ISOLATION AND CHARACTERIZATION OF STRETCHIN-MYOSIN LIGHT CHAIN KINASE MUTANTS IN DROSOPHILA MELANOGASTER

DISSERTATION

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By

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Muscle function depends upon the molecular interaction of myosin and actin. This interaction and the function of each molecule are tightly regulated and have been extensively studied. In *Drosophila*, the indirect flight musculature (IFM) is a powerful model to study muscle structure and function as these muscles are dispensable for life under laboratory conditions. Furthermore, disruption of these muscles leads to a flightless behavior.

Flies with mutations in the muscle regulatory light chain (MLC2) that cannot be phosphorylated at the conserved Myosin light chain kinase (Mlck) target sites are flightless, but the IFM is normal. Flight impairment is due to an altered stretch activation response, thus phosphorylation of MLC2 at the Mlck target sites is important for flight.

In *Drosophila*, the *Stretchin-Mlck* (Strn-Mlck) gene encodes several Mlck-like isoforms with kinase activity as well as other isoforms lacking this domain. Analysis of the gene in this work has shown that it is expressed in both muscle and nonmuscle cell types and that some isoforms show tissue specific expression patterns.

In order to understand what role Strn-Mlck plays in MLC2 regulation, mutants were isolated. Three new mutants were identified and the previously described *curved* mutant was shown to be an allele. Phenotypic and molecular analysis of the mutants provided considerable insight. Strikingly, *Strn-Mlck* mutants lacking kinase activity are
viable. However, the mutants show a recessive flightless phenotype and defects in wing position. Electron micrographs demonstrated the flight musculature is intact, therefore, the flightless phenotype is most likely due to the wing position defect.

As mentioned, expression analysis showed Strn-Mlck is expressed in nonmuscle cells. The myosin nonmuscle regulatory light chain is encoded by *spaghetti squash* (*sqh*). Mutations in nonmuscle RLC at the conserved Mlck target sites are lethal. Finding that mutants lacking Strn-Mlck kinase activity are viable suggests other Mlcks exist in flies and function redundantly. Preliminary genetic studies have linked Strn-Mlck with another Mlck, CG1776 (also known as *Mlk-2*). This Mlck-like kinase has been implicated in morphogenesis in nonmuscle tissue culture cells. In the future, it will be important to analyze these two Mlcks in depth at the genetic level. Double mutants may reveal functions for these enzymes that are masked in the single mutants analyzed to date.
Dedicated to my family
and the *Angels* in my life

In memory of the kind stranger
who, through organ donation,
gave me the Gift of Life
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CHAPTER 1

INTRODUCTION

Muscle can be viewed as a biological force-producing machine, where different proteins interact to produce movement. In order to understand how muscles can produce such force, it is necessary to identify all the proteins involved and elucidate the role they play in muscle contraction. Research on muscle has progressed quickly in the last few decades and many more of the proteins involved in muscle contraction have been identified. Some are very well characterized, while others remain to be studied further.

Our laboratory has previously contributed to this field. Specifically, we have used the indirect flight muscles of *Drosophila melanogaster* to analyze the role of tropomyosin (Kreuz et al., 1996), and the role of phosphorylation of the myosin regulatory light chain (MLC2) by *Stretchin-Myosin light chain kinase (Strn-Mlck)* (Tohtong et al., 1997; Tohtong et al., 1995) in muscle contraction. Our lab showed that MLC2 phosphorylation was required for full stretch activation of the muscle and that in the absence of MLC2 phosphorylation at the conserved Strn-Mlck phosphorylation sites, flight is dramatically affected (Tohtong et al., 1995). The focus of my research is to understand the regulatory and structural role of *Strn-Mlck* by a genetic and molecular biology approach. To this
end I have isolated and characterized the first mutants in a multicellular organism for the
Strn-Mlck gene.

In this chapter I present a review of the current knowledge about genes that
regulate muscle structure and function to facilitate an understanding of this research and
put it into context. I will first introduce a broad view of muscle structure and function
including regulation of muscle contraction and the role myosin light chain kinase plays in
this function. I will then review Drosophila musculature and its use as a model system to
study muscle. Lastly, I will give a summary of what is currently known about the Strn-
Mlck gene in Drosophila.

