupon contact can be made with the sarcoplasmic reticulum surrounding each myofibril thus triggering the release of Ca^{2+}, which then diffuses towards the contractile regulatory elements. Hydrolysis of ATP occurs forming ADP and inorganic phosphate (Pi). These products remain tightly bound to myosin and are only released when Ca^{2+} is present. The strong interaction between myosin and actin is prevented by the inhibitory component TnI. Calcium binding to TnC relieves TnI induced inhibition, possibly by causing a movement in TM, thus allowing productive myosin-actin interaction to take place. The binding of myosin to actin may be a two step process; initially weak binding occurs whereby the myosin head assumes the correct orientation and binds to a new actin monomer. Upon tight binding, phosphate is released and the power stroke occurs. The removal of products is also postulated to be a two step process, initially involving release of phosphate, followed by release of ADP (reviewed by Chalovich, 1993;

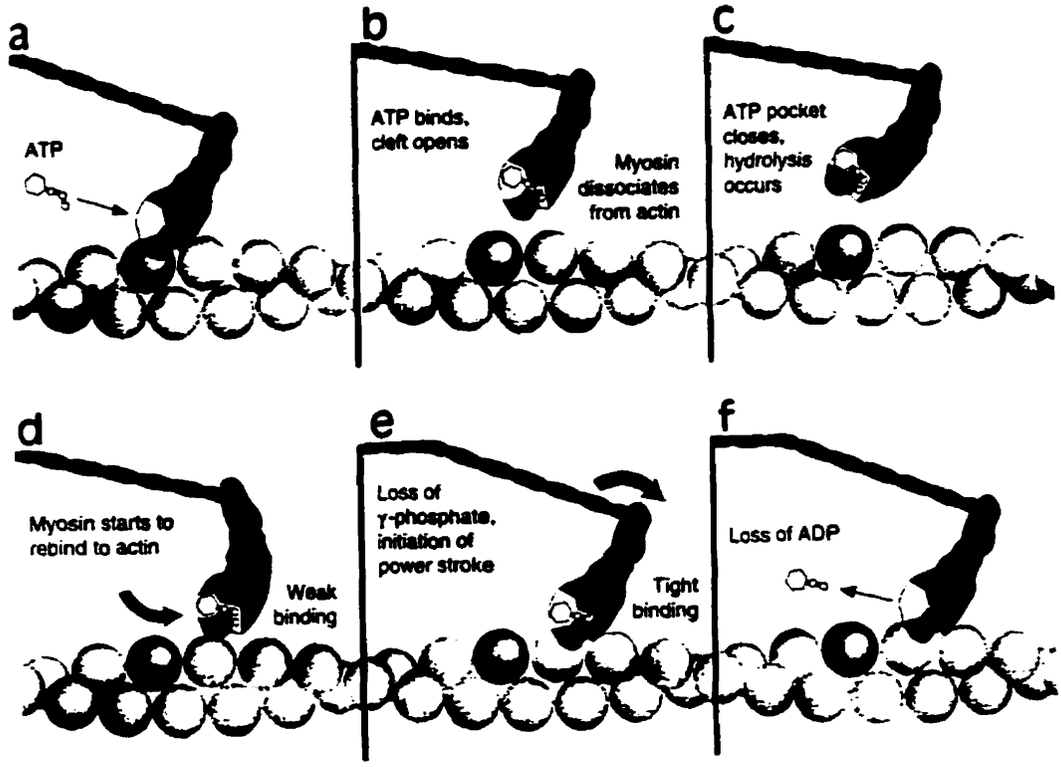


Fig. 4. Schematic representation of the contractile cycle. Actin is denoted by the string of spheres. This figure depicts conformational changes thought to occur in the myosin head during the contractile cycle. In the absence (a) of nucleotide, myosin is bound to actin. Upon binding of ATP, myosin dissociates. A conformational change then takes place that may involve closure of the ATP binding pocket. This step induces the hydrolysis of ATP (c). Myosin moves by diffusion and interacts weakly with a new actin molecule (d) before isomerization to a tight binding state can occur (e). Simultaneous to the formation of a tight complex between actin and myosin, the phosphate is released and the power stroke begins, followed by release of ADP (f). Taken from Rayment and Holden, (1994).

Adelstein and Eisenberg, 1980; Rayment and Holden, 1994; Spudich , 1994).

The preceeding cycle features three popular theories of muscle contraction, which address different parts of the contractile cycle, (1) the sliding filament hypothesis, (2) the steric blocking model and (3) the swinging crossbridge model. Proposed by Huxley in 1954, the sliding filament hypothesis envisages that in a contracting muscle the thin and thick filaments slide past each other with no change occurring in the length of the filaments. The steric blocking model proposes that in a relaxed muscle TM lines the groove of the actin filaments and physically blocks the interaction of myosin and actin. Upon activation, TM is proposed to move deeper into the actin groove and away from its blocking position, thus allowing the interaction of myosin and actin. The swinging crossbridge model, also developed by Huxley, postulates that myosin heads interact with actin at two distinct angles, thus must vary their tilt angle to generate force production.

Purpose of Study

It is clear that protein heterogeneity, is a common way to introduce great diversity into a system while at the same time preserving features that are essential for function. Early experiments have documented the relationship between speed of shortening of the muscle and myosin Mg^{2+}ATPase activity (Bárány, 1967). It therefore follows that factors that influence the ATPase rate of a certain muscle will contribute to differences in performance. Thus, isoforms of the Tn subunits and TM are prime candidates for providing such diversification. Since many studies have related changes in Ca^{2+} sensitivity (Nassar et al., 1991; Reiser et al., 1992), Ca^{2+} binding

(McAuliffe et al., 1990), TM and TnC binding (Pan and Potter, 1992) and myosin Mg^{2+}ATPase activity (Tobacman, 1988) to heterogeneity of TnT, it is reasonable to assume that variations in this protein will give rise, in part, to differences between fast and slow muscles. While fast skeletal muscle proteins have been fairly well characterized, slow muscle proteins have been studied considerably less. One major reason for this is that commonly used research animals lack a plentiful supply of homogeneous slow muscle. Furthermore, most mammalian slow muscles are interspersed with fast type fibers. Thus, the large scale preparation of slow muscle specific proteins has been hampered. The use of round bodied fish such as salmonids has proven advantageous in regard to isolating TM from slow muscle (Heeley et al., 1995). Such organisms possess a unique segregation of slow and fast fibers. The slow muscle is confined to a band that runs from the head to the tail and is sandwiched between the fast tissue and the skin (Johnson et al., 1975). This arrangement allows for relatively easy separation of slow muscle with less than 5% contamination of fast type fibers. The isoform pattern of salmonid TM (Heeley et al., 1995) was determined to be much simpler than that of mammals and birds. Therefore, salmonid TnT was investigated in the hope that the isoform pattern would also be relatively simple. With purification procedures and analysis of TM function already carried out, studies of the TM-TnT interaction could be performed relatively easily.

The present work was initiated to catalog the TnT isoform content of slow and fast myotomal muscle, and to carry out preliminary characterization studies of slow and fast muscle TnTs from fish. For the first time, slow muscle TnT has been purified in amounts necessary for biochemical studies.

Others have reported nucleotide sequence data for slow TnT from human muscle (Gahlmann et al., 1987; Samson et al., 1994) and chicken muscle (Yonemura et al., 1996) but have not provided protein data to complement their sequencing results. In addition to preliminary protein characterization, this work includes the nucleotide sequence of a slow TnT isoform.

Chapter 2

MATERIALS AND METHODS

Preparation of Troponin (Tn)

In round bodied fish, such as Atlantic salmon *(Salmo salar)*, the slow (darker toned) muscle is located along the lateral line and can be easily separated from the fast (lighter toned) muscle. Slow type muscle is present in appreciable amounts, therefore Tn could be prepared from both tissue types according to the method of Ebashi (Ebashi et al., 1971). Muscle was minced through a grinder equipped with a medium sized plate and suspended in 3 volumes of 0.15M sodium phosphate, pH 6.5 at 4°C, 0.3M KCl, 0.88mM adenosine triphosphate (ATP), 1mM phenylmethylsulfonic acid (PMSF). Following centrifugation, the residue was washed with 3 volumes of 2mM $NaHCO_3$, 20mM KCl, centrifuged and washed with deionized water. The residue from this centrifugation was extracted with 2 volumes of 50mM sodium acetate, pH 4, 0.6M LiCl, 1mM DTT, 1mM PMSF for 1.5 hours at 4°C. Tropomyosin (TM) was removed by isoelectric precipitation at pH 4.6. The complex was salted out between 40% and 60% saturated $(NH_4)_2SO_4$ and dialyzed. Both the supernatant and resuspended pellet were lyophilized separately. Occasionally, myofibrils were prepared first (Perry and Corsi, 1958) by blending chilled muscle with five (5) volumes of 0.1M KCl, 5mM EDTA, 39mM potassium borate, pH 7.1 at 2°C, 1mM PMSF. The myofibrils were subjected to four cycles of resuspension followed by centrifugation. Troponin was then isolated as described (Ebashi et al., 1971).

Troponin from slow muscle was also prepared by the method of Potter (1982). Carefully dissected slow muscle was homogenized in a household blender in 5 volumes of (5mM Tris-HCl, pH 8 at 4°C, 25mM KCl, 0.1% (v/v) Triton X-100, 1mM PMSF), the slurry was centrifuged and resuspended at least five times or until all residual lipid had been washed away. An acetone powder was prepared by washing the pellet three times with three volumes of cold ethanol followed by cold acetone treatment. The dried powder was extracted overnight in 15 volumes of 25mM Tris-HCl, pH 8 at 4°C, 1M KCl, 0.1mM $CaCl_2$, 0.1mM DTT, 1mM PMSF. Following centrifugation, the pH of the supernatant was adjusted to 4.6 and stirred on a magnetic stirrer for 30 minutes to precipitate tropomyosin. The pH of the supernatant was then adjusted to 7.8 and the Tn was salted out at 40% followed by 60% $(NH_4)_2SO_4$. The pellet from the 60% salt cut was redissolved in and dialyzed against Q Sepharose Fast Flow start buffer (50mM Tris-HCl, pH 8 at room temperature, 6M urea, 1mM DTT, 0.1% NaN_3) and applied directly to the Q Sepharose Fast Flow resin. Samples were obtained from commercial outlets, usually Fishery Products International Seafood Shops.

Preparation of Tropomyosin (TM)

The muscle samples were minced through a grinder with a medium sized plate and dehydrated with cold ethanol and acetone. Tropomyosin was extracted from the dried powder by washing in a high salt solution (1M KCl, 25mM Tris-HCl, pH 8.0 at 4°C, 0.25mM DTT, 0.5mM EDTA), for 1-2 hours on a magnetic stirrer, at 4°C (Cummins and Perry, 1973). Following centrifugation, the residue was washed with the same solution for 15

minutes. The supernatants from both spins were combined and the concentration of the solution was adjusted to 1-2mg/mL. The pH of this solution was decreased to 4.6. The isoelectric precipitate was collected by centrifugation, resuspended in hydroxylapatite column start buffer (1M NaCl, 50mM sodium phosphate pH 7.0 at room temperature, 0.01% (w/v) NaN_3, 1mM DTT) and applied to a hydroxylapatite column (2.5 x 10-25 cm). Tropomyosin was eluted isocratically with two column volumes of the above buffer containing 250mM sodium phosphate. In an attempt to limit proteolysis, the protease inhibitors diisopropylfluorophosphate (DFP) and PMSF both at a concentration of 1mM were included in the slow preparations. The isolated protein was judged to be 95% homogeneous by SDS PAGE. The slow TMs usually contained ~5% fast TM due to an inability to completely separate out all the fast fibres.

Electrophoretic Methods

Electrophoresis was performed on a Bio-Rad mini-Protean II apparatus (Bio-Rad, Richmond, Ca). All gels were 0.75mm thick and consisted of an acrylamide/N,N-methylene-bis-acrylamide (w/v) ratio of 37.5:1. Protein bands were visualized by staining in a shaking bath containing 0.2% (w/v) Coomassie Brilliant Blue R-250 (Kodak) in 50% (v/v) ethanol, 10% (v/v) acetic acid and then destaining in 20% (v/v) ethanol, 10% (v/v) acetic acid. SDS PAGE was carried out by the method of Laemmli (1970) on 10% (for TM) or 15% (for Tn) polyacrylamide slabs, with a 3% stacking gel at 100-200V constant voltage.

Whole muscle extracts were prepared by homogenizing cardiac, slow

and fast muscle (0.1-0.2g) in a 10X (v/w) ratio of saturated urea with a polytron (Brinkman, Westburg, N.Y.). The extracts were clarified by centrifugation in a microcentrifuge (Brinkman). An aliquot (usually 20-100μL) was mixed with an equal volume of sample buffer (65mM Tris-HCl, pH 6.8, 1.3%(w/v) SDS, 13%(v/v) glycerol and a small amount of Bromophenol Blue (Schwarz-Mann Biotech). Usually 10μL of protein was loaded onto each lane.

Immunoblotting

Western transfer from SDS polyacrylamide gels to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) was carried out in a Bio-Rad mini Trans Blot Electrophoresis Transfer Cell. The blotting conditions were 60V for 2.5 hours in 10mM CAPS pH 11 at room temperature, 10% (v/v) methanol. To check whether or not complete transfer had occurred, the SDS gel used for blotting was stained in Coomassie Brilliant Blue and then destained to visualize any remaining protein bands. Membranes were blocked in 3% bovine serum albumin (BSA) or 3% casein and washed in 20mM Tris-HCl pH 7.5, at room temperature, 0.5M NaCl. Immobilized proteins were probed with one of two antibodies, CT3-mouse-anti-rabbit-cardiac-TnT, a gift from Dr.J. Lin (University of Iowa, Iowa) or F-24-mouse-anti-chicken-fast-TnT, a gift from Dr.G.Dhoot (Royal Veternary College, London, England). Both antibodies were diluted 1:2000 with antibody buffer (0.05% Tween 20, 20mM Tris-HCl, pH 7.5, 0.5M NaCl). Reactive proteins were detected with 1:3000 dilution of goat anti-mouse IgG-alkaline phosphatase (Bio-Rad) and a solution of 0.1M $NaHCO_3$, 1.0mM $MgCl_2$, pH 9.8 at room temperature,

0.15mg/mL 5 - bromo - 4 - chloro - 3 - indolyl - phosphate and 0.3mg/mL 4-Nitroblue tetrazolium chloride (Boehringer Mannheim, Germany) (Towbin, 1979).

Chromatography

Ion Exchange Chromatography

The troponin subunits were separated on a Q Sepharose Fast Flow (Pharmacia) anion exchange resin equilibrated with 6M urea, 50mM Tris-HCl, pH 8 at room temperature, 1mM EDTA, 1mM DTT. Subsequent to sample application, the column was washed with at least two column volumes of start buffer followed by elution with ~500mL of the above buffer containing 0-0.5M NaCl gradient. The flow rate was ~50mL/h and ~5 mL fractions were collected. Components of the complex were identified by SDS PAGE, dialyzed against water and lyophilized. Some preparations of slow TnTs were dialyzed against, and stored frozen in, a high salt buffer (0.5M NaCl, 50mM Tris-HCl, pH 7.5) in an attempt to increase solubility of these proteins. Occasionally a S Sepharose Fast Flow (Pharmacia) chromatography was employed to purify slow TnTs.

Immobilized Metal Affinity Chromatography

Metal ion chelation chromatography was used to separate TnTs from whole Tn. The filtrate of a 20mg/mL solution of $FeCl_3$ in 1mM HCl was passed over a Chelating Sepharose Fast Flow resin equilibrated with deionized water. The resin (1cmx6cm) was washed with water and then equilibrated with start buffer (6M urea, 50 mM imidazole, pH 7 at room

temperature). Following sample application, the column was washed with two column volumes of start buffer. Proteins were eluted with 60mL of a 0-0.5M NaCl gradient. The flow rate was ~20mL/h, 2mL fractions were collected. Protein was detected by absorbance measurements at 280nm and SDS PAGE analysis.

Affinity chromatography

Affinity columns were prepared according to manufacturer's specifications using CNBr activated Sepharose 4B (Pharmacia). The Sepharose was washed with 2L of 1mM HCl and suction filtered until semi dry. Fast and slow TM affinity columns were prepared by dissolving purified protein (10mg/mL) in coupling buffer (0.1M $NaHCO_3$, pH 8.3, 0.5M NaCl) and rotating each solution with an aliquot of the resin (12mL) end over end for 1 hour at 4°C. Coupling of the TM was verified by SDS gel analysis and absorbance measurements (at 280nm) before and after the coupling step. The resin was washed with coupling buffer followed by 0.1M Tris-HCl pH 8 at 4°C, and then three cycles of alternating pH buffers (0.1M sodium acetate, pH 4 at 4°C, 0.5M NaCl) and (0.1M Tris-HCl, pH 8 at 4°C, 0.5M NaCl). Fast and slow muscle TnTs were loaded onto the corresponding TM affinity column (1cmx6cm) equilibrated in 10mM imidazole, pH 7 at 4°C, 0.15M NaCl, 0.25mM EDTA, 0.5mM DTT. Troponin Ts were eluted by addition of 60mL of a 0.15-0.6M NaCl gradient. The flow rate was 6mL/h or less and 1mL fractions were collected. Protein was detected by absorbance measurements at 230nm and SDS PAGE analysis.