Muscle types

Muscle is one of the most important and abundant tissues in animals. Movement,
whether voluntary (walking, flying, etc.) or involuntary (pumping of the heart, digestive
tract movements, etc.), requires specific cell types to convert chemical energy into
mechanical work. In vertebrates three types of muscle can be found: skeletal, cardiac and
smooth muscle.

**Skeletal muscle:** This muscle type is responsible for the voluntary movements of
the body. Skeletal muscle is so named because it is the muscle that attaches to the
skeleton. It is also called striated muscle, because when viewed under the microscope,
the muscle fibers give an appearance of cross banding caused by alternating light and
dark bands, giving it a striped or striated appearance. Striated muscles are composed of
bundles of multinucleated cells called myofibers. The major elements in myofibers are
the myofibrils, which are the contractile elements of the muscle cells and are composed
of contractile proteins, primarily myosin and actin. Each myofibril has a cylindrical
shape and can be several centimeters long running parallel to the longitudinal axis of the
myofiber. This striped (striated) appearance of the muscle is caused by alternating dark
A bands and light I bands. The A bands and I bands are so called because when the
myofibrils are viewed using polarized light, the anisotropic band (A band) is denser and
appears more bright, while the isotropic (I band) is less dense and appears dark.

With higher resolution and the development of the electron microscope, the
studies of muscle structure improved dramatically. The banding appearance of striated
muscle is distinct (Figure 1.1). Further, the light I bands are seen to be bisected by an
electron dense material called the Z disk (or Z line). Each Z disk demarcates the ends of
the functional units of the myofibril, called the sarcomere. These sarcomere units are
repeated over the entire length of the myofibril. Adjacent to the light I band of a
sarcomere is the darker A band. In the center of each A band there is a less dense band
called the H zone. The H zone is also bisected by a slightly more electron dense region
called the M line.

Early studies of the muscle ultrastructure indicated that the striated appearance of
the sarcomeres was due to the arrangement of two sets of filaments (called myofilaments)
in the myofibrils (Huxley, 2000a; Huxley, 2000b; Huxley & Niedergerke, 1954b; Huxley
& Hanson, 1954; Huxley, 2000c). These myofilaments lie parallel to each other and
overlap in some regions of the sarcomere. One of these myofilaments, the thin filament,
extends from the Z disk toward the center of the sarcomere, passing through the I band
and A band, but not the H zone. The other set of filaments, the thick filaments, are found
Figure 1.1 Skeletal muscle structure. A. Electron micrograph showing the banding appearance of skeletal (striated) muscle. B. Diagram of a sarcomere showing the dark A bands and light I bands corresponding to the electron micrograph in (A). The banding appearance is due to the overlapping arrangement of the thin (actin) and thick (myosin) filaments.
Figure 1.1 Skeletal muscle structure.
in the A band, but do not extend into the I band nor the Z discs. Therefore the I bands are composed entirely of thin filaments, while the A bands correspond to regions of overlap of these two myofilaments. The H zones are composed entirely of thick filaments. Examination of cross sections through the A bands where the thick and thin filaments overlap show that the filaments are arranged in a hexagonal pattern with six thin filaments surrounding one thick filament.

**Cardiac muscle:** Cardiac muscle appears to have features resembling both skeletal and smooth muscle (see below). Cardiac muscle forms the contractile walls of the heart and is responsible for pumping blood as the heart beats. Similar to skeletal muscle, cardiac muscle shows a striation pattern of the muscle fibers due to the arrangement of the thin and thick filaments. However, unlike skeletal muscle, the cells have a single nucleus and the muscle fibers are branched so that they often touch or run into adjacent fibers. This is important for the function of the heart as it allows for the synchronization of contractions. Because of this branching of the cardiac muscle cells, the cardiac muscle may not appear as striated as skeletal muscle. Similar to smooth muscle, cardiac muscle is under involuntary control.

**Smooth muscle:** Smooth muscle is responsible for involuntary movements like gut peristalsis, and thus it is found in the walls of the digestive tract and other internal organs. Smooth muscle is so called because it lacks striations and thus it is considered to be the most primitive muscle structurally, resembling most closely nonmuscle cells. The cells have a single nucleus and are elongated and spindle shaped. Smooth muscle does
contain thin and thick filaments, however their arrangement in the cell is not like the ordered structures of the skeletal muscle, forming into myofibrils. Instead, the filaments are loosely arranged into a contractile apparatus, which runs roughly parallel to the long axis of the cells.

Myofilament composition and structure

The banding pattern of striated muscle visualized under the light microscope has been observed for centuries (Needham, 1971). However, it took until the middle of the 20th century to understand that the banding pattern reflected a specific arrangement of contractile filaments in the myofiber. Hansen and Huxley showed in 1953 that myosin was exclusively located in the A bands (Huxley, 2000a; Huxley, 2000b; Huxley, 2000c). When they used a solution that selectively eliminated myosin from the myofibers, they found that the A bands disappeared from the sarcomeres. Thus, the electron micrograph studies of that time helped elucidate the composition of the sarcomere. The I band is composed of thin filaments alone and the H zone composed of thick filaments alone while the more electron dense area or A band is composed of overlapping thick and thin filaments.

Thick filaments: The major component of thick filaments is myosin. The muscle protein myosin II is a hexameric molecule consisting of two heavy chains with molecular masses of ~200 kDa each, and two sets of light chains, with molecular masses of ~20 kDa each (Figure 1.2). One set of light chains, called the regulatory light chains (MLC2), is phosphorylated by Ca²⁺/Calmodulin-dependent MLCKs (myosin light chain

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Figure 1.2 Diagrammatic representation of the structural composition of the thick filament. A. Diagram of a myosin hexamer composed of two globular heads at the N-terminus and a coiled coil α-helical tail (rod region) at the c-terminus. Each myosin head has a pair of light chains attached. B. Representation of the structural organization of thick filament. The myosin molecules assemble in an antiparallel fashion starting at the center. The globular heads protrude from the filament and form crossbridges with the thin filaments. ELC, essential light chain; MLC2, regulatory light chain.
Figure 1.2 Diagrammatic representation of the structural composition of the thick filament.
kinases) and so are also referred to as phosphorylatable light chains. The other set of light chains are the essential light chains (ELC1, 3). The C terminus of the myosin heavy chains assemble into a coiled-coil $\alpha$-helical tail structure also called the myosin rod region, while the N terminal portions of the heavy chains form globular head regions (also called subfragment S1). One pair of light chains binds to each S1 globular head. The myosin molecules assemble into thick filaments in an antiparallel fashion starting in the middle of the filament (M line) and adding outwardly. The S1 globular head regions protrude from the surface of the thick filaments at regular intervals and form crossbridges to the thin filaments. The S1 fragments contain the actin binding domain and the catalytic domain for ATP hydrolysis and thus interact with the thin filaments to generate force and muscle contraction through a sliding filament mechanism.

**Thin filaments:** Thin filaments are attached at the Z line and run along the sarcomere through the I band and into the A band, where they interact with the thick filaments, but do not extend into the H zone. Thin filaments assemble from 3 major components: actin, tropomyosin and the troponin complex (Figure 1.3).

Actin molecules (globular actin or G actin) associate spontaneously to form a two-stranded helical structure called F actin, the backbone of the actin filament. They are polar structures as they assemble into the helical conformation in a specific orientation. Because of this polarity, actin filaments, which extend from the Z lines, do so in opposite directions. Thus, thin filaments in a sarcomere have opposite directionality. This polarity is important for the sliding mechanism of muscle contraction (see below).
Figure 1.3 Structural organization of the thin filament. Thin filaments assemble from three major components: actin, tropomyosin and troponin complex. Globular actin molecules associate into a two-stranded filament. Tropomyosin binds along the length of the actin molecule providing stability to the thin filament. The troponin complex consists of TnI, TnC and TnT and there is one troponin complex per tropomyosin molecule.
Tropomyosin (Tm) is a rod shaped molecule. It is a dimer of two α-helical chains configured into a coiled-coil similar to the tail region of myosin II. Each tropomyosin binds along the length of the actin filament fitting into the grooves of the actin filament and spanning 7 actin monomers, providing additional stability and stiffness to the thin filament. In addition to actin, tropomyosin also binds troponin in order to regulate muscle contraction in vertebrate skeletal muscle.

Troponin consists of a complex of 3 polypeptides. Troponin C (TnC) so called because it has Ca$^{2+}$ binding activity providing skeletal and cardiac muscle with Ca$^{2+}$ sensitivity for muscle contraction. TnC has a dumbbell shape. Troponin I (TnI) or inhibitory, so called because it inhibits the actomyosin ATPase. TnI is the component that keeps the troponin complex together as it binds TnC and TnT as well as actin. Troponin T (TnT) is so called because of its association with tropomyosin, it is considered the actual structural ‘glue’ for the Tn-Tm interaction. There is one troponin complex associated with each tropomyosin molecule in the actin filament.