Amino Acid Analysis

The various TnTs (~1mg) were hydrolyzed in 1mL of 6M HCl, containing 0.05% (v/v) phenol. Hydrolysates were analyzed on a Beckman Model 121 MB Amino Acid Analyser using Benson D-X8.25 Cation Xchange Resin, bed size 200 x 2.8mm. A single-column, three buffer sodium-citrate elution method was used. The flow rate was 8 mL/h with buffers and column temperature as per Beckman 118/119 Cl AM 001 application notes. Quantitation of the results was done using a Hewlett-Packard Computing Integrator Model 3395A. All analyses were done in triplicate at 24, 48 and 72 h. Serine and threonine were extrapolated back to time zero and tyrosine was taken from the 24h sample. Values for valine and isoleucine were taken from the 72h sample. Tryptophan was measured by hydrolysis in mercaptoethane sulphonic acid (Penke et.al., 1974). Methionine was determined as methionine sulfone and half-cystine as cysteic acid after oxidation in performic acid prior to acid hydrolysis (Moore, 1963). Occasionally half-cystine was also analyzed by determination of carboxymethyl-cysteine, following reaction with iodoacetic acid in 6M guanidine-HCl (Crestfield et.al., 1963) and acid hydrolysis, at 150°C, for 4h in the presence of a small amount of DTT. The compositions were calculated relative to the alanine number which gave a molecular mass closest to that predicted by mass spectrometry.

Cyanogen Bromide Digestion

Troponin Ts (typically 2mg) were dissolved in 70% formic acid to a final concentration of ~ 1-2mg/mL and treated with 200 fold excess of cyanogen bromide (CNBr) (Kodak) over methionine. The reaction was

carried out overnight at room temperature in the dark. Following digestion, the sample was diluted 15 fold with water and lyophilized (Gross, 1967).

Alkaline Phosphatase Digestion

Lyophilized Tn (~60 mg) was dissolved in 150mM NaCl, 50mM Tris-HCl, pH 8.00, 10mM $MgCl_2$. Alkaline phosphatase (Worthington) at a ratio of 1:200 (enzyme:substrate) was added to the protein solution, mixed well and incubated at 37°C for at least 1 hour. The solution was dialyzed against cold water and lyophilized.

Microsequence analysis

Automated Edman degradation was performed on electroblotted samples of intact and CNBr treated TnTs. This was carried out using an Applied Biosystems pulsed-liquid sequencer, model 475A equipped with an on-line phenylthiohydantion analyzer, model 120A and also an Applied Biosystems pulsed-liquid sequencer, model 473A, equipped with a microgradient phenylthiohydantion analysis. Analyses were performed at the Biotechnology service centre, Dept. of Clinical Biochemistry, University of Toronto, Toronto, Ontario.

Preparation of cDNA library

All reagents were of molecular biology grade, restriction enzymes were purchased from either Pharmacia or Promega.

A slow muscle cDNA library was prepared by Donna M. Jackman using a Superscript™ plasmid system for cDNA synthesis and cloning (Gibco BRL)

following manufacturer's specifications. Total RNA was isolated from a sample of slow muscle tissue (0.85g) excised from a freshly killed brown trout (*Salmo trutta*). Using an affinity oligo (dT) cellulose column, mRNA (2mg) was prepared, and a (polyT)*NotI* primer adapter was added to provide a substrate for both reverse transcriptase and RNase H during first strand synthesis and to facilitate ligation to the vector following second strand synthesis. The second strand was synthesized by nick translational replacement of the mRNA. The resulting cDNAs were ligated into pSport (4.1kb) and transformed into *Escherichia coli.* (*E.coli.*) DH5α competent cells and plated on Luria Broth (LB: 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L BactoAgar) ampicillin (100μg/mL) plates. Approximately 1100 colonies, were individually transferred from petri plates to 96 well Microtiter (Fisher Scientific) plates in TYPGN (2% tryptone, 1% yeast extract, 0.8% glycerol, 5g/L Na_2HPO_4, 10g/L KNO_3) media, and stored at -70°C (Brown and Knudson, 1991). Duplicate plates were prepared by inoculation with a sterile, inverted box of pipette tips.

Screening of cDNA Library

Clones from duplicate plates were immobilized on nylon Hybond-N^+(Amersham) nucleic acid transfer membranes using a dot blot apparatus. Membranes were prewet with 10% SDS. Clones were transferred using an octapipette and were immobilized to the membrane by suction. The cells were lysed using 100μL of (0.5 M NaOH, 1.5 M NaCl) and neutralized by addition of 100μL of (0.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 at room temperature) (Brown and Knudson, 1991). The membranes were rinsed in 4X SSC (0.15M NaCl,

15mM sodium citrate) then air dried.

An 18 base mixed oligonucleotide (Queen's Oligonucleotide Synthesis Facility) was designed from protein sequence data derived from microsequencing of fragments from CNBr digested TnT, corresponding to residues 108-113 (KKDEDE). The sequence of the oligonucleotide was: ($AA^{A}_{G}AA^{A}_{G}GA^{C}_{T}GA^{A}_{G}GA^{C}_{T}GA^{A}_{G}$). The oligonucleotide (50pmol/mL) was radiolabelled using bacteriophage T4 polynucleotide kinase (PNK) (Pharmacia), $\gamma\ ^{32}P$ ATP (Mandel) (12.5mCi/mL) and PNK buffer (50mM Tris-HCl,pH 7.6, 10mM MgCl, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA). Following incubation at 37°C for 45 minutes the reaction was heated to 68°C to inactivate PNK. Unincorporated radioactive phosphate was removed by ethanol precipitation of the DNA. The pellet was washed with 70% ethanol and added to 10 mL of prehybridization solution (5XSSPE [150mM NaCl, 10mM sodium phosphate, pH 7.4, 1.25mM EDTA], 5X Denhardt's Reagent [0.5% of each of Ficoll, polyvinylpyrrolidone and BSA], 0.5% SDS). Prior to hybridization, the membranes were incubated with prehybridization solution for 2 hours at 42°C. Hybridization of the membranes with the TnT probe was carried out overnight at 42°C. Following hybridization, the membranes were washed with a series of solutions of varying stringency. All washes were carried out at room temperature for 3 minutes unless otherwise stated. The membranes were first washed in 2X SSPE, 0.5%SDS, followed by 2X SSPE, 0.1% SDS. The membranes were then subjected to two cycles of washes of 0.1X SSPE, 0.5% SDS, the first at room temperature and the second at 42°C. Finally the membranes were rinsed in 0.1X SSPE, covered with plastic wrap and exposed ~6 hours at -70°C with an intensifying screen (Sambrook et al.,

1989).

Positives from dot blot hybridization were verified by Southern hybridization (Southern 1975). The insert was excised from the plasmid using *Hind III* and *EcoRI*, subjected to agarose gel electrophoresis and alkaline (0.4M NaOH) transferred to a nylon membrane. Hybridization conditions and stringency washes were identical to those for dot blot hybridizations. Prior to probing, the membrane was neutralized by shaking in 0.2M Tris-HCl, pH 7.5, 2X SSC (150mM NaCl, 15mM sodium citrate).

DNA Isolation and Sequencing

Pelleted cells from overnight cultures (5mL) of positive clones were resuspended in 200μL of GTE (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA, 100μg/mL RNase A), 400μL of 0.2N NaOH, 1%SDS and 300μL of KAcF (5M potassium acetate, 88% formic acid). Following centrifugation, the supernatant was extracted with an equal volume of phenol:chloroform (1:1). DNA was extracted from the aqueous layer using 0.5 volume 7.5M ammonium acetate and 2 volumes of ice cold ethanol (Sambrook et al., 1989). DNA purification was carried out using a Wizard™ minicolumn (Promega) attached to a 5mL syringe (Becton-Dickson). Usually 1mL of the resin was mixed with the DNA sample and applied to the column. The DNA was washed with 2mL of 80% isopropanol and eluted with hot (65°C) TE (10mM Tris, pH 8, 1mM EDTA).

Dideoxynucleotide sequencing (Sanger, 1977) was performed using T7™ Sequencing kit (Pharmacia) according to manufacturer's specifications, using $\alpha^{35}S$ dATP (Mandel). Sequencing gels were run at 32 Watts at a

temperature ~50-55°C in TBE (0.1MTris-HCl, pH 8.3, 0.1M boric acid, 2mM EDTA). Following electrophoresis, gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid, dried and exposed overnight at room temperature.

Subcloning

Inserts were excised from plasmid pSport (Gibco BRL) using *Hind III* and *EcoRI* and were subjected to agarose gel electrophoresis in 0.5TBE, 50μL/L ethidium bromide at 80V for 4-5 hours. DNA bands were eluted from the gel and purified by phenol:chloroform extraction followed by ethanol precipitation. Restriction digests of purified inserts were carried out using *Sau3A* and *RsaI* for actin and *PstI* and *SacI* for TnT. Isolated fragments were ligated into pUC 18 or 19 with compatible ends (*BamHI* for *Sau3A*, *SmaI* for *RsaI*, *SacI* for *SacI* and *PstI* for *PstI*) using T4 DNA ligase (Pharmacia) and ligation buffer (66mM Tris-HCl, pH 7.5, 6.6mM $MgCl_2$, 10mM DTT, 0.5mM ATP). This reaction was left at 16°C for 3-14 hours and was then used to transform *E. coli.* DH5α competent cells, diluted with 0.1M $CaCl_2$ for 1 hour on ice. The cells were heat shocked at 42°C for 90 seconds, 1mL of LB was added and the cells were incubated at 37°C for 1hour. Following low speed centrifugation, the cells were plated on LB ampicillin plates and incubated at 37°C overnight. Colonies were grown in 5mL of LB (10g/L tryptone, 5g/l yeast extract, 5g/L NaCl), 100μg/mL ampicillin, the DNA was isolated and digested with *HindIII* and *EcoRI* and electrophoresed on a 0.7%-1.2% agarose gel (depending on the expected size of the insert) to verify presence of the correct insert. The clones were then sequenced as described.

Chapter 3

RESULTS

Preparation of Troponin T

Preparation of troponin T involved a two step procedure. First, the whole Tn complex was prepared (Ebashi et al., 1971) then the subunits of Tn were separated chromatographically under denaturing conditions. Using Ebashi's method (Ebashi et al., 1971), both the yield and the purity of Tn from fast myotomal muscle was variable. The yields were (mg of protein/kg of tissue used): 178-300mg/kg of the precipitate fraction and 889-917mg/kg of the supernatant fraction. To try and address the problem of purity, Tn was extracted, by the Ebashi method, from myofibrils, prepared using the method of Perry and Corsi (1958). Generally, this Tn was purer than before, however lower amounts were obtained (88-190mg/kg precipitate and 88-367mg/kg supernatant fraction). For the preparation of Tn from slow myotomal muscle neither of the above procedures was entirely adequate. Low yields were a major problem, in part, due to the higher lipid content of slow muscle. Lipid droplets may have prevented sedimentation of protein during centrifugation. In fact, "flakes" of protein remained in the supernatant at the highest concentration of ammonium sulfate. This problem was not alleviated by longer centrifugation times. In an attempt to remove lipid, Triton X-100 was introduced into preparations of slow muscle Tn, using the method of Potter (1982). The isolated Tn was slightly less pure than the Ebashi prepared material but better yields were obtained (300mg/kg precipitate fraction and 400mg/kg supernatant fraction).

Troponin subunits were prepared from batches of whole Tn that were shown to be of reasonably high purity by SDS PAGE, by carrying out chromatography under denaturing conditions (6M urea). Fast muscle TnTs were prepared by passing whole fast Tn over a Q Sepharose Fast Flow anion exchanger at pH 8.00 (Fig. 5). For preparation of slow TnT, best results were obtained with a two step approach, involving separation using Q Sepharose Fast Flow resin (Fig. 6A), followed by chromatography on S Sepharose Fast Flow resin (cation exchanger) (Fig. 6B).

Electrophoretic analysis of purified TnC revealed that both slow and fast muscle each contain a single, tissue specific form of the protein. The isoform distribution of TnI is presently unresolved. The electrophoretic results also show that the fast isoforms of TnI and TnC (-Ca^{2+}) comigrate on SDS gels when the TnC is not complexed with Ca^{2+}. However, Ca^{2+} induces a shift in the mobility of TnC, thus enabling the resolution of these two components by SDS PAGE (Fig. 7, lanes 1 & 2). Similar observations have been made with other Ca^{2+} binding proteins, including mammalian TnC (Head and Perry, 1974) but this is the first time that TnC has been shown to comigrate with TnI. The electrophoretic mobility of slow TnC was also affected by Ca^{2+}, but to a lesser extent than what was noted for the fast isoform (Fig. 7, lanes 7&8). The difference in the Ca^{2+} induced mobilities may be partly explained by the fact that slow TnC has lost the ability to bind Ca^{2+} at site I (Collins et al., 1977), whereas fast muscle TnC can bind two moles of Ca^{2+}. The difference in Ca^{2+} binding properties has been shown to be of significance (Sweeney et al., 1990). The remainder of this section will now deal with troponin T.

Fig. 5. Chromatographic separation of Atlantic salmon fast muscle Tn. Whole Tn (25mg: 10mg/mL) was loaded onto a Q Sepharose Fast Flow column (1 cm x 7 cm) in 6M urea, 15mM Tris/HCl, pH 8.0 at room temperature, 1mM EDTA, 1mM DTT. Elution was carried out at room temperature with a 0-0.5 M NaCl gradient (total volume = 150 mL). The flow rate was 10mL/h and 1.5 mL fractions were collected. (Inset) Coomassie Blue stained SDS polyacrylamide gel (15%, w/v). Samples were prepared by adding an equal volume of sample buffer (65mM Tris/HCl, pH 6.8, 1.3% w/v SDS, 13% v/v glycerol, bromophenol blue) to the fraction number indicated. The abbreviations are: I = TnI; C = TnC; 1F, 2F and 3F = TnT 1F, 2F and 3F.

1. starting material (fast Tn)
2. 7
3. 17
4. 23
5. 30
6. 35
7. 45
8. 58
9. 80
10. 113

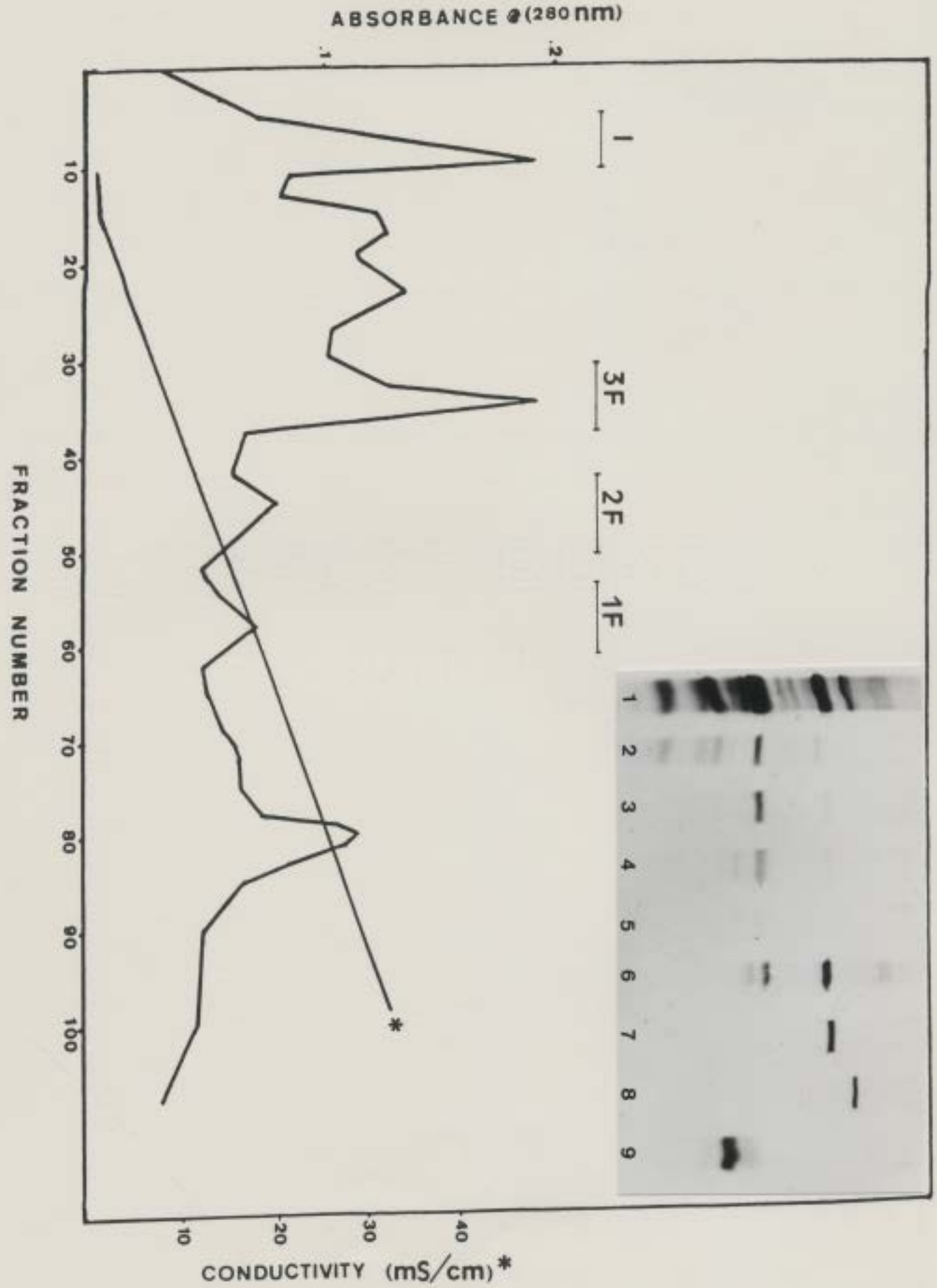

ABSORBANCE @ (280 nm)
1
2
I
3F
2F
1F
FRACTION NUMBER
10
20
30
40
50
60
70
80
90
100
*
10
20
30
40
CONDUCTIVITY (mS/cm)*
1
2
3
4
5
6
7
8
9

Fig. 6 A. **Chromatographic separation of Atlantic salmon slow muscle Tn.** Crude whole Tn from the 60% ammonium sulfate salt cut (Absorbance 280nm = 1.057) was dialyzed against Q Sepharose Fast Flow start buffer (6M urea, 50mM Tris/HCl, pH 8.00, 1mM EDTA, 1mM DTT) and then loaded onto Q Sepharose Fast Flow resin (2.5cm x 8cm). Elution was carried out at room temperature with a 0-0.5M NaCl gradient (total volume = 450mL). The flow rate was 70mL/h and 5 mL fractions were collected. (Inset) Coomassie Blue stained polyacrylamide gel (15%, w/v). Samples were prepared by adding an equal volume of sample buffer to the fraction number indicated. The abbreviations are: I = TnI; C = TnC; 1F, 2F and 3F = TnT 1F, 2F and 3F.