**Sliding filament mechanism**

The early studies of muscle ultrastructure (Hanson & Huxley, 1953; Huxley, 2000a; Huxley, 2000b; Huxley & Niedergerke, 1954a; Huxley & Niedergerke, 1954b; Huxley & Hanson, 1954; Huxley, 1953; Huxley, 2000c) elucidated the composition of the sarcomeres. The banding pattern observed under the microscope reflected a specific arrangement of the thin and thick filaments in the muscle fibers. The two sets of myofilaments run parallel to each other and overlap in distinct regions of the sarcomere.
Figure 1.4. The sliding filament mechanism of muscle contraction. Diagram representing a sarcomere in the relaxed (top) and contracted (bottom) states. The overlapping thick and thin filaments slide past each other keeping the length of the filament constant but increasing the overlap between them. The sliding is coordinated by the myosin head crossbridges attaching and detaching from the thin filaments in a cyclical fashion.
Figure 1.4. The sliding filament mechanism of muscle contraction.
The spatial arrangement of the two sets of myofilaments was the element for the development of the sliding filament mechanism (Figure 1.4) to explain muscle contraction (Huxley, 2000a; Huxley, 2000b; Huxley & Niedergerke, 1954b; Huxley & Hanson, 1954; Huxley, 2000c). The sliding filament model explains muscle contraction as the movement of the thick and thin filaments past each other in the region of overlap in the sarcomere. These observations stem from data that showed that during contraction there is a shortening of the I bands and Z zone while the length of the A bands as well as the distance between the Z lines and the edge of the H zone remained constant. This shows that during contraction the length of the thick and thin filaments remained constant and the sarcomeres were shortened by the thick and thin filaments sliding past each other increasing the overlap between them as they do so. This sliding is coordinated by the extensions or crossbridges protruding from the thick filaments that attach and detach from the thin filaments in a cyclical fashion, and is driven by the hydrolysis of ATP.

More recent crystallography studies have elucidated the atomic structure of the myosin head (S1 fragment, Figure 1.5 A) and actin. Rayment et al. proposed a model for muscle contraction that combined the sliding filament model with the crystallography results (Rayment et al., 1993a; Rayment et al., 1993b). From the structure of myosin S1, Rayment et al. suggest there is a hinge or lever-arm located between the catalytic domain (which attaches to actin) and the light chain binding domain (the long α-helix to which the light chains are attached). The lever-arm mechanism proposes there is a tilting or change in angle of this lever-arm relative to the catalytic domain which pulls on the
Figure 1.5 Space filling model of the myosin motor (S1 fragment) and representation of the myosin motor cycle, lever arm model. A. Space filling model of the S1 fragment of chicken smooth muscle. The myosin heavy chain is composed of three domains colored in red, green and blue. The regulatory light chain (RLC) is colored purple, and the essential light chain (ELC) is in yellow. The lever-arm region (bold line) undergoes a conformational change that causes the myosin head to move along the actin filament. The molecule is oriented with the actin binding site to the left and the actin filament would run perpendicular to the myosin head. Adapted from (Rayment et al., 1993a; Rayment et al., 1993b) B. The crossbridge cycle, lever arm model as elucidated by the Rayment group. ATP hydrolysis causes a conformational change of the myosin molecule that displaces the myosin head along the actin filament, the so-called lever-arm model. Release of Pi initiates the power stroke or the return of the myosin molecule to its original state (see text for details).
Figure 1.5  Space filling model of the myosin motor (S1 fragment) and representation of the myosin motor cycle, lever arm model.
myosin filament, thus explaining how the crossbridges produce force and make the filaments slide past each other.

The structure suggests that the sliding movement of thick and thin filaments is generated by the mechanism illustrated in Figure 1.5 B. At the beginning of the cycle, there is no nucleotide bound and the myosin head is tightly bound to the actin filament and the cleft in the catalytic site is closed (Step 1). ATP binding to the catalytic site opens the cleft disrupting the interaction between actin and myosin but allowing a weak binding state (Step 2). The cleft closes around the ATP molecule and this causes a change of configuration of the myosin head. This change in the myosin head or lever arm causes a change in the curvature of the molecule such that the C terminus of the myosin head moves along the filament relative to the actin binding site. All this is driven by ATP hydrolysis, but the ADP and Pi remain bound to the myosin head, and stronger binding to actin produces cleft closing (Step 3). Binding of the myosin head to a new site on the actin filament and cleft closure releases the Pi and this triggers the power stroke (Step 4). The power stroke is the force generating change in shape that restores the myosin head to its original conformation. This opens up the active site pocket and releases the ADP (Step 5) and the myosin head is again bound tightly to the actin filament returning to its original state.

**Role of myosin regulatory light chain phosphorylation**

Phosphorylation of myosin regulatory light chain (RLC) by myosin light chain kinase (MLCK) has been shown play a role in the contraction of muscle cells and certain processes in nonmuscle cells, however the actual role that the MLCK mediated
prosphorylation plays varies depending on the cell type. In vertebrates, phosphorylation of RLC is sufficient to initiate contraction in smooth muscle cells and regulate various processes in nonmuscle cells while in skeletal muscle it plays more of a modulatory role (Figure 1.6). However, there is a significant difference between vertebrate skeletal muscle and the striated muscle of flies. Specifically, phosphorylation of RLC in the indirect flight muscle (IFM) of the fly has been shown to be critical for the stretch activation response required for flight (Tohtong et al., 1995), demonstrating that while in vertebrates phophorylation of RLC only slightly enhances the activation initiated by calcium release, in fly IFM, full stretch activation and hence, flight ability, require RLC phosphorylation.

**Myosin Light Chain Kinase (MLCK) in muscle contraction**

Myosin light chain kinases (MLCKs) are Ca\(^{2+}\)/ Calmodulin (Ca\(^{2+}\)/ CaM) dependent serine/threonine kinases responsible for the phosphorylation of myosin RLC by catalyzing the transfer of a phosphate group from MgATP to a specific site of myosin RLC (Gallagher et al., 1997; Kamm & Stull, 2001). Calmodulin (CaM) is a ubiquitous Ca\(^{2+}\) binding protein involved in the regulation of many processes (Means, 2000). MLCK (Figure 1.7) has a kinase domain consisting of a catalytic domain followed by a regulatory domain (with a pseudosubstrate and CaM binding sequences) that is implicated in the regulation of the phosphorylation of the substrate via a relief of autoinhibition. The pseudosubstrate contains residues that are similar to the sequences of the substrate (RLC). These autoinhibitory sequences bind to the active site thus inhibiting availability of the active site to the substrate. When Ca\(^{2+}\)/ CaM is present, the
Figure 1.6 Role of Myosin Regulatory Light Chain phosphorylation.

Phosphorylation of myosin regulatory light chain (RLC) by myosin light chain kinase (MLCK) has been implicated in muscle contraction and several nonmuscle processes. In smooth muscle, MLCK mediated phosphorylation of RLC is required for contraction. The same mechanism drives several cellular processes in nonmuscle cells. In striated muscle, phosphorylation of RLC is not the driving force behind muscle contraction as it only serves a modulatory role. Figure adapted from (Kamm & Stull, 2001).
Figure 1.6  Role of Myosin Regulatory Light Chain phosphorylation.
Figure 1.7 A schematic representation of various Myosin light chain kinases (MLCKs) and related proteins. Several motifs are found in MLCK and other related kinases. The *Drosophila* MLCK is encoded by *Strn-Mlck* and shares many structural elements with the vertebrate skeletal and smooth muscle MLCKs and the giant titins. Closely related kinases from *C. elegans, Aplysia* and *Dictyostelium* are shown. See text for details. Figure adapted from (Kamm & Stull, 2001).
Figure 1.7  A schematic representation of various Myosin light chain kinases (MLCKs) and related proteins.
complex binds to the CaM binding sequences (which overlap the pseudosubstrate sequences) activating the enzyme and releasing the inhibition thus making the active site available for binding of RLC and its phosphorylation (Kamm & Stull, 2001).