1. starting material (slow Tn)
2. 131
3. 139
4. 146
5. 153
6. 158
7. 162
8. 177
9. 189
10. TnT 2S + slow TnI (standards)

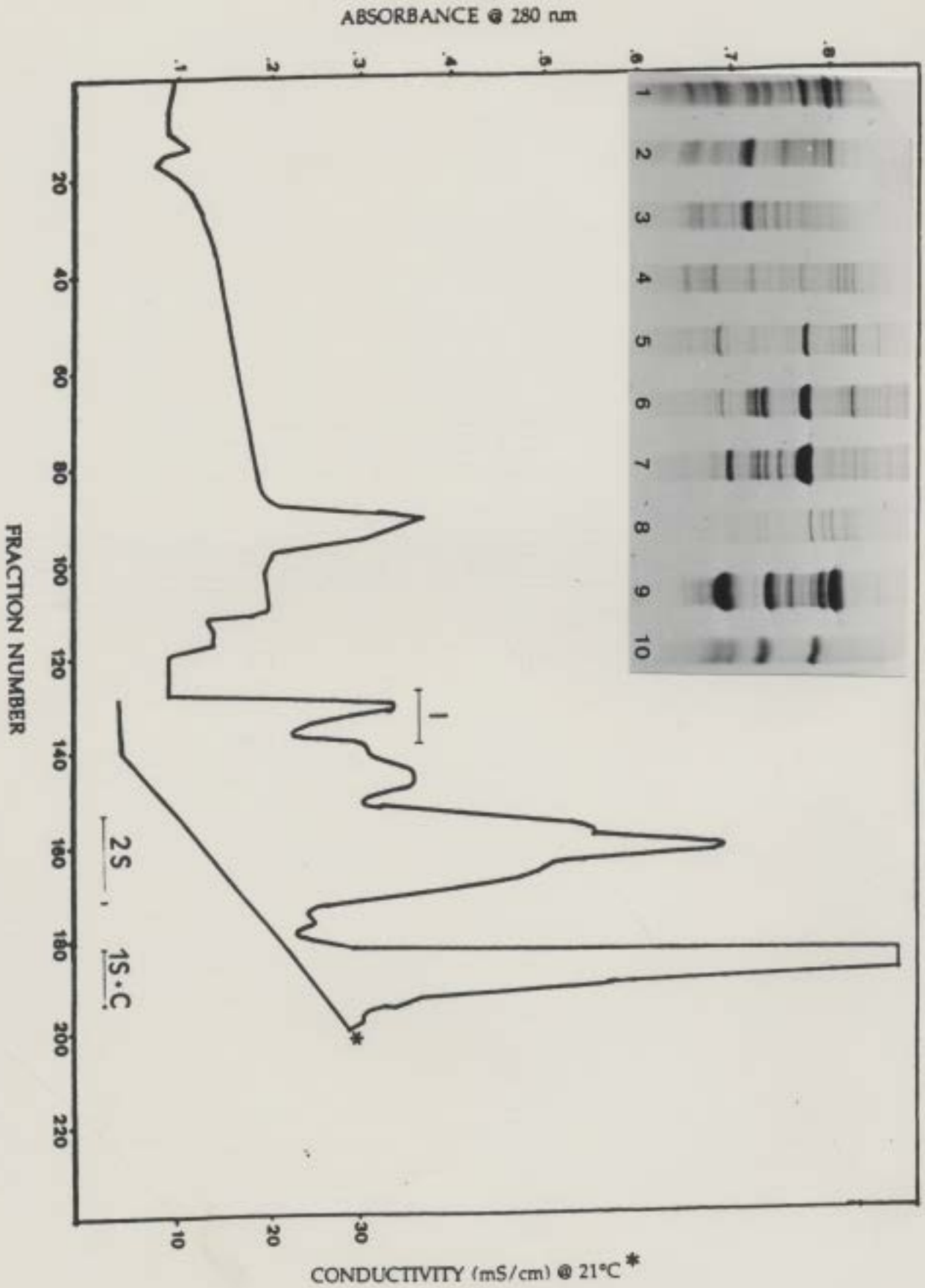
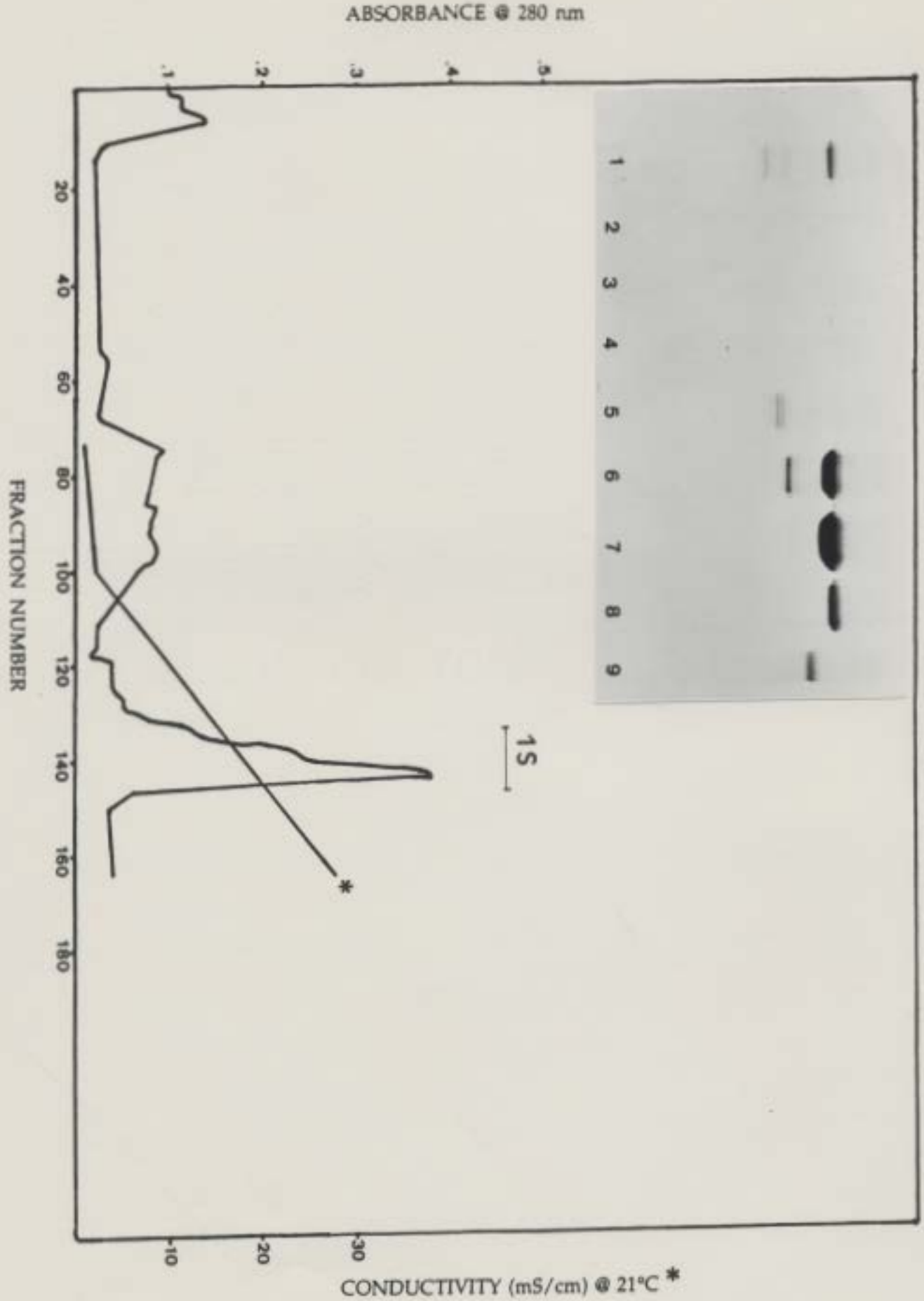

ABSORBANCE @ 280 nm
.1
.2
.3
.4
.5
.6
.7
.8
1
2
3
4
5
6
7
8
9
10
FRACTION NUMBER
20
40
60
80
100
120
140
160
180
200
220
2S
1S·C
*
10
20
30
CONDUCTIVITY (mS/cm) @ 21°C *

Fig. 6 B. Purification of the higher molecular weight TnT isoform from slow muscle. Fractions 184-200 from the Q Sepharose Fast Flow separation were dialyzed against the chromatography start buffer (6M urea, 50mM Tris/HCl, pH 8.0, 1mM EDTA, 1mM DTT) and loaded onto a S Sepharose Fast Flow column (2.5 cm x 7 cm). Elution was carried out at room temperature with a 0-0.5M NaCl gradient (total volume = 375mL). The flow rate was 100mL/h and 4.5 mL fractions were collected. (Inset) Coomassie Blue stained SDS polyacrylamide gel (15%, w/v). Gel samples were prepared by adding an equal volume of sample buffer to the fraction indicated.

1. starting material
2. 7
3. 75
4. 95
5. 120
6. 139
7. 144
8. 147
9. TnT standard (isoform 2S)


ABSORBANCE @ 280 nm
1
2
3
4
5
20
40
60
80
100
120
140
160
180
FRACTION NUMBER
1 2 3 4 5 6 7 8 9
1S
*
10
20
30
CONDUCTIVITY (mS/cm) @ 21°C *

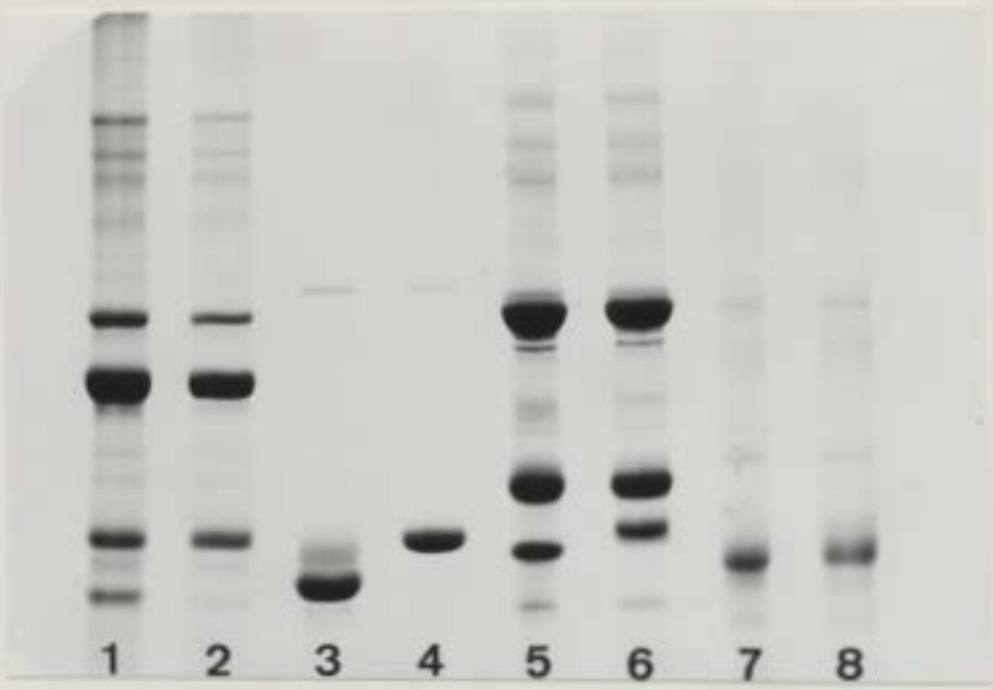


Fig. 7. SDS PAGE analysis of Atlantic salmon troponin and troponin C in the presence and absence of calcium. A 15% (w/v) SDS polyacrylamide gel was used. *Rabbit Tn is included for comparison.*

1. fast Tn + 5mM $CaCl_2$
2. fast Tn + 5mM EDTA
3. fast Tn C + 5mM $CaCl_2$
4. fast Tn C + 5mM EDTA
5. rabbit Tn + 5mM $CaCl_2$
6. rabbit Tn + 5mM EDTA
7. slow Tn C + 5mM $CaCl_2$
8. slow Tn C + 5mM EDTA

Characterization of TnT Isoforms

Proteins were identified as isoforms of TnT based on the following criteria; copurification with troponin, amino acid composition, partial protein sequence data from CNBr fragments of purified proteins, recognition by anti-TnT antibodies and interaction with TM on affinity resins.

The presence of multiple peaks in the TnT elution region of chromatographic profiles provided the first indication that multiple isoforms of TnT were present in the swimming muscles of Atlantic salmon. Three peaks corresponding to fast TnTs and two corresponding to slow TnTs were apparent for separations using Q Sepharose Fast Flow chromatography (Figs. 5 & 6). These have been denoted TnT 1F, 2F & 3F and TnT 1S & 2S based on increasing mobility on SDS polyacrylamide gels (Fig. 8, see also Figs. 5 & 6 insets). Owing to the close spacing of the bands, isoforms 2F & 3F are only discernible on SDS polyacrylamide gels (sub 15% w/v polyacrylamide) at low protein concentrations. This is clearly demonstrated in Fig. 8, lane 4. Judging from the relative mobilities of the bands, 1F is of higher apparent Mr than the major isoform of rabbit fast TnT while 2F and 3F are of lower apparent Mr. It is possible that additional minor isoforms of both slow and fast TnT are present in myotomal muscle but escape detection due to their low abundance. Without exception, the relative proportions, as assessed by SDS PAGE and absorbance measurements, of both fast and slow TnTs did not differ between chromatographic separations under denaturing conditions. The relative proportions of fast TnTs were (from highest to lowest); TnT 3F > 2F > 1F. Conversely, the higher apparent Mr slow TnT isoform, TnT 1S, was always present in significantly greater amounts than TnT 2S.

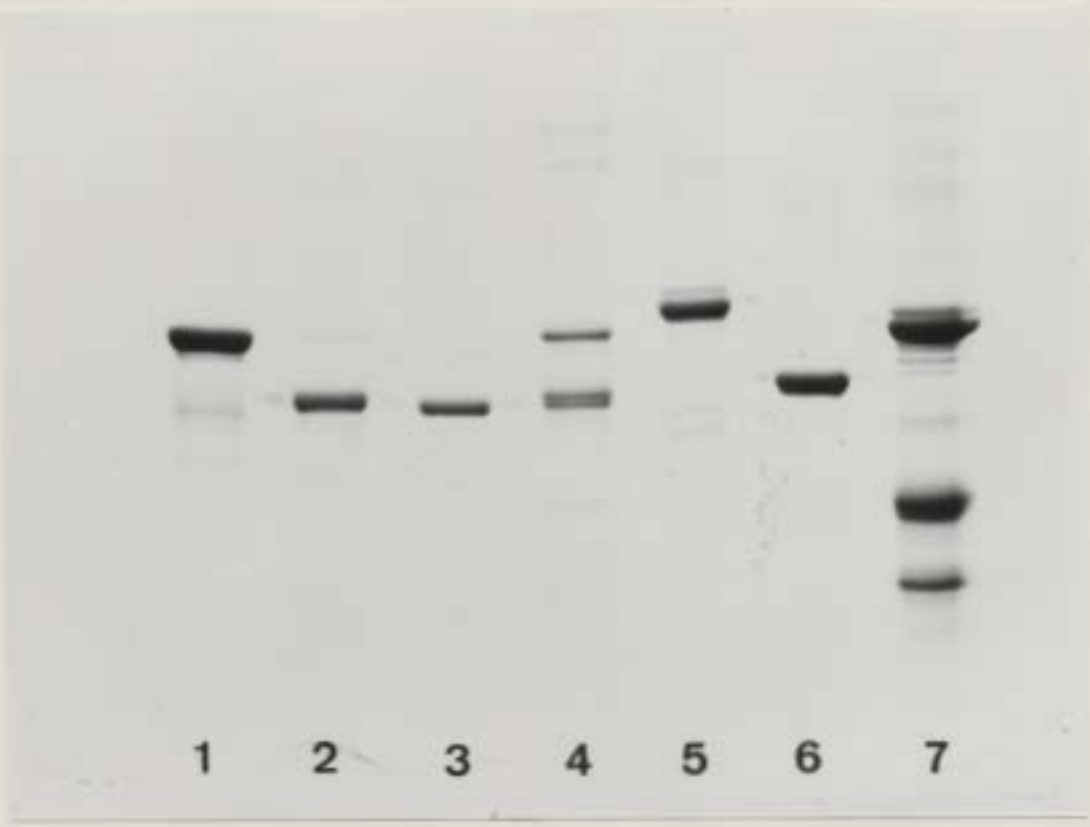


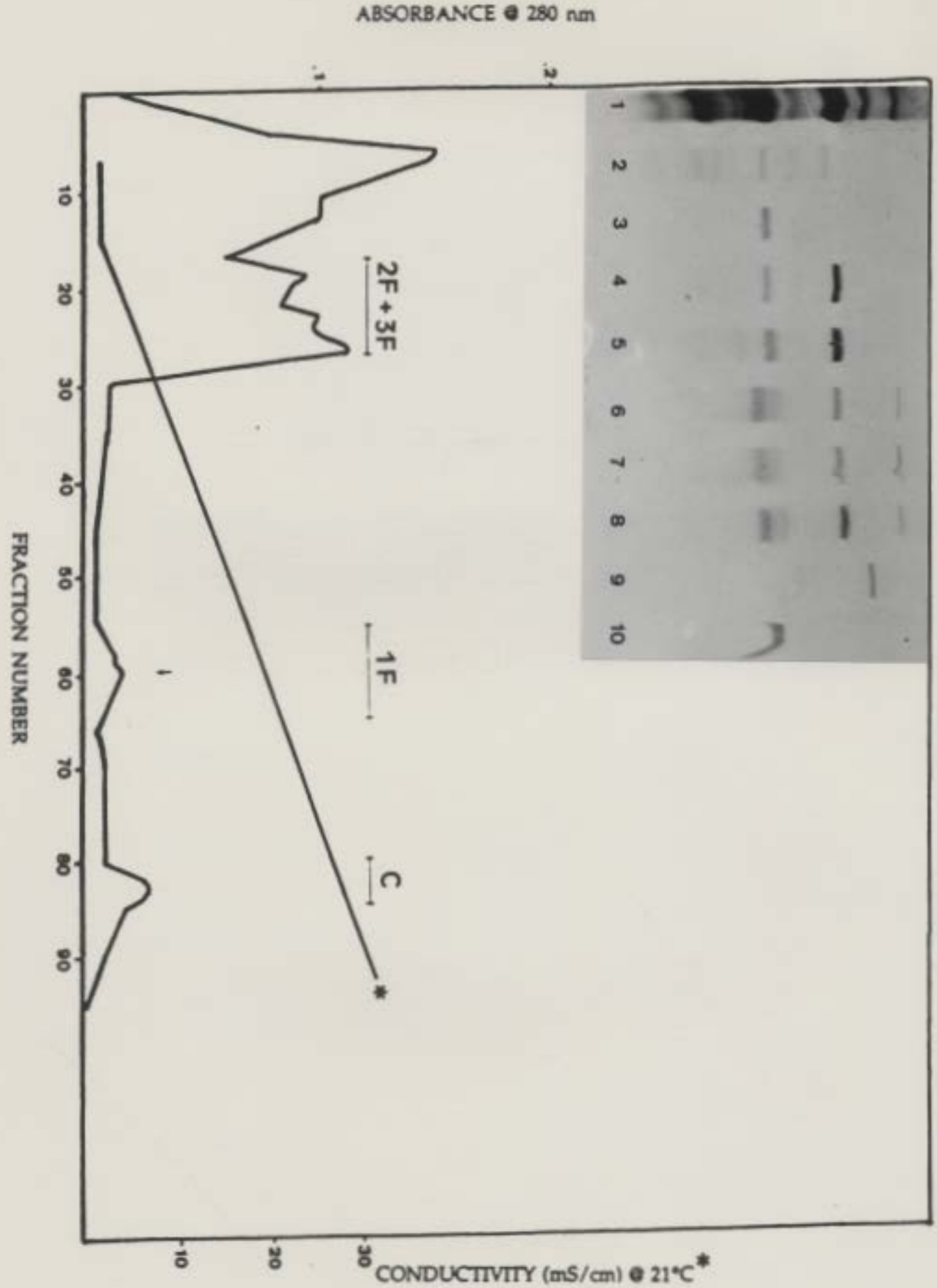
Fig. 8. SDS PAGE analysis of slow and fast isoforms of TnTs from Atlantic salmon. A 14% (w/v) SDS polyacrylamide gel was used.

1. Tn T 1F
2. Tn T 2F
3. Tn T 3F
4. Tn T 1F+2F+3F
5. Tn T 1S
6. Tn T 2S
7. Rabbit Tn (included for comparison)

Mammalian TnT isoforms have been identified previously by chromatography (Gusev, et al., 1983; Briggs et al., 1987; Heeley, 1994). Since, these studies showed that some of the elution peaks arose from the partial phosphorylation of the TnTs, it was necessary to investigate whether or not fish TnTs were similarly modified. To this end, chromatography was carried out on whole fast Tn that had first been treated with alkaline phosphatase. When phosphatase treated fast Tn was subjected to Q Sepharose Fast Flow chromatography, the affinity of TnTs 2F and 3F for the anion exchanger was weaker than for the untreated control (compare Figs. 5 and 9). Judging from the mobilities of 2F and 3F (Fig.9 inset) it is clear that no proteolysis had occurred. By contrast, TnT 1F from treated and untreated Tns eluted in the same position in the salt gradient as determined by conductivity measurements (~ 18mS/cm for the fraction of maximum absorbance). From these results, it is possible to conclude that isoforms 2F and 3F are phosphoproteins whereas isoform 1F is not. However, since the chromatography data is not quantitative, it is possible that TnTs 2F and 3F differ only in their extent of phosphorylation. This is to say that they may not be distinct isoforms. A number of lines of evidence are available to support the view that 2F and 3F differ in their primary structure and, therefore are true isoforms. First, differences in phosphate content do not alter the mobility of mammalian TnTs on SDS polyacrylamide gels (Heeley, 1994) and this is also the case for the fish TnTs (Fig. 10). Second, when large amounts of fast Tn (>300mg) were loaded onto a Q Sepharose Fast Flow resin, in the presence of urea, isoform 3F appeared in both flow through and retained fractions, while isoform 2F eluted in two different positions in the salt gradient (Fig. 11). The presence of

Fig. 9. Chromatographic separation of Atlantic salmon fast muscle Tn treated with alkaline phosphatase. A dephosphorylated sample of whole Tn (6mg, 10mg/mL) was applied to a Q Sepharose Fast Flow column (1cm x 7cm) in 6M urea, 15mM Tris/HCl, pH 8.0, 1mM EDTA, 1mM DTT. Elution was carried out at room temperature with a 0-0.5M NaCl gradient (total volume = 150mL). The flow rate was 10mL/h and 1.5mL fractions were collected. Conductivity measurements were taken every fifth tube and are indicated by *. (Inset) Coomassie Blue stained SDS polyacrylamide gel (15%, w/v). Samples were prepared by combining 50μL of fraction number indicated and 50μL of sample buffer. The elution position of TnT 1F is marked with an arrow. The abbreviations are: C = TnC; 1F, 2F and 3F = TnT 1F, 2F and 3F.

1. starting material (dephosphorylated fast Tn)
2. 6
3. 13
4. 17
5. 19
6. 22
7. 23
8. 27
9. 60
10. 83


ABSORBANCE @ 280 nm
1
2
FRACTION NUMBER
10
20
30
40
50
60
70
80
90
2F + 3F
1F
C
I
10
20
30
CONDUCTIVITY (mS/cm) @ 21°C*
1 2 3 4 5 6 7 8 9 10

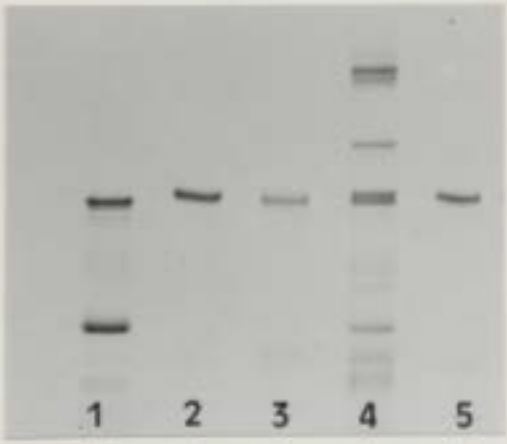


Fig. 10. SDS PAGE analysis of phosphorylated and unphosphorylated TnTs. The samples are from a large scale separation of whole fast muscle Tn (supernatant fraction) on Q Sepharose Fast Flow media under denaturing conditions (see Fig. 11 for further explanation). The samples were dialyzed and lyophilized prior to electrophoretic analysis on a 14% (w/v) SDS polyacrylamide gel.

1. pooled fractions 12-31
2. pooled fractions 101-113
3. pooled fractions 114-129
4. fast Tn
5. pooled fractions 130-144

Fig. 11 A. Large scale separation of Atlantic salmon fast muscle Tn. Whole Tn from the supernatant fraction (385mg) was loaded onto a Q Sepharose Fast Flow column (2.5cm x 7cm) in 6M urea, 30mM Tris-HCl, pH 8.00 at room temperature, 1mM EDTA, 1mM DTT. Elution was caried out at room temperature with a 0-0.5M NaCl gradient (total volume = 800mL). The flow rate was 50mL/h and 3.5 mL fractions were collected. Conductivity measurements were taken every fifth tube and are indicated by *. SDS PAGE analysis of fractions is included in Fig. 11B on page 60. The abbreviations are: I = TnI, C = TnC, 1F 2F and 3F = TnT 1F, 2F and 3F.

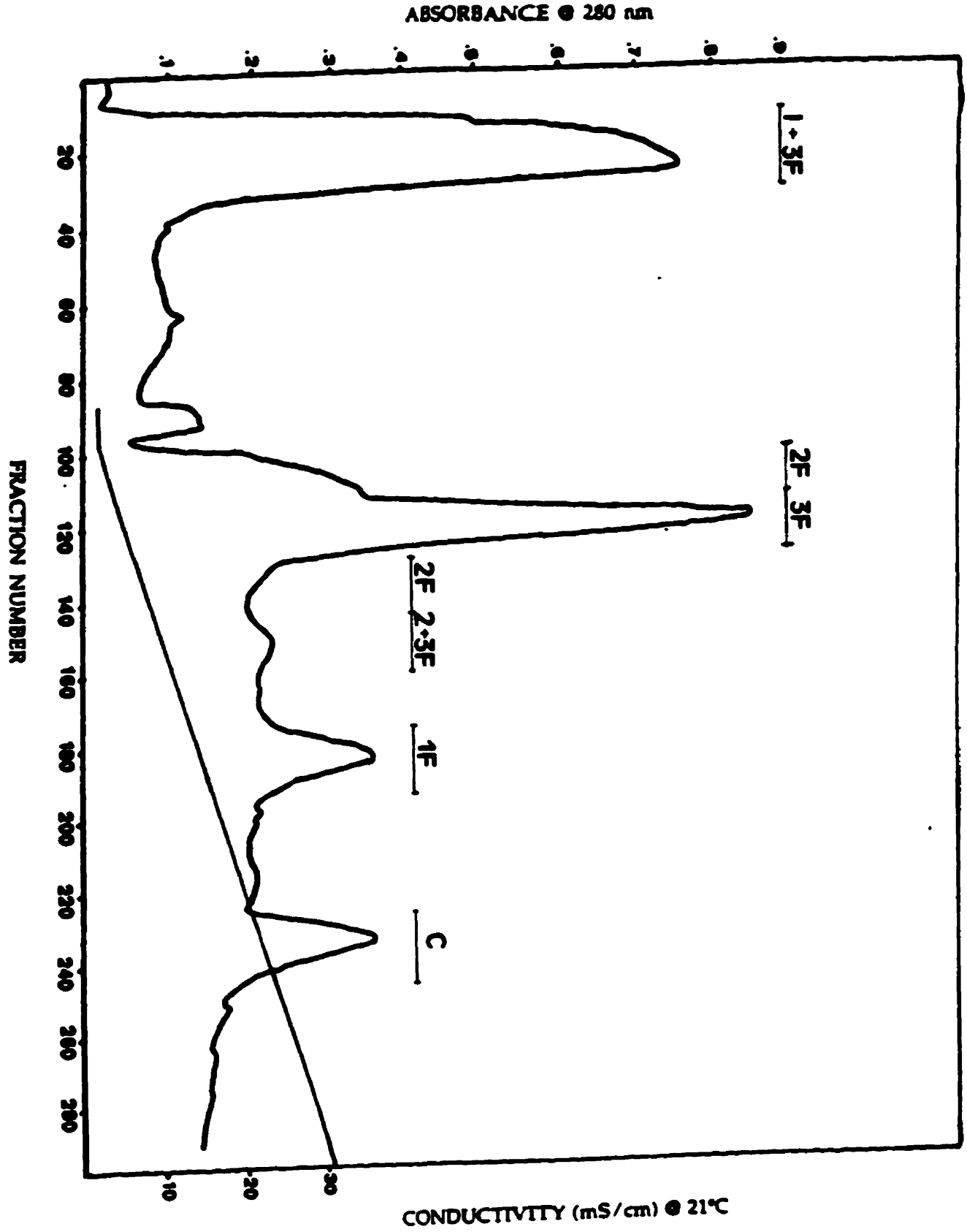

ABSORBANCE @ 280 nm
1+3F
2F 3F
2F 2+3F
1F
C
FRACTION NUMBER
CONDUCTIVITY (mS/cm) @ 21°C

Fig. 11 B. Coomassie Blue stained SDS polyacrylamide gel (15% w/v) of fractions from a large scale separation of fast Tn by Q Sepharose Fast Flow chromatography. Samples were prepared for electrophoresis by heating an equal volume of sample buffer and the fraction indicated.

Gel (A).

1. starting material
2. 13
3. 27
4. 64
5. 83
6. 101
7. 111
8. 121
9. 128
10. standards (TnTs 1F, 2F,3F & fast TnI)

Gel (B).

1. starting material
2. 131
3. 153
4. 185
5. 198
6. 216
7. 234
8. 254
9. standards


A
1 2 3 4 5 6 7 8 9 10


B
1 2 3 4 5 6 7 8 9

TnT in these fractions was confirmed by immunoblotting with an anti-fast TnT antibody (Fig. 12). This finding indicates that 2F and 3F exist in two pools each having different affinities for the resin. Following further purification of some of the TnT containing fractions by immobilized metal ion chromatography (profiles not shown), phospho-amino acid analysis was carried out. For each isoform, the pool that interacted more weakly with the Q Sepharose Fast Flow resin was found to contain no phosphorylated amino acids, while phospho-serine was detected in the pool that interacted more strongly. This result demonstrates that fast myotomal Tn consists of partially phosphorylated TnT 2F and 3F (and unphosphorylated TnT 1F) and furthermore, 2F and 3F are distinct forms of the protein. Dephosphorylation experiments were not attempted with slow myotomal Tn owing to the problems associated with preparing this particular Tn (see Materials and Methods). However, based on the results of partial amino acid analysis TnT 2S is not phosphorylated.

Amino Acid Analysis

The amino acid compositions of the purified slow TnT and fast TnTs are presented in Table 2. For TnT 1S, both the measured as well as the predicted compositions are included. In addition, rabbit fast muscle TnT isoform 2F from Pearlstone et al. (1977) is included for comparison. It is clear from the data, that the TnTs possess similar compositions with some exceptions. For example, all isoforms contain one tryptophan with the exception of TnT 1S which contains two. Differences are also apparent in the proline contents. The number of residues (mole/mole protein) are: four

Fig. 12. Immunoblot analysis of TnTs. Western immunoblot analysis of salmonid TnTs using an anti-fast TnT antibody (courtesy of Dr. T. Dhoot). Pooled fractions from a Q Sepharose Fast Flow chromatographic separation were dialyzed against water and lyophilized (see legend to Fig. 11 for more details). Samples were prepared for electrophoresis by dissolving ~0.1mg of freeze dried powder in 100μL sample buffer. Usually 2-5μL were loaded onto a 14% (w/v) polyacrylamide gel. Only the stained PVDF membrane is shown. Muscle extracts were prepared by homogenizing with a Brinkman polytron (setting 4) for ~1-2 min., 0.1g of muscle in 1.0 mL of saturated urea. The extract was clarified by centrifuging in a microfuge for 1min. at 14000g. Usually 50μL of the supernatant was added to an equal volume of sample buffer and heated in the presence of SDS, 5-10μL were loaded onto the gel. Positive reactions were detected using goat anti-mouse alkaline phosphatase conjugated second antibody as outlined in Materials and Methods.

1. whole fast muscle extract
2. pooled fractions 12-31
3. pooled fractions 101-113
4. pooled fractions 114-129
5. pooled fractions 130-144
6. pooled fractions 145-160
7. TnT standard (isoform 3F)

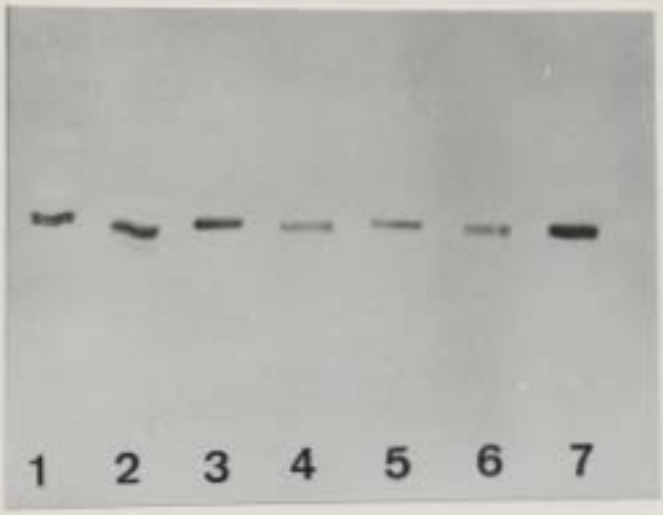

1
2
3
4
5
6
7

Table 2. Amino acid compositions of salmonid skeletal troponin Ts. The number of each amino acid have been calculated relative to alanine to yield a total molecular mass closest to that measured by mass spectrometry (TnT 1F: 30054, 2F: 27312, 3F: 26817 and TnT 2S: 28584). The molecular mass of TnT 1S (32362) was calculated from the inferred amino acid sequence. The masses as well as the amino acid compositions of TnT 2F, 3F and 2S were determined by Thorben Bieger. In the case of TnT 1S the composition is calculated to give a total number of amino acids closest to that predicted by nucleotide sequencing. In addition, the inferred amino acid composition of TnT 1S (denoted by an *) is also provided. With respect to the Asx and Glx values of TnT 1S*, the first number in the column corresponds to Glu or Asp, while the second denotes Gln or Asn. The composition of rabbit TnT, corresponding to isoform 2F of fast skeletal muscle, is from Pearlstone et al., 1977.