In vertebrates two distinct genes code for the skeletal and the smooth muscle/nonmuscle (sm/nm) MLCKs. The skeletal muscle MLCK contains a catalytic domain with the regulatory region but no other motifs are found (Figure 1.7). In contrast, the smooth muscle/nonmuscle MLCK has several repeated motifs along with the kinase domain. The sm/nm MLCK has Fibronectin type III (Fn3) motifs and immunoglobulin C-2 (Ig) motifs. These repeated motifs are also found in other muscle proteins with kinase activity like vertebrate titins and invertebrate twitchin/projectin. These motifs are thought to be important for protein-protein interactions. The sm/nm MLCK also has the PEVK motif, a repeated motif rich in proline (P), glutamic acid (E), valine (V) and lysine (K), and is implicated in the elasticity of the sarcomere (Champagne et al., 2000; Fyrberg et al., 1992; Labeit et al., 1992; Labeit et al., 1990). There are also actin binding domains. Three different isoforms are encoded by this gene. A short and a long form differ in the number of Ig domains and actin binding domains. The third isoform, telokin, is a small protein consisting of the C-terminal Ig domain and is thought to be involved in binding of MLCK to myosin (Gallagher et al., 1997; Kamm & Stull, 2001).

Figure 1.7 also shows other kinases with homology to the vertebrate MLCKs that share many of the motifs found in them. Vertebrate titins are giant proteins implicated in muscle elasticity. In C. elegans and Drosophila the titin homologues are called twitchin and projectin respectively. The Dictyostelium MLCK differs from the other MLCKs in that it is Cam independent (Kamm & Stull, 2001). In Drosophila, MLCK is encoded by
the *Stretchin-Mlick* gene (Champagne *et al*., 2000; Kojima *et al*., 1996; Tohtong *et al*., 1997).

**Drosophila musculature**

All insect muscle is striated and therefore has basic similarities to vertebrate striated muscle. However three general types of skeletal muscle are recognized. The first type is the supercontractile muscle of the larva and the adult. It is present in the gut and viscera, including the heart and the larval intersegmental (or body wall) muscles. The dominant feature of these muscles is the ability to contract to 80% of its resting length, thus the term ‘supercontractile’. Modifications for this supercontraction can be observed in the structure of the Z-lines, which are perforated, allowing the thin and thick filaments of adjacent sarcomeres to penetrate.

The second type of muscle in *Drosophila* is the tubular muscle of the adult. Tubular muscle makes up the majority of the adult muscle and includes the leg muscles; jump muscle (the tergal depressor of the trochanter or TDT muscle, the largest tubular muscle) and direct flight muscles (function to adjust the position of the wings). Ultrastructural analysis shows that the myofibrils are rectangular (have a tube-shape appearance) and closely packed. The myofibrils exhibit an obvious striated pattern because the Z-lines are precisely aligned in registry with adjacent myofibrils. Overall, the myofilaments are less organized with 8-12 thin filaments surrounding each thick filament.
The third type of muscle in *Drosophila* is the fibrillar indirect muscle of the adult. This is characteristic of the indirect flight muscles (IFM), which constitute 80% of the mass of the adult thorax. The ‘fibrilar’ nature of these muscles is due to the large size of these fibers and because the fibers are easy to separate into its component myofibrils. Each myofiber contains thousands of myofibrils. The highly organized cylindrical myofibrils contain precisely arranged myofilaments consisting of 6 thin filaments surrounding each thick filament, and the filaments are arranged in a highly ordered hexagonal array.

The IFM are the muscles that provide the power for flight by moving the wings indirectly by deformation of the thoracic exoskeleton. They are called ‘indirect’ flight muscles because they are attached to the cuticle and thus are only indirectly connected to the wing. The fibrillar muscle is also termed ‘asynchronous’ flight muscle because the frequency of contraction is usually independent or in excess of the frequency of motor-nerve input. This feature is necessary for insect flight muscle to achieve the high wing beat frequencies needed to attain lift and power flight (in *Drosophila* the wings need to beat at a frequency of 240 times/s to achieve flight (Maughan & Vigoreaux, 1999)). In order to achieve full activation, stretch activation is also required.

The IFM consists of six pairs of dorsal longitudinal muscles (DLM), which depress the wings by longitudinal arching of the thorax, and seven pairs of dorsoventral muscles (DVM), which elevate the wings. Just before flight, the fly jumps. The jump, caused by the contraction of the TDT, stretches the DLM. The DLM senses this stretch, which causes a delayed rise in tension (the so called stretch activation response) and the muscle contracts. This in turn activates the DVM in the same fashion. As one set of
muscle elevates the wing and the other depress the wing, this oscillatory movement of the thorax produces the high power necessary for flight.