	TnT 1F	TnT 2F	TnT 3F	TnT 1S*	TnT 1S	TnT 2S	Rabbit
ASX	20.6 (21)	20.4 (20)	20.3 (20)	20, 6	28.9 (29)	24.8 (25)	20
THR	5.8 (6)	6.4 (6)	6.1 (6)	11	10.9 (11)	9.4 (9)	6
SER	11.3 (11)	13.3 (13)	14.0 (14)	10	7.84 (8)	8.6 (9)	9
GLX	55.9 (56)	47.9 (48)	46.6 (47)	48 18	66.5 (66)	50.6 (51)	57
PRO	16.7 (17)	4.4 (4)	3.8 (4)	13	13.9 (14)	7.0 (7)	9
GLY	7.0 (7)	8.4 (8)	7.3 (7)	9	10.4 (10)	12.3 (12)	8
ALA	24	17	17	26	25	19	26
VAL	6.5 (6)	6.6 (7)	6.8 (7)	10	10.3 (10)	6.9 (7)	11
MET	5.8 (6)	4.0 (4)	4.0 (4)	6	6.3 (6)	6.4 (6)	5
ILE	10.2 (10)	8.3 (8)	8.7 (9)	10	9.8 (10)	9.1 (9)	8
LEU	17.0 (17)	17.8 (18)	17.7 (18)	18	18.2 (18)	18.1 (18)	19
TYR	2.2 (2)	1.9 (2)	1.9 (2)	2	2.2 (2)	2.0 (2)	4
PHE	5.3 (5)	5.4 (5)	5.1 (5)	3	5.2 (5)	5.1 (5)	5
HIS	5.8 (6)	6.6 (7)	6.4 (6)	6	5.3 (5)	5.3 (5)	6
LYS	39.9 (40)	42.7 (43)	42.3 (42)	37	39.4 (39)	35.6 (36)	39
ARG	21.2 (21)	23.5 (23)	23.5 (23)	23	21.9 (22)	20.8 (21)	25
TRP	1.1 (1)	1.2 (1)	1.2 (1)	2	1.7 (2)	1.3 (1)	2
1/2 CYS	0.2 (0)	0.2 (0)	0.2 (0)	0	0.4 (0)	0.5 (0)	0
TOTAL	257	234	232	278	282	242	259

(2F&3F), seven (2S), thirteen (1S) and seventeen (1F). In TnT from other organisms, proline is located almost exclusively at the N-terminus, a region of the molecule that is known to be hypervariable (Breitbart et al., 1985). The high content of this amino acid in higher molecular weight isoforms could originate from differences in this part of the molecule. In addition, Glx (Glu + Gln) values were higher in TnT 1F and TnT 1S than in the lower molecular weight forms. If this is due to increases in glutamic acid, it would again be consistent with heterogeneity within the N-terminal region. None of the isoforms contained cysteine, which is the case for most TnTs from other species sequenced to date, with the exception of bovine cardiac TnT (Leszyk et al., 1987) which contains a cysteine at position 39 (TnT-1) and quail and chicken TnT which have a cysteine at position 234 (Hastings et al., 1985). The function of this amino acid, if any, within TnT, has yet to be determined.

Microsequence Analysis

Microsequencing of intact purified TnTs revealed that all isoforms, with the exception of TnT 1F, were blocked at the N-terminus. Fragments generated from CNBr cleavage of purified TnTs were electrophoresed on a 15% w/v SDS polyacrylamide gel and electroblotted onto a PVDF membrane. Microsequence analysis was performed at the sequencing facility at the Hospital for Sick Children at Toronto. Included in Table 3 are the determined sequences as well as their maximum alignment with TnTs from other species. The number of sequenced amino acids for each isoform is: 1F = thirty five; 2F and 3F = fifteen; 1S and 2S = nineteen. The high degree of similarity revealed in Table 3 is very strong evidence that the proteins from

Table 3. Alignment of partial protein sequences of salmonid TnTs with published TnT sequences. The partial sequence data were derived from microsequencing of CNBr digests of purified TnTs, with the exception of the N-terminal sequence of TnT 1F which was obtained when the untreated protein was sequenced. The N-terminal sequence of TnT 1S (EVPAEEDAAPEEEPEPEPEPEP) is derived from the nucleotide sequence (Fig.21) and is included for comparison. The TnT sequences are coded as follows. TnT 1F, 2F and 3F: salmonid fast myotomal TnTs; TnT 1S and 2S: salmonid slow myotomal TnTs; RB fast: residues 70-93, 152-166 of rabbit fast TnT (Pearlstone et al., 1977); RB car: residues 97-120, 178-192 of rabbit cardiac TnT (Pearlstone et al., 1986); CH ski: residues 62-85, 144-158 of chicken skeletal fast muscle TnT (Bucher, et al., 1989); HU slow: amino acids 76-99, 158-169 of human slow TnT (Gahlmann et al., 1987). Microsequencing of TnT 2F, 3F and 2S was carried out by Thorben Bieger, these are included for comparison.

TnT 1F	EVAPEAAEEPEAEPEPEPE?E		GSNYSSHLQKADSKR
TnT 2F			GSNYSSHLQKADSKR
TnT 3F			GSNYSSHLQKADSKR
TnT 1S	EVPAEEDAAPEEEPEPEPEPEP	AELTSLIESHFVQRKKDED	
TnT 2S		AELTSLIESHFVQRKKDED	
RB fast		MELQALIDSHFEARKKEEEELVAL	GANYSSYLAKADQKR
CH ski		IELQALIDSHFEARRKEEEELVAL	GASYSSYLAKADQKR
HU slow		LELQTLIDVHFEQRKKEEEELVAL	GAHFGGTLVKAEQKR
RB car		NELQTLIEAHFENRKKEEEELVSL	MMHFGGYIQKQA

which the fragments are derived are indeed TnTs. It was difficult to align the N-terminal sequence (EVAPEAAEEPEAEPEPEPEE) of TnT 1F to TnTs from other species. This could be due to the fact that the fragment selected for sequencing emanates from the hypervariable N-terminal region. On this point it is perhaps significant that the partial amino acid sequence of TnT 1F shares some similarity with the N-terminal region of TnT 1S derived from nucleotide sequencing (see later section on the nucleic acid sequence of TnT). Furthermore, the high glutamic acid and proline content of the sequence from TnT 1F corroborates the amino acid compostition data of TnT 1F and TnT 1S.

The sequencing results also lend credence to the proposal that the lower molecular weight isoforms are not breakdown products of the higher molecular weight forms. As stated earlier, all of the fish TnTs, with the exception of TnT 1F, are N-terminally blocked. Thus, if proteolysis had taken place it would most likely occur at a C-terminal site(s). Since the relative proportion of the TnTs does not vary between preparations and does not appear to alter during any given preparation, the breakdown would have to occur both rapidly and quantitatively at an early stage in the isolation procedure. This is considered to be unlikely. Furthermore, two bands of identical mobility to TnT 2F and 3F are recognized by an anti-fast TnT antibody in whole muscle extracts under conditions where proteolysis is less likely. The fragments of TnT 1F, 2F and 3F selected for sequencing are identical. Likewise, the fragments of TnT 2S and 1S that were selected for analysis clearly possess identical sequences. These observations may indicate that same fiber type isoforms arise from a single gene. However the

possibility remains that isoforms 2F and 3F arise from partial processing at the N-terminal end followed by "capping" by the blocking group.

Immunostaining Studies

All three TnT isoforms purified from fast muscle were recognized by a mouse anti-rabbit fast TnT antibody (donated by Dr. T. Dhoot, Royal Veternary College, London, England) as was rabbit fast skeletal muscle TnT (Fig. 13). On the other hand, the antibody did not react with either TnT 1S, 2S or cardiac TnT (Fig. 13, lanes 1, 3 & 8).

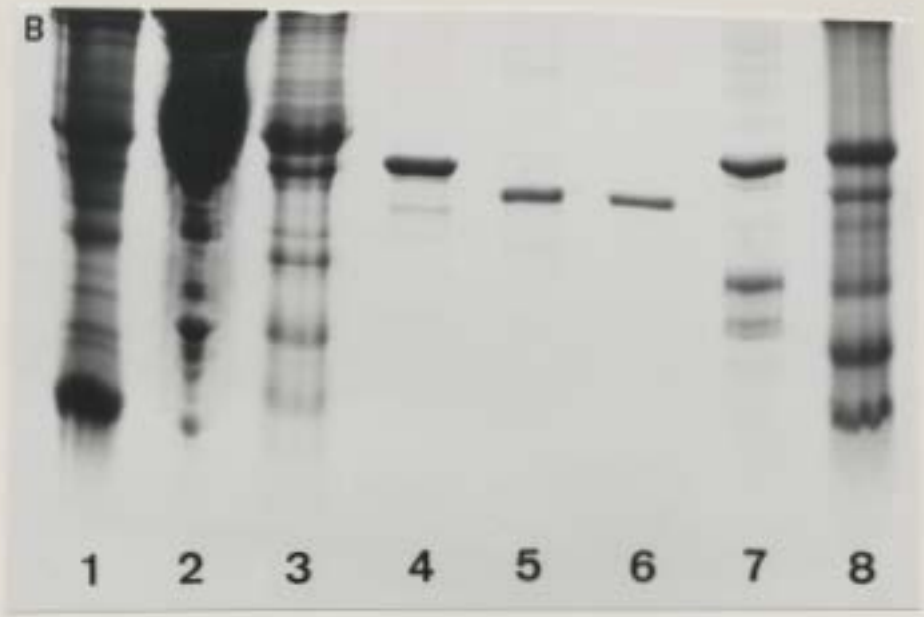
When a whole fast muscle homogenate was subjected to SDS PAGE and then reacted with the anti-fast TnT antibody, only one major band was detected (Fig. 13, lane 2). This band most likely corresponds to both TnT 2F & 3F. The separation of these isoforms may have been diminished by the large amount of protein loaded onto the gel (see Fig. 13B, lane 2), combined with the concentration of polyacrylamide used (15% w/v). The highest molecular weight species (TnT 1F) is not obviously apparent in immunoblots of whole fast muscle extracts however one of the faint bands could correspond to TnT 1F (Fig. 13A, lane 2). Low abundance or insufficient extraction of the protein may account for the lack of reactivity of TnT 1F in this experiment.

Components other than TnT 1F, 2F and 3F were recognized by the fast TnT antibody. High molecular weight immunopositive species are apparent in lanes 5, 6 and 7 of Fig. 13, and are especially pronounced in lane 7 (rabbit Tn). In view of their high apparent molecular weight, it is unlikely that these bands represent additional isofroms of TnT. In addition, a small band can be visualized running just underneath the highest molecular weight TnT (Fig.

Fig. 13. Immunoblot analysis of salmonid muscle extracts and purified proteins using an anti-fast TnT antibody. Western immunoblot analysis of salmonid TnTs with anti-fast TnT antibody (courtesy of Dr. T. Dhoot). A 15% (w/v) SDS polyacrylamide gel was used. Muscle extracts were prepared as described in the legend of Fig. 12. Positive reactions were detected using goat anti-mouse alkaline phosphatase conjugated second antibody as outlined in Materials and Methods. The lack of reactivity of TnT 1F in whole muscle homogenates may be explained by the weaker affinity of this isoform for the antibody as well as the low abundance of this protein in the muscle. (A) Stained PVDF membrane. (B) Replicate gel ran at the same time as the gel used for Western transfer but stained with Coomassie Blue.

1. slow muscle extract
2. fast muscle extract
3. cardiac muscle extract
4. TnT 1F
5. TnT 2F
6. TnT 3F
7. rabbit Tn
8. slow Tn


A
1 2 3 4 5 6 7 8


B
1 2 3 4 5 6 7 8

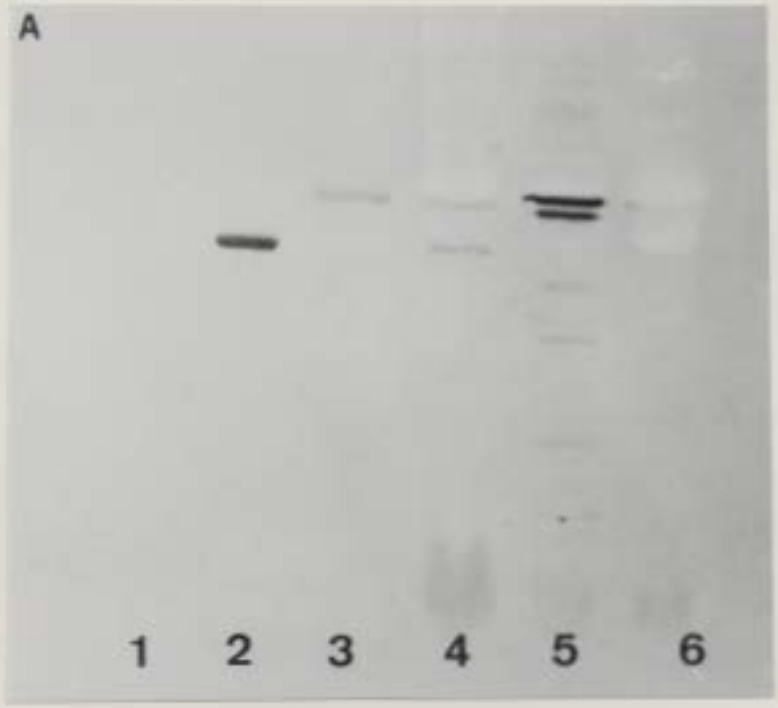
13, lane 4). This may represent an additional isoform that has previously not been detected or it may be a breakdown product of TnT 1F. Nonspecific binding is also a possibility even though the membranes were blocked with either BSA or casein. Furthermore, these components could also be impurities that cross react with the first antibody. On this point it is worth noting that glyceraldehyde-3-phosphate dehydrogenase shares a common antigenic region with TnT (Sanders et al., 1987).

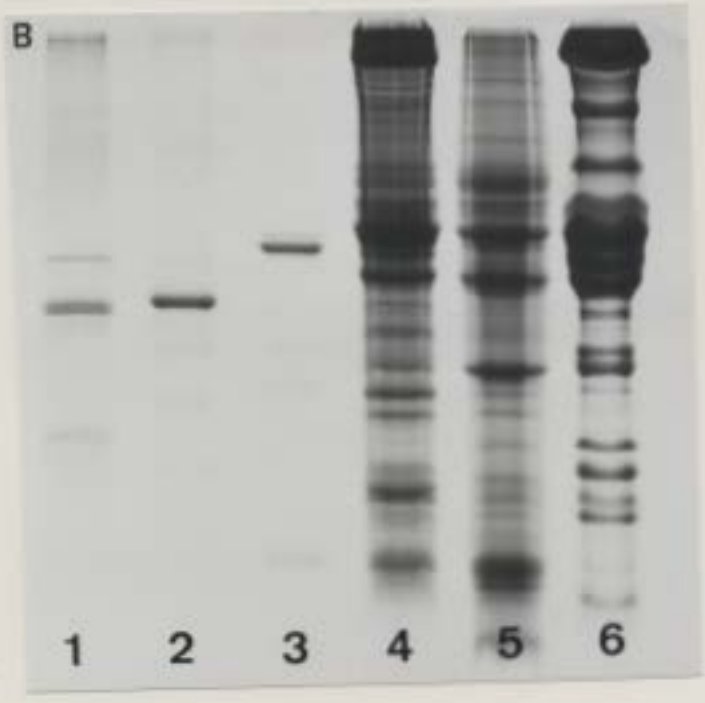
A single faint band comigrating with TnT 2F and 3F was detected in slow muscle Tn (Fig.13, lane 8). Since the slow isoforms are not recognized by this antibody this band most likely corresponds to TnT 2F and 3F which originates from a low level of cross contamination of fiber types incurred during dissection of the myotomal muscles. The fact that no such band appears in the slow muscle homogenates, where greater care could be taken to exclude any fast type fibers, suggests this is the case.

Salmonid slow muscle and cardiac muscle TnTs were identified by a mouse anti-rabbit cardiac TnT (donated by Dr. J. Lin, University of Iowa, Iowa) while the fast TnTs were not (Fig. 14). This result indicates that slow and cardiac TnT share a common epitope that is not present in fast TnT (Fig.14). The reaction of purified TnT 1S and 2S was not identical however. The 2S isoform exhibited a much stronger reaction than TnT 1S (Fig. 14, lane 2 & 3). This demonstrates that the epitope necessary for antibody recognition is not the same for these two TnTs and therefore TnT 2S can not be a breakdown product of TnT 1S. Also apparent from Fig. 14 is that there are at least two cardiac muscle specific isoforms. It is interesting that TnT 1S migrates in close proximity to the cardiac TnTs. Nonetheless, it is clear that cardiac TnT and

Fig. 14. **Immunoblot analysis of muscle extracts and purified proteins using an anti-cardiac TnT antibody.** Western immunoblot analysis of salmonid TnTs with CT3 anti-cardiac TnT antibody (courtesy of Dr. J. Lin). A 15% (w/v) SDS polyacrylamide gel was used. Muscle extracts were prepared as described in the legend to Figure 12. Bands were recognized by a goat anti-mouse IgG alkaline phosphatase conjugated second antibody. (A): Stained PVDF membrane. (B): Replicate gel ran at the same time as the gel used for Western transfer, but stained with Coomassie Blue.

1. fast Tn
2. TnT 2S
3. TnT 1S
4. slow muscle extract
5. cardiac muscle extract
6. fast muscle extract


A
1
2
3
4
5
6


B
1
2
3
4
5
6

slow TnT are not identical because they are not recognized by the antibody to equal extents: cardiac TnT exhibited a much stronger staining intensity. Therefore cardiac TnT and TnT 1S must be considered distinct isoforms.

Affinity Chromatography

All TnT isoforms were retained by affinity columns containing immobilized TM providing another line of evidence supporting the assignment of these proteins as TnTs. In these experiments, following the application of a subsaturating amount of TnT to the column, no protein was evident in the flow through. The interaction was specific for TnT since BSA did not bind to either fast TM or slow TM affinity columns (Table 4). The absolute binding ability of all TnTs to their respective TM column is included in Table 4.

Due to the low solubility of purified TnT 1F and 1S it was not possible to test the binding affinity of these isoforms for immobilized TM. However, the experiment could be performed with an unseparated mixture of fast TnTs (TnT 1F+2F+3F) that had been prepared by chromatography of whole fast Tn on Chelating Sepharose Fast Flow resin. Although the reason for the enhanced solubility of TnT 1F is unknown, when this material was applied to the fast TM affinity column, all the components in the mixture were retained by the resin. Unfortunately, several unsuccessful attempts were made to adsorb TnT 1S and mixtures of TnT 1S and 2S to the column. It is likely that during preparation of the sample for chromatography, which included dialysis, the protein precipitated under the low ionic strength conditions of the experiment. No protein was detected either in the flow through material,

Table 4. Binding of slow and fast TnTs to their corresponding TM affinity resins. Affinity chromatography was carried out in 10mM imidazole, pH 7.0 at 4°C, 0.15M NaCl, 0.25mM EDTA, 0.01% sodium azide, 1mM DTT. The salt gradient was 0.15-0.6M NaCl (total volume = 60mL) and 1.2 mL fractions were collected, at a flow rate of 6mL/h. These are the results of three experiments with the exception of BSA which was applied to the column only once. Several unsuccessful attempts were made to adsorb TnT 1S or mixtures of TnT 1S and 2S to the resin. The likely explanation for this is the low solubility of the protein, since no protein was detected in the flow through or in the gradient volume.

Ligand	Sample applied	Retained by column
fast TM	TnT 2F	yes
fast TM	TnT 3F	yes
fast TM	TnT 1F+2F+3F	yes
fast TM	BSA	no
slow TM	TnT 2S	yes
slow TM	TnT 1S	?
slow TM	BSA	no

in the elution volume or in the high salt wash.

Nucleotide Sequencing

Troponin T

A full length TnT clone (Genbank accession number U84037) was isolated from a brown trout (*Salmo trutta*) slow muscle cDNA library prepared by Donna M. Jackman, using a mixed oligonucleotide probe radiolabelled at its 5' end with $\gamma^{32}P$. Brown trout was used because a live adult salmon was unavailable. The probe was designed to match a conserved region (residues 108-113) of TnT based on the microsequencing results displayed in Table 3 and sequence comparisons of TnTs from other species (Table 3). Due to the high degree of degeneracy of the probe, low stringency and short incubation and washing steps were employed often resulting in high background levels. In such cases membranes were subjected to a second cycle of wash steps and were exposed a second time using fresh X-ray film.

Ten possible positives, including 24C12 (Fig. 15) were chosen from the probed dot blots and were Southern hybridized (Fig. 16). The inserts were excised by restriction endonuclease digestion with *EcoRI* and *HindIII*, loaded onto a 0.7% agarose gel, alkaline transferred to a nylon membrane and reprobed using the mixed oligonucleotide probe. Two bands (Fig. 16, lanes 10 and 11) were recognized corresponding to 24C5 and 24C12. Plasmids were prepared from both clones and sequenced from the 5' and 3' ends. A BLAST search was carried out of GenBank and EMBL using the algorithm of Altschul et al., 1990. Clone 24C5 was found to be a partial clone coding for myosin heavy chain while 24C12 coded for TnT. Digestion with *EcoRI* and *HindIII* revealed 24C12 was ~1100bp (Fig. 17).

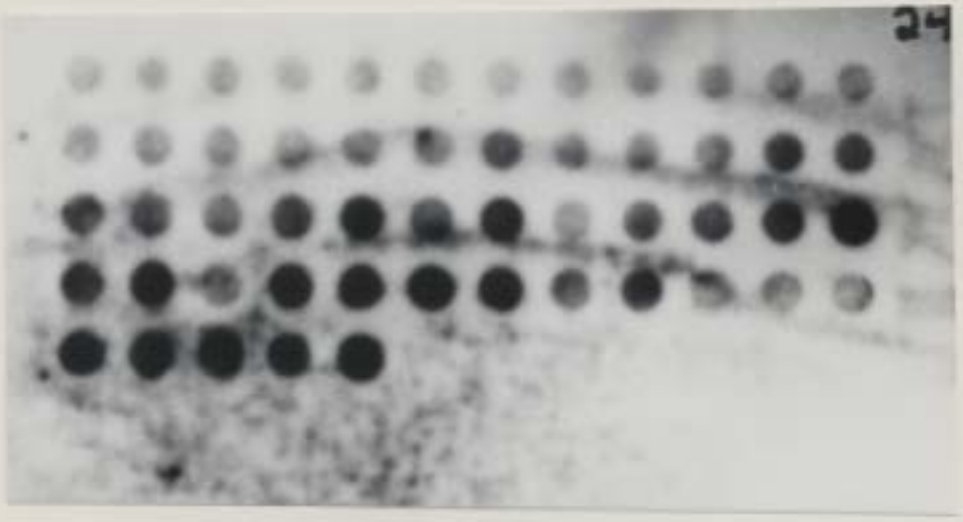


Fig. 15. Identification of possible TnT clones by detection with a radiolabelled mixed oligonucleotide probe. The probe was designed to match a conserved region based on protein data analyses. The filters were washed with varying concentrations (see Materials and Methods) of SSPE (150mM NaCl, 10mM sodium phosphate, 1.25mM EDTA) and SDS at 42°C and exposed for ~ 8h at -70°C. Due to the low stringency, a number of positives were obtained, some of these were further analyzed by Southern hybridization.

Fig. 16. Southern blot analysis of selected possible TnT clones. Approximately 10μg of DNA from a number of possible positives (from Fig. 15) was digested with restriction enzymes *HindIII* and *EcoRI* to release the insert. The DNA was size fractionated on a 0.7% agarose gel, alkaline transferred onto a nylon (Hybond N^+) membrane, and hybridized with the mixed oligonucleotide probe. Each lane is labelled using clone nomenclature based on ELISA plate number. The molecular weight marker used was λ DNA digested with *HindIII* and *EcoRI;* which generated the following fragments: 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831 and 564 bp.

1. Molecular weight marker
2. 14B4
3. 14F6
4. 14G2
5. 14H4
6. 15E2
7. 21G1
8. 23C10
9. 23G4
10. 24C5
11. 24C12


24C12
1 2 3 4 5 6 7 8 9 10 11

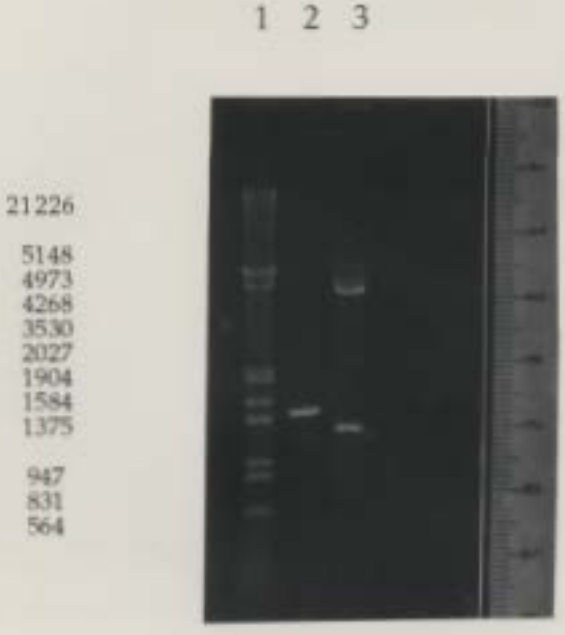


Fig. 17. Electrophoretic analysis of restriction endonuclease digestion of TnT clone 24C12 and actin clone 15H3. Both were incubated with *EcoRI* and *HindIII* and left at 37°C for 4-14 h, mixed with running dye and loaded onto a 0.7% agarose gel. Running conditions were 80V for 4h. The band at ~4.1 kb in lane 3 is the plasmid pSport. The molecular weight markder used was λ DNA digested with *HindIII* and *EcoRI*. See the legend to Fig. 16 for fragment sizes generated.

1. Molecular weight marker, (λ DNA digested with *Hind III* and *EcoRI*)
2. 15H3
3. 24C12

Trial digests involving a number of restriction enzymes revealed that only *PstI* and *SacI* liberated fragments of DNA suitable for subcloning (Fig. 18). Digestion with *PstI* generated a 860 bp fragment (Fig. 18) which was subcloned into the pUC 19 cloning vector with *PstI* / *HindIII* compatible ends and sequenced using M13 reverse sequencing primer (Pharmacia). Restriction endonuclease digestion with *SacI* liberated two fragments; 650 bp and 430 bp (Fig. 19). A map of the restriction enzyme digestion sites is shown in Fig. 20. The fragments isolated from digestion with *SacI* were cloned into pUC 19 with compatible ends *SacI* / *EcoRI* (430 bp) and *SacI* / *HindIII* (650 bp) and were sequenced. To completely sequence both DNA strands, two primers (Fig. 21) were synthesized by GIBCO BRL. The sequence of each of these, in the 5' to 3' direction was; TnT1: GTC AGC CAG AAT CTT CTT and TnT2: AAG AAG ATT CTG GCT GAC. The complete nucleotide sequence, as well as the predicted amino acid sequence is presented in Fig. 21. The coding region has 834 nucleotides which translate to 278 amino acids with a predicted molecular weight of 32362, and a predicted charge of ~ -3 at pH 7.0.

Following completion of nucleotide sequencing, comparative sequence analyses were carried out using both BLAST (basic local alignment search tool) (Altschul et al., 1990) and the CLUSTAL V program (Higgins et al., 1992). These analyses demonstrated that the clone codes for TnT. In addition, clone 24C12 was shown to be more similar to cardiac TnT than to slow or fast TnT (Fig. 22). This may be due to the low number and source of slow TnT sequences available in the database. Since N-terminal sequencing of a CNBr fragment of TnT 1S and 2S yielded a sequence that is 100% identical to residues 94-112 of the predicted sequence, this clone is deemed to be a slow

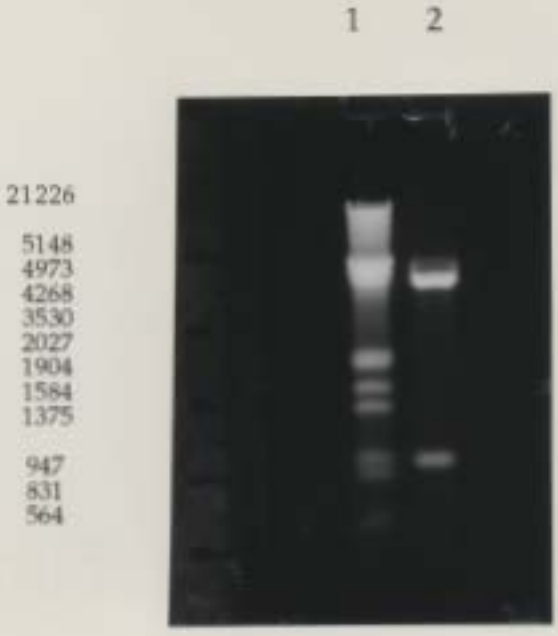


Fig. 18. Electrophoretic analysis of clone 24C12 digested with *PstI*. Clone 24C12 was digested with *HindIII* and *PstI* and loaded onto a 0.7% agarose gel. The band at ~4.1 kb in lane 2 is the plasmid pSport. See the legend to Fig. 16 for fragment sizes of molecular weight marker

1. Molecular weight marker (λ DNA digested with *HindIII* and *EcoRI*)
2. 24C12 digested with *PstI* and *HindIII*

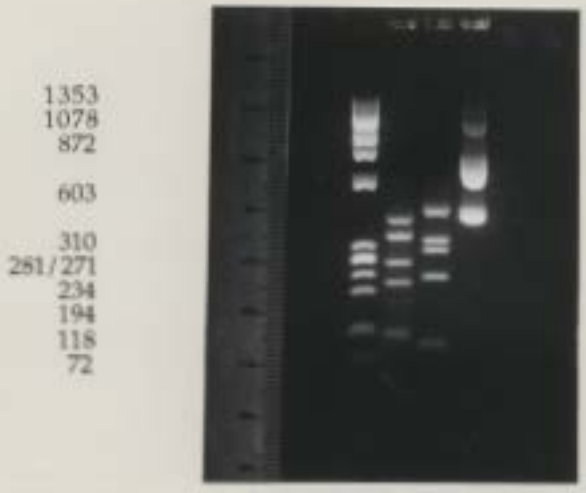


Fig. 19. Electrophoretic analysis of digests using restriction endonucleases for subcloning of 24C12 and 15H3. Inserts of 24C12 and 15H3 were liberated by digestion with *HindIII* and *EcoRI*, fractionated on a 0.7% agarose gel, excised, eluted from the gel by centrifugation and purified by phenol: chloroform extraction, followed by ethanol precipitation. The purified inserts were digested with the appropriate restriction endonuclease and loaded onto a 1.2% agarose gel. The molecular weight marker used was øX174 DNA digested with *HaeIII* , which generated the following fragments: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp.

1. Molecular weight marker (øX174 digested with *HaeIII*)
2. 15H3 digested with *RsaI*
3. 15H3 digested with *Sau3A*.
3. 24C12 digested with *SacI*.

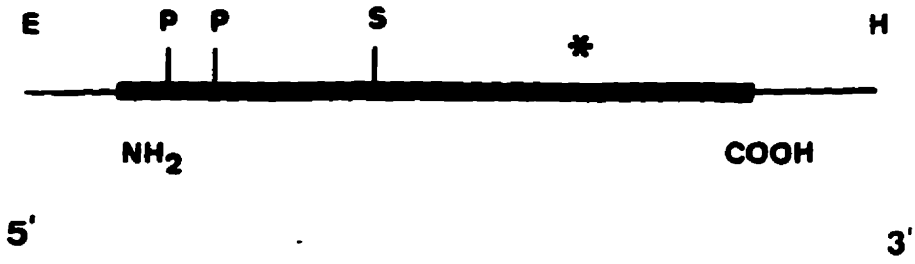


Fig. 20. Restriction endonuclease digest map of the full length brown trout slow muscle TnT cDNA clone 24C12. The fine line represents 5' and 3' untranslated regions. The thick line represents coding region (834 bp). Only restriction endonucleases used for subcloning are included. Restriction enzymes are denoted by a single letter; P = *Pst I*, S =*Sac I*, H = *HindIII* and E =*EcoR I*. Synthetic primers were prepared to region indicated by *. The scale is 1cm = 100 base pairs.

Fig. 21. The complete nucleotide sequence and derived amino acid sequence of the full length slow myotomal muscle TnT cDNA clone, 24C12. The clone was identified using a γ ^{32}P radiolabelled mixed oligonucleotide probe ($AA^{A}/_{G}AA^{A}/_{G}GA^{C}/_{T}GA^{A}/_{G}GA^{C}/_{T}GA^{A}/_{G}$) derived from a region (underlined portion) of TnT 1S determined by microsequencing of TnT fragments. The nucleotide sequence was determined completely on both DNA strands by sequencing from synthetic primers (indicated by •••) and by cloning fragments, generated by restriction endonuclease digestion, into pUC 18 cloning vectors and sequencing as described in Materials and Methods. The amino acid sequence deduced from the coding sequence is written beneath the nucleotide sequence in single letter code. The sequence represented by residues 94-112 corresponds exactly to the sequence obtained by microsequencing of a CNBr fragment (Table 3). The asterisk indicates the termination codon.

```
  aaagctggtacgcctgcaggtaccggtccggaattcccggtcgacccacgcgtccgga

aagaataagaccggtccatttctctttcccggtggggtttggaggtaatccagtataaac

ATGGCAGATGAAGAAAATGAGGAGGAAGTTGAGGTTGAGGTCCCAGCAGAGGAAGATGCT
M  A  D  E  E  N  E  E  E  V  E  V  E  V  P  A  E  E  D  A          20

GCTCCTGCAGAGGAAGAACCAGAACCAGAGCCCGAGCCAGACATAGAAGAGCCCACTGCA
A  P  A  E  E  E  P  E  P  E  P  E  P  D  I  E  E  P  T  A          40

GTAGAAGAAACACCTGCAGAAGAAGCCAGTGGAGAGACTCAAGATTCCAAAGCCAAACCA
V  E  E  T  P  A  E  E  A  S  G  E  T  Q  D  S  K  A  K  P          60

AAAAGTTTTATGCCAAATGTTGCCCCTCCAAAGCTACCAGAGGGAGATGGTAAAGTCGAT
K  S  F  M  P  N  V  A  P  P  K  L  P  E  G  D  G  K  V  D          80

TTTGACGACCTGCACAGGAAGCGTCAAGAGAAGGACATGGCTGAGCTGACCTCTCTGATT
F  D  D  L  H  R  K  R  Q  E  K  D  M  A  E  L  T  S  L  I         100

GAGTCTCACTTTGTCCAGAGGAAGAAAGATGAAGATGAGCTCATCAGTCTCGTCAACAGG
E  S  H  F  V  Q  R  K  K  D  E  D  E  L  I  S  L  V  N  R         120

ATCGAGAAGCGTCGTACCGAGAGGGCAGAGCAGCAGAGGATTCGTGCTGAGCAAGAGAAA
I  E  K  R  R  T  E  R  A  E  Q  Q  R  I  R  A  E  Q  E  K         140

GAGAGGCAGGCCCGTCTAGCCGAAGAGAAAGAGAGGAGGGAGCAAGACGAACAGCGTAGG
E  R  Q  A  R  L  A  E  E  K  E  R  R  E  Q  D  E  Q  R  R         160

AAGCACGATGATGACGCCAAGAAGAAGAAGGCCCTCACTACCATGACTCACACGTACGGT
K  H  D  D  D  A  K  K  K  K  A  L  T  T  M  T  H  T  Y  G         180

GGCATCCAACAGAAGCAAGAGGGCAAGAAGGGAGCCAAGAAGCAGACGGAGAGAGAAAAG
G  I  Q  Q  K  Q  E  G  K  K  G  A  K  K  Q  T  E  R  E  K         200
   .................*
AAGAAGAAGATTCTGGCTGACAGAAGGAAGGCTCTGATCATCGACCATCTCAACGAGGAC
K  K  K  I  L  A  D  R  R  K  A  L  I  I  D  H  L  N  E  D         220

AAACTCAAGGAGAAGGCCAATGAGCTGTGGCAGTGGATGATGGAGCTGGAGGCGAGAAGT
K  L  K  E  K  A  N  E  L  W  Q  W  M  M  E  L  E  A  R  S         240

TCGACCCTCAGCGAGAAACTGAAGAAGCAGAAATACGACATCAATCAGCTTCTGGCCCGT
S  T  L  S  E  K  L  K  K  Q  K  Y  D  I  N  Q  L  L  A  R         260

GTCCAGGACCACCAGAGTGCAAAAGGCAGAGGCAAGGCCAAGGCGGTCAGGAGAtagtga
V  Q  D  H  Q  S  A  K  G  R  G  K  A  K  A  V  R  R  *            278

cctggaggatgaatcttcaggactgagacgccagatgatgacgaggacgttgtgtgtcct

gtatcagcgtgcatgcaaatgctatggaaatgtactgttatcacggtcactgtttgcatg

aagctagaaaccaataaaggacgttgcttttg
```