**Drosophila IFM as a model system to study muscle function**

For over half a century *Drosophila* indirect flight muscle (IFM) has been successfully used to study muscle function. These muscles are only required for flight, thus a mutation affecting the IFM can be isolated and maintained in the lab without affecting viability. The IFM are large muscles taking up most of the mass of the fly’s thorax and the myofibrils are highly organized. This facilitates structural studies of the contractile proteins. *Drosophila* has facilitated the study of contractile proteins (Bernstein et al., 1993), and numerous mutations in these genes have been characterized allowing analysis of the function of muscle proteins. The major myofibrillar proteins identified in *Drosophila* IFM are shown in (Figure 1.8). There are many that are conserved proteins throughout species (myosin, actin), while others are specific to the IFM of insects (flightin).

**Drosophila contractile proteins**

**Myosin heavy chain**

A single gene located in chromosome position 36 B1-2 encodes the *Drosophila* muscle myosin heavy chain gene (MHC) (Mogami et al., 1986). The gene contains 29 exons, some of which are alternatively spliced to produce multiple muscle specific MHC transcripts (Bernstein et al., 1983; Bernstein et al., 1986; Collier et al., 1990; Kronert et al., 1991; Rozek & Davidson, 1983; Wassenberg et al., 1987). To date, 15 MHC protein
Figure 1.8 Major myofibrillar proteins identified in *Drosophila* IFM. The overall structure of the sarcomere is similar to the skeletal muscle of other species. However, there are some modifications of proteins that are specific to the fly system, including extensions on conserved proteins (regulatory light chain, MLC2) or proteins only found in *Drosophila* IFM (flightin). See text for details. Figure taken and adapted from (Maughan & Vigoreaux, 1999).
Figure 1.8  Major myofibrillar proteins identified in *Drosophila* IFM.
isoforms are known to be present in the fly during muscle development. At least one of these isoforms is IFM specific, while other muscle types accumulate various other isoforms. There is also a unique isoform of MHC, the myosin rod protein (MRP) that is produced from the MHC gene through the use of an alternative transcriptional promoter. The MRP has a unique N-terminal extension coupled to the MHC rod domain and is a myofilament component in some muscle types as it co-localizes with myosin to the thick filaments (Standiford et al., 1997).

There is also a single gene (zipper) located in chromosome position 60 E9 encoding the nonmuscle myosin heavy chain (Ketchum et al., 1990; Kiehart & Feghali, 1986; Kiehart et al., 1989). The transcription unit spans over 20 kb and, like the sarcomeric MHC gene, encodes multiple transcripts through alternative splicing (Ketchum et al., 1990; Mansfield et al., 1996). Nonmuscle MHC has been implicated in many processes including oogenesis, cytokinesis during embryogenesis, morphogenesis and adult metamorphosis (Edwards & Kiehart, 1996; Halsell et al., 2000; Young et al., 1991; Young et al., 1993).

**Myosin light chains**

The myosin molecule consists of a myosin head encoded by the MHC gene and 2 classes of myosin light chains attached to each myosin head: a myosin regulatory light chain (RLC, MLC2) and an essential (alkali) light chain (ELC). In *Drosophila*, as is the case with the MHC gene, both MLC2 and ELC have two genes each that encode the sarcomeric and the nonmuscle proteins.
The sarcomeric RLC is encoded by the *Myosin light chain 2 (MLC2)* gene located at position 99E1-3, while the nonmuscle RLC protein is the product of the *spaghetti-squash (sqh)* gene located at position 5D6 (Bernstein et al., 1993; Yamashita et al., 2000).

The *Mlc2* gene is composed of three exons encompassing 2 kb of DNA that encode two developmentally regulated transcripts of 1.1 and 1.4 kb through the use of two different polyadenylation sites, producing a single polypeptide of 20 kDa. In vertebrates, phosphorylation of RLC is sufficient to initiate contraction in smooth muscles and nonmuscle cells, while in skeletal muscle it plays more of a modulatory role. Even though the *Drosophila* MLC2 transcript is expressed in all muscle types, mutation of the MLCK target sites to alanines affects only the IFM (Tohtong et al., 1995). Specifically, phosphorylation of MLC2 in the IFM has been shown to be critical for the stretch activation response required for flight (Tohtong et al., 1995). This demonstrates a significant difference between vertebrate skeletal muscle and indirect flight muscle of flies