Fig. 22. Comparison and alignment of published TnT sequences with brown trout slow muscle TnT 1S. The abbreviations are as follows; RS FAST- rabbit skeletal fast muscle (Pearlstone et al., 1977); CH CAR- chicken cardiac TnT (Pearlstone et al., 1986); HU SLOW- human slow TnT (Samson et al., 1994). Gaps, represented by * have been introduced to maximize sequence similarity. Dashes indicate identity with RS FAST.

```
RS FAST      S D * E E * V E H V E E E * * A E E E A P S P A      20
CH CAR     M - - S - - V - - E Y - Q - Q * * - - - Y V E E G      23
HU SLOW    M - - T - - Q * * E Y - - - Q P * - - - A A E E *      21
TnT 1S     M A - * - - N E - E - - V - V P - - - D A A * - -      23

RS FAST    * * E * V H E P A P E H V V P * E E V H E E E * *      39
CH CAR     Q E D Q - D - E E E - T E E T T A - E Q - D - T K      48
HU SLOW    E E - A P E - - E - V A * E - E - - * * * * * * *      38
TnT 1S     E E E P E P - - E - D I E E - T A * - * E E * T *      44

RS FAST    * * * * * * * * * * * * * * * * * * * K P R *      42
CH CAR     A P E Q G G E G D R E Q E P G E G E S K P - - K P      73
HU SLOW    * * * * * * * * * * * * * * * * * * R P - - S R      44
TnT 1S     * P A E E A S G E T * Q * * * * * D S K A - - K S      62

RS FAST    * * * K L T * A P K I P E G E * K V D F D D I Q K      62
CH CAR     F M P N - V * P - - - - - D - - * R L - - - - - - H R      96
HU SLOW    P V V P P L I P - - - - - - - - * R - - - - - - - H R      68
TnT 1S     F M P N * V A P - - L - - - D G - - - - - - - L H R      86

RS FAST    K R Q N K D L M E L Q A L I D S H F E A R K K E E      87
CH CAR     - - M E - - - N - - - - - - - E A - - - S - - - - - -     121
HU SLOW    - - M E - - - L - - - T - - - V - - - Q - - - - - -      93
TnT 1S     - - - E - - M A - - T S - - E - - - V Q - - - D -     111

RS FAST    E E L V A L K E R I E K R R A E R A E Q Q R I R A     112
CH CAR     - - - I S - - D - - - Q - - - - - - - - - - - - - S     146
HU SLOW    - - - - - - - - - - - - R - - S - R - - - - - - F - T     118
TnT 1S     D - - I S - V N - - - - - - - T - - - - - - - - - - -     136

RS FAST    E K E R E R Q N R L A E E K A R R E E E D A K R R     137
CH CAR     - R - K - - - A - M - - - R - - K - - - E - R K K     171
HU SLOW    - - - - - - - - A K - - - - - M - K - - - E - - K -     143
TnT 1S     - Q - K - - - A - - - - - - - E - - - Q D E Q R - K     161

RS FAST    A E E D L K K K K A L S S M G A N * Y S S Y L A K     161
CH CAR     - - K E A R - - - - - F - N - * L H * F G G - M Q -     194
HU SLOW    - - D - A - - - - - V - - N - - - H * F G G - - V -     167
TnT 1S     H D D - A - - - - - - - T T - * T H T - G G I Q Q -     185

RS FAST    A D Q K R G * K K Q T A R E M K K K I L A E R R K     185
CH CAR     S E K - G - * - - - - E - - K - - - - - - S - - - - -     218
HU SLOW    - E - - - - - * - R - - G - - - - - V R - - S - - K -     191
TnT 1S     Q E G - K - A - K Q T E R E K - - - - - - - D - - -     210
```

```
RS FAST     P L N I D H L S D E K L R D K A K E L W D T L Y Q     210
CH CAR      - - - - - - - - - E D - - - - - - - - - - Q - I R D     243
HU SLOW     - - D - - Y M G E - Q - - E - - Q - - S - W I H -     216
TnT 1S      A - I - - - - N E D - - K E - - N - - - Q W M M E     235

RS FAST     L E T D K F E F G E K L K R Q K Y D I M N V * R A     234
CH CAR      - - A E - - D L Q - - F - - - - - E - * N V L - N     267
HU SLOW     - - S E - - D L M A - - - Q - - - E - * N V L Y N     240
TnT 1S      - - A R S S T L S - - - - K - - - - - * N Q L L -     259

RS FAST     R V E M L A * K F * * S K K * A G T T A K G K V G     255
CH CAR      - - S D H Q * - V K G - - A * - * * R G - T M - -     288
HU SLOW     - I S * H - Q - - * * * R - G - - * * * - - R - -     258
TnT 1S      - - Q D H Q S * A K G * * * * * * * * * R - - A K     274

RS FAST     G R W K                                               259
CH CAR      - - - -                                               292
HU SLOW     - - - -                                               262
TnT 1S      A V R R                                               278
```

isoform. The inferred amino acid composition of clone 24C12 more closely resembles the measured amino acid composition of TnT 1S than any other isoform. Furthermore, judging from the high amounts of proline and glutamic acid it is clear that 24C12 can not correspond to isoform 2S. In addition, the predicted molecular mass of clone 24C12 is 32362 which is significantly greater than the molecular masses of TnT isoforms 1F, 2F, 3F and 2S (see legend to Table 2) determined by mass spectrometry. Thus, TnT 1S is concluded to be encoded by this clone and to be specific to slow myotomal muscle. The use of brown trout is considered valid because it is closely related to Atlantic salmon. It is anticipated that TnT isoforms from salmonids will be highly similar if not identical as reported for TM (Heeley et al, 1995; Jackman et al., 1996). In the case of TnT 1S, this prediction is supported by the good agreement between the amino acid analysis of Atlantic salmon TnT 1S and the predicted composition of brown trout TnT 1S as well as the identity of residues 94-112.

The predicted primary structure of TnT 1S will now be discussed. Some of the more interesting features of the inferred amino acid sequence are summarized in Table 5. Like other TnTs, this isoform contains a high proportion (134 of 278 or ~48%) of charged residues. The N-terminal region is highly acidic (Fig. 21). In fact, 26 of the first 55 amino acids are acidic. Interestingly, only 4 aspartic acids residues are present within this region. The C-terminal domain (residues 221-278) is predominantly basic comprising twice as many basic as acidic residues.

Another aspect of the primary structure is that all 13 proline residues occur within the first 73 amino acids. Most TnTs contain less than 1% of their

Table 5. Summary of features of the inferred amino acid sequence of TnT 1S. The amino acid sequence is derived from the nucleic acid sequence of a full length slow muscle TnT clone 24C12. The complete nucleic acid sequence as well as the inferred amino acid sequence can be found in Fig. 21.

Property or feature	Example	Comments
N-terminus	residues 1-55: 26 acidic, 0 basic	highly negatively charged, likely to affect structure
C-terminus	residues 221-278: 7 acidic $15^1/_2$ basic	positively charged
central portion	residues 61-220: 35 acidic, $45\ ^1/_2$ basic	highly charged
Proline rich N-terminal region	all 13 Pro occur within the first 73 amino acids	likely to affect structure
Tryptophan	two Trp at residues 230 and 232, none at C-terminus	other TnTs have 1 Trp in similar position
Cysteine	None of the isoforms contained Cys	Similar for most TnTs
Glycine	Clusters at positions: 51, 75 & 77; 180, 181, 188 & 191; 269 & 271	Noted for other TnTs esp. chicken cardiac; likely to affect structure
Methionine	evenly distributed	suitable fragments may be generated
Lack of serine 1	Serine replaced by alanine	Probably not a phospho-protein

proline residues in the C-terminal half of the protein, with the exception of human slow TnT (clone H22h) which has an insertion (residues 204-219) containing 4 Pro, 1 Cys and 1 Trp at the C-terminus (Gahlmann et al., 1986). At the present time it is unknown how differing proline contents will affect the structure of the N-terminal portion of TnT. Clearly, large numbers of proline would be predicted to prevent the formation of helical segments.

Of the major salmonid TnTs from fast and slow myotomal muscle, only isoform TnT 1S contains two tryptophans, both of which are very closely positioned in the sequence (residues 230 and 232). Most other TnTs, with the exception of quail fast skeletal muscle TnT (residues 205 & 207) (Hastings et al., 1985) have a single tryptophan at an analogous position in addition to tryptophan at the penultimate C-terminal residue. As stated above, TnT 1S does not contain a C-terminal tryptophan. This is an interesting finding since the C-terminal sequence is highly conserved. The major salmonid TnT isoforms (TnT 1F, 2F, 3F, 1S and 2S) do not contain any cysteine as assessed by amino acid analysis (Table 2). In the case of TnT 1S this is verified by nucleotide sequencing. It is also evident that no histidine residues are found in the N-terminal half of the protein. Instead this amino acid is distributed fairly evenly throughout the central and C-terminal domains.

Six methionine residues are present in TnT 1S (positions 1, 64, 93, 175, 233 and 234). With the exception of the C-terminal domain where two methionines are juxtaposed (residues 233 and 234) the other residues are evenly distributed throughout the sequence. This will allow for the generation of relatively large fragments of TnT 1S by digestion with CNBr. N-terminal fragments arising from peptide bond cleavage at methionine 64

or 93 could be purified to determine the functional significance of this region. Purification of the fragment spanning residues 94-175 would be useful in studying the interaction of TnT and TM, since this region corresponds to a fragment of rabbit fast TnT (residues 71-151) which has been implicated in TM binding (Mak and Smillie, 1981).

With the exception of the N-terminal domain which is highly negatively charged and the extreme C-terminus which is mostly positively charged, basic and acidic residues are clustered together in other parts of the molecule. Examples include residues 107-113: RKK DEDE and residues 163-170, DDD A KKKK (Fig. 21). A string of highly polar residues will likely affect the secondary structure of the molecule as would the clusters of glycine residues that are found throughout the sequence. For example, three glycines occur at postions 51, 75 and 77, and four are found within an eleven amino acid stretch from 180-191 (positions: 180, 181,188 and 191) and two glycines are found at the C-terminus at positions 269 and 271.

In most TnTs the N-terminal serine (position 1) is phosphorylated *in vivo*. Since the N-terminal serine has been replaced by an alanine, TnT 1S is unlikely to be a substrate for a kinase, at least, *in vivo* (Fig. 21).

Using the CLUSTAL V sequence alignment program TnT 1S was compared to other slow TnT sequences. The % identities counting deletions as differences and ignoring N-terminal extensions were; human slow muscle TnT (Samson et al., 1994) versus chicken slow muscle TnT (Yonemura et al., 1996) 80%, while TnT 1S versus chicken and human were 47.7% and 47.9% respectively. Clearly the salmonid slow muscle TnT isoform is equally divergent from mammals and birds.

Significant similarity exists between a number of TnTs from other organisms and TnT 1S especially over the last two-thirds of the molecule. The high degree of conservation stems from the preservation of important structural and functional domains. This region is implicated in a number of protein-protein interactions. For example TM is known to bind to residues 71-151 (Mak and Smillie, 1981; Pearlstone and Smillie, 1977) while TnI interacts with residues 152-209 (Ohtuski, 1975; Pearlstone and Smillie, 1978) (rabbit TnT nomenclature). Inspection of the sequence alignment data (Fig. 22) reveals that all four TnTs (rabbit skeletal TnT, rabbit cardiac TnT, human slow TnT and TnT 1S) are conserved over this region. TnT 1S shares 81% sequence similarity (as defined by Pearlstone et al., 1986) with rabbit skeletal fast muscle TnT residues 71-151 and 68% similarity with amino acids 152-209. Residues 229-242 of rabbit fast TnT are generated by mutually exclusive splicing of two exons producing two similar sequences (Breitbart et al., 1985). These correspond to amino acids 255-266 of TnT 1S and are poorly conserved (28% similarity). This is significant since these residues fall within a portion of the molecule that contains a binding site for TnC and TM (Mak and Smillie, 1981; Ohtuski, 1975; Pearlstone and Smillie, 1977; 1978; White et al., 1987; McLachlan and Stewart, 1976). Thus, it is possible that substitutions within this region may produce TnTs having unique regulatory properties.

Several reports have documented heterogeneity at the N-terminus of TnT that arises from the differential splicing of a number of small exons (Wilkinson et al., 1984; Breitbart et al., 1985; Medford et al., 1984; Cooper and Ordahl, 1985; Jin et al, 1992; Jin and Lin, 1989). For a number of reasons the prediction of exon boundaries in TnT 1S is not straightforward. One of the

complicating factors is that TnTs from different sources vary in length. Thus, the exon number designations are not the same in different TnTs. For example, exon 4 of rabbit fast TnT does not have the same sequence as exon 4 of rabbit cardiac TnT. In addition, several studies (Breitbart et al., 1985; Cooper and Ordahl, 1985) have shown that the N-terminal region of TnTs of mammals and birds is a 'hot spot' in terms of sequence substitutions. However, comparative sequence analysis reveals that residues 82-222 share some similarity to amino acids 58-197 of rabbit skeletal TnT and 85-218 of rabbit cardiac TnT. Thus, the exon delineations (10-14) (Breitbart et.al.,1985; Cooper and Ordahl, 1985) may be analogous in this region. In addition amino acids 1-11 are similar to residues 1-9 of rabbit skeletal TnT and may be analogous to exons 2 and 3 of rabbit skeletal TnT (Breitbart et.al., 1985). Further sequencing of the slow TnT gene is necessary before the exon boundaries of the slow TnT gene can be defined.

Actin

During exploratory sequencing of the slow muscle cDNA library, a full length actin clone was isolated (Fig. 17). Restriction endonucleases *ScaI*, *Sau3AI* and *RsaI* were used to generate fragments suitable for subcloning (Fig. 19). A map of the restriction sites is shown in Fig. 23. Digestion with *ScaI* generated a 900 bp fragment which was subcloned into pUC 19 with compatible ends *ScaI* / *HindIII* and sequenced. Both *Sau3AI* and *RsaI* generated a number of smaller fragments. In both cases an aliquot of the digest was ligated into pUC 19 with all possible compatible ends, for example, *Sau3AI* / *HindIII*, *Sau3AI* / *EcoRI*, and *Sau3AI* / *Sau3AI*. Following

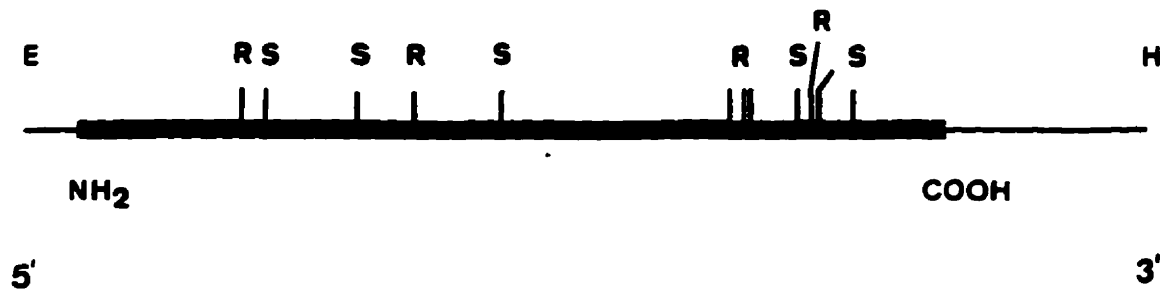


Fig. 23. **Restriction endonuclease digest map of the full length brown trout slow muscle actin cDNA clone, 15H3.** The fine line represents 5' and 3' untranslated regions. The thick line represents coding region (1131 bp). Only restriction endonucleases used for subcloning are included. Restriction enzymes are denoted by a single letter; S = *Sau3A*, R = *RsaI*, H = *HindIII* and E=*EcoR I*. The scale is 1cm = 100 base pairs.

transformation into *E.Coli.* DH5α competent cells, the plasmids were isolated and their inserts were sequenced. A BLAST search was carried out of GenBank and EMBL using the algorithm of Altschul et al., 1990. Owing to the highly conserved nature of actin the fragments could be matched easily.

Clone 15H3 encodes actin of 377 amino acids with a predicted molecular weight of 41852. Based on comparative analyses the sequence is believed to encode a class II α skeletal muscle actin. The nucleotide sequence as well as the inferred amino acid sequence is provided in Fig. 24. Illustrated in Fig. 25 is a comparison of actin sequences from rabbit skeletal and cardiac muscle, carp skeletal muscle and clone 15H3. As expected a great degree of sequence conservation is apparent. However, it is also clear that clone 15H3 is slightly more similar to cardiac than to fast muscle actin from mammals and birds.

Fig. 24. **The complete nucleotide sequence and derived amino acid sequence** of the **full length actin cDNA clone, 15H3.** The nucleotide sequence was determined by cloning restriction fragments into pUC 19 and sequencing as described in Materials and Methods. The amino acid sequence deduced from the coding sequence is written beneath the nucleotide sequence in single letter code. Based on sequence comparisons, this clone is believed to be an α actin of Class II. The asterisk indicates the termination codon. The polyadenylation signal is in bold.

```
                                                                cccacgcgtcgg

ggactgtaacccaccgcctcctgtccagctctccagggcccacagtaaccagactcaacc

ATGTGTGACGAGGAAGAGACAACAGCCTTGGTATGCGACAACGGCTCAGGACTGGTGAAG
M  C  D  E  E  E  T  T  A  L  V  C  D  N  G  S  G  L  V  K          20

GCTGGCTTCGCCGGTGACGATGCCCCCAGGGCAGTGTTCCCCTCCATTGTGGGGCGCCCC
A  G  F  A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  R  P          40

CGTCATCAGGGGGTGATGGTGGGTATGGGTCAGAAAGACTCCTATGTAGGAGACGAGGCC
R  H  Q  G  V  M  V  G  M  G  Q  K  D  S  Y  V  G  D  E  A          60

CAGAGCAAGAGAGGTATCCTGACCCTCAAGTACCCCATTGAGCACGGCATCATAACTAAC
Q  S  K  R  G  I  L  T  L  K  Y  P  I  E  H  G  I  I  T  N          80

TGGGACGACATGGAGAAGATCTGGCATCATACCTTCTACAATGAGCTTCGTGTGGCACCT
W  D  D  M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P         100

GAGGAGCACCCTGTCCTGCTCACTGAGGCCCCACTCAACCCCAAGGCCAACAGAGAGAAG
E  E  H  P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  E  K         120

ATGACCCAGATCATGTTTGAGACCTTCAACGTGCCAGCTATGTATGTGGCCATCCAGGCT
M  T  Q  I  M  F  E  T  F  N  V  P  A  M  Y  V  A  I  Q  A         140

GTGCTGTCCCTGTACGCCTCTGGTCGTACCACAGGTATTGTGCTGGACGCTGGCGATGGT
V  L  S  S  Y  A  S  G  R  T  T  G  I  V  L  D  A  G  D  G         160

GTGACCCACAACGTGCCCGTATATGAGGGTTATGCCTTGCCCCATGCCATCATGAGACTG
V  T  H  N  V  P  V  Y  E  G  Y  A  L  P  H  A  I  M  R  L         180

GACTTGGCTGGCAGAGACCTGACTGACTACCTGATGAAGATCCTCACTGAGAGAGGCTAC
D  L  A  G  R  D  L  T  D  Y  L  M  K  I  L  T  E  R  G  Y         200

TCTTTCGTCACCACCGCTGAGAGAGAGATTGTGCGTGACATCAAGGAGAAGCTGTGCTAC
S  F  V  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y         220

GTGGCTCTGGACTTTGAGAATGAGATGGCCACCGCTGCCTCCTCCTCCTCTCTGGAGAAG
V  A  L  D  F  E  N  E  M  A  T  A  A  S  S  S  S  L  E  K         240

TCCTATGAGTTGCCCGATGGTCAGGTCATCACCATCGGTAACGAGAGGTTCCGTTGCCCA
S  Y  E  L  P  D  G  Q  V  I  T  I  G  N  E  R  F  R  C  P         260

GAAACCCTCTTCCAGCCCTCCTTCATTGGCATCGAGTCCGCTGGTATCCATGAGACCACA
E  T  L  F  Q  P  S  F  I  G  M  E  S  A  G  I  H  E  T  T         280

TACAACGGCATCATGAAGTGCGACATTGACATCCGTAAGGACCTGTACGCCAACAATGTC
Y  N  G  I  M  K  C  D  I  D  I  R  K  D  L  Y  A  N  N  V         300

TTGTCCGGTGGTACCACCATGTACCCAGGTATCGGTGACCGCATGCAGAAGGAAATCACA
L  S  G  G  T  T  M  Y  P  G  I  G  D  R  M  Q  K  E  I  T         320
```

```
GCCCTGGCCCCCAGCACAATGAAGATCAAGATGATTGCCCCCCCCTGAGCGTAAGTACTCA
A  L  A  P  S  T  M  K  I  K  M  I  A  P  P  E  R  K  Y  S          340

GTCTGGATCGGCGGCTCCATCCTGGCCTCCCTGTCCACCTTCCAGGCCATGTGGATCAGC
V  W  I  G  G  S  I  L  A  S  L  S  T  F  Q  A  M  W  I  S          360

AAAGATGAGTATGAGGAGGCCGGACCCTCAATCGTCCACAGAAAGTGCTTCtaatttctc
K  D  E  Y  E  E  A  G  P  S  I  V  H  R  K  C  F  *               377

tctccaactgtcaactgtccccaaatatttgctctctgtctattctcatgtgtctctaat

ccccacaaataacccattgtaattgtttactatttgtgcaacgttcccaaagaatctgta

ttatcagtctctaatccacaaataccattgtaattgtttactatttgtgcaacgttccca

aagaatctgtattatcaggatattgctaaataaattaattctgacttttg
```

Fig. 25. Alignment of the inferred protein sequence of brown trout actin (clone 15H3) with other published actin sequences. The abbreviations are as follows; BT SKL- brown trout skeletal muscle actin; CARP SKL carp skeletal muscle actin (Watabe et al., 1995); RB SKL: rabbit skeletal muscle actin (Elzinga et al., 1973); HU CAR human cardiac muscle actin (Hamada, et al., 1982). Residues identical to brown trout skeletal muscle actin are represented by a dash (-).

```
BT SKL      MCDEEETTAL VCDNGSGLVK AGFAGDDAPR AVFPSIVGRP RHQGVMVGMQ
CARP SKL     -DD------ ---------- ---------- ---------- ----------
RB SKL         --D----- ---------- ---------- ---------- ----------
HU CAR         -D------ ---------- ---------- ---------- ----------

BT SKL      KDSYVGDEAQ SKRGILTLKY PIEHGIITNW DDMEKIWHHT FYNELRVAPE
CARP SKL    ---------- ---------- ---------- ---------- ----------
RB SKL      ---------- ---------- ---------- ---------- ----------
HU CAR      ---------- ---------- ---------- ---------- ----------

BT SKL      EHPVLLTEAP LNPKANREKM TQIMFETFNV PAMYVAIQAV LSSYASGRTT
CARP SKL    ---T------ ---------- ---------- ---------- --L-------
RB SKL      ---T------ ---------- ---------- ---------- ----------
HU CAR      ---T------ ---------- ---------- ---------- ----------

BT SKL      GIVLDAGDGV THNVPVYEGY ALPHAIMRLD LAGRDLTDYL MKILTERGYS
CARP SKL    ---------- ---------- ---------- ---------- ----------
RB SKL      -----S---- -----I---- ---------- ---------- ----------
HU CAR      -----S---- -----I---- ---------- ---------- ----------

BT SKL      FVTTAEREIV RDIKEKLCYV ALDFENEMAT AASSSSLEKS YELPDGQVIT
CARP SKL    ---------- ---------- ---------- ---------- ----------
RB SKL      ---------- ---------- ---------- ---------- ----------
HU CAR      ---------- ---------- ---------- ---------- ----------

BT SKL      IGNERFRCPE TLFQPSFIGM ESAGIHETTY NGIMKCDIDI RKDLYANNVL
CARP SKL    ---------- ---------- ---------- -S-------- ----------
RB SKL      ---------- ---------- ---------- -S-------- ---------M
HU CAR      ---------- ---------- ---------- -S-------- ----------

BT SKL      SGGTTMYPGI GDRMQKEITA LAPSTMKIKM IAPPERKYSV WIGGSILASL
CARP SKL    ---------- A--------- ---------I ---------- ----------
RB SKL      ---------- A--------- ---------I ---------- ----------
HU CAR      ---------- A--------- ---------I ---------- ----------

BT SKL      STFQAMWISK DEYEEAGPS IVHRKCF
CARP SKL    ----Q---T- Q--D----- -------
RB SKL      ----Q---T- Q--D----- -------
HU CAR      ----Q----- ---D----- -------
```

Chapter 4

Discussion

Sarcomeric proteins display a tremendous capacity for diversity. Numerous isoforms arise from the use of multiple genes, multiple promoters and alternative splicing of individual genes. Although, the existence of contractile isoforms has been well documented much less is known about how variants of any one given component differ in their functionality. A very good case in point is slow skeletal muscle TnT, the biochemical properties of which have not been studied to date. As stated earlier (see introduction), this is a direct reflection of the inability to prepare sufficient quantities of the protein from conventionally used laboratory animals.

The current work has addressed this problem by taking advantage of the reservoir of slow muscle present within the myotome of salmonid fish. For the first time, a quantitative isolation of TnT specific to this muscle type has been carried out. Slow and fast myotomal muscles were found to be completely distinct from each other in terms of their respective contents of TnT. In view of this fact (i.e. that TnT exists as muscle type specific isoforms, as reported for TM (Heeley and Hong, 1994; Heeley, et al. 1995), salmonid fish are emerging as a suitable experimental model in which to investigate contractile protein heterogeneity. However, it must be noted that the efforts to characterize the heterogeneity of TnT were limited to those isoforms that are present in greatest amounts (that is, major forms). This task will always be

made more difficult by the possible existence of minor isoforms. Post translational modifications, such as phosphorylation and proteolysis can further complicate analysis. Indeed, the muscle researcher may never be able to characterize, or even detect all of the forms of any individual contractile protein.

During the course of this work, three fast TnT isoforms and two slow TnT isoforms were identified by a number of defining characteristics, including copurification with whole troponin, amino acid compositions, sequence similarity, immunoreaction with anti-TnT antibodies and interaction with immobilized TM on affinity resins. The TnT isoforms differ in both size as determined by SDS PAGE and mass analysis, and in charge as assessed by ion exchange chromatography. As stated earlier, all TnT isoforms are distinct and exhibit a tissue-specific distribution. The lower molecular weight TnT isoforms (2F and 3F) from fast muscle were partially phosphorylated, while TnT 1F, 1S and 2S were not.

Another outcome of this thesis was the determination of the complete nucleic acid sequence of brown trout TnT 1S. This information, together with previous research on TnT, is of obvious use in discussing the potential regulatory properties of isoform 1S. Troponin T is responsible for relaying conformational information from TnC to TM. From previous work, TnT is thought to interact with TM at two sites (Mak and Smillie, 1981; Pearlstone and Smillie, 1977; 1978; White et al., 1987; McLachlan and Stewart, 1976). At one of these sites, the N-terminal region of TnT (residues 71-151 rabbit skeletal TnT numbering scheme) interacts with the C-terminal part of TM, spanning the overlap domain of adjacent TM molecules and therefore may

contribute to thin filament directed regulation. In addition, recent evidence suggests that TnT plays an active role in the regulation of muscle contraction through direct interaction with TnC (Potter et al., 1995). Heterogeneity, within the N-terminal region is anticipated to produce isoforms of TnT having different regulatory properties. A number of studies (Nassar et al., 1991; McAuliffe et al., 1990; Tobacman and Lee, 1987; Tobacman et al., 1988) have shown that even small changes or insertions in the amino terminal sequence of TnT have measurable effects on the regulatory properties of the thin filament. Experiments describing the biochemical implications of different salmonid TnTs are likely to reveal differences in slow and fast TnT-containing Tn or myofilaments, as well as tissue specific variations. The introduction of a number of negatively charged residues as in TnT 1S could have a weakening effect on the interaction with TM due to electrostatic repulsions with the negatively charged C-terminal domain of TM. Furthermore, the abundance of proline residues at the N-terminus which, in turn, may affect formation of secondary structure may also affect the TnT-TM interaction. Although the hypervariable region is slightly outside the binding site of TM, long range effects could be exerted on the Tn-TM complex affecting the Ca^{2+} sensitivity (Ca^{2+} concentration at 50% of the maximal ATPase activity) of the thin filament (Tobacman and Lee, 1987). The differences in Ca^{2+} sensitivity are attributed mostly to TnT either directly by influencing Ca^{2+} binding to the low affinity sites of TnC or by altering the conformation of the troponin complex to indirectly affect the TnC-Ca^{2+} interaction.

For these reasons the heterogeneity of TnT, and how it is generated, has been of great interest. It is still not clear, however, whether or not

salmonid TnTs arise from separate genes or are the products of alternative splicing. In other species, separate TnT genes exist for each muscle type. Additional TnT isoforms are generated by differential splicing of these genes. Troponin T is therefore an excellent model for the study of splicing reactions (Breitbart et al., 1985). Unfortunately, little is presently known about how splicing is regulated. Some of the signals used to identify exons include the presence of purine rich sequences, split codons, and acceptor and donor consensus sequence sites that flank each exon and serve to mark the splice site (Breitbart et al. 1985). Nonetheless, the degree of precision of the splicing events suggest they are more stringently regulated. Recently, Ramchatesingh et al., (1995) described the presence of highly conserved consensus sequences called splicing enhancers that are actually contained within exon 5 of the cardiac TnT gene. Splicing factors (SR proteins) directly interact with the enhancer to activate excision of the upstream intron. The reaction is specific and there appears to be a correlation between conservation of this sequence and splicing efficiency (Ramchatesingh et al., 1995). SR proteins may also be involved in directing small nuclear ribonucleic proteins (snRNPs) during assembly of the spliceosome. In other work, splicing factors have been shown to bind to both the 5' and 3' ends of the exon and serve to define the splice sites (Robberson et al., 1990).

Future Experiments:

The development of methods for the purification of muscle type specific isoforms of TnT has laid the groundwork for future studies which are outlined below.

It would be of interest to define the exon boundaries of the brown trout slow muscle TnT gene. This could be achieved by preparing a genomic DNA library and determining both coding and intervening sequences. In addition, determining the coding sequence of TnT 2S would also provide insight into the exon pattern of the slow TnT gene.

Using the information obtained from the sequence data of TnT 1S, it will be possible to generate fragments corresponding to important functional domains. For example, an N-terminal fragment arising from peptide bond cleavage at methionine 64 or 93 could be purified to study the effects of a highly acidic N-terminal domain. In addition, a fragment spanning residues 94-174 could be purified and used in functional assays. This fragment is of interest since it corresponds to residue 71-151 of rabbit skeletal TnT which have been implicated in TM binding. It may also be possible to express fragments of TnT 1S in a bacterial host cell. The advantage here is that segments of TnT 1S of interest can be studied even if appropriate cleavage sites are lacking. Fragments generated by chemical cleavage or bacterial expression may be more soluble than the intact protein.

A number of attempts to obtain the molecular weight, by mass spectrometry, of TnT 1S were unsuccesful. The reason for this is unknown. It could possibly be due to the low solubility of this isoform. In the future, attempts should be made to identify alternative solutions that may enhance solubility of TnT 1S while at the same time being compatible with the mass spectrometer. Alternatively, it may be possible to determine the mass of 1S by analysis of whole Tn.

Further experiments are needed to improve the preparation of whole

Tn. This would permit studies involving whole Tn to be performed circumventing the lengthy process of reconstituting Tn from the individual subunits. A second major advantage is that the solubility of the TnTs, especially the highly insoluble TnT 1S and TnT 1F, is greatly increased when part of the Tn complex. If purification of whole Tn containing the different isoforms of TnT is not possible, Tn can be reconstituted by mixing stoichiometric amounts of TnI, TnC and TnT in the presence of urea followed by dialysis.

From the preliminary work performed on the Tn subunits of slow and fast muscle it is now possible to begin biochemical assays to determine the biochemical basis of differences in slow muscle and fast muscle physiology. The use of fish muscle provides a unique opportunity to study slow muscle specific proteins. The advantages include a readily available supply of fish, a homogeneous supply of slow tissue, and a relatively simple isoform pattern.

It is predicted that Tn containing different TnT isoforms or TnT fragments will exhibit distinct functional properties. A number of studies designed to investigate this are possible, including Mg^{2+}ATPase assays, Ca^{2+} binding studies and analysis of the TnT-TM interaction. Myosin Mg^{2+}ATPase assays involve reconstitution of the major myofibrillar proteins and the radioactive or colormetric quantitation of released phosphate. In this way, all of the TnT isoforms could be analyzed.

A second functional property of the thin filament assembly that may be easily studied is the Ca^{2+} binding ability of the reconstituted thin filament. This may be carried out using equilibrium dialysis (Potter and Gergely, 1975) or assays that make use of fluorescent probes (Johnson and Smillie, 1978).

To completely characterize differences among TnT isoforms an investigation of the interaction of TnT with TM is necessary. This can be studied by affinity chromatography (Pearlstone and Smillie, 1977; 1978; Heeley, 1994), viscometry (Heeley, 1989; 1994) and again by using fluorescent probes (Lehrer, 1978) .

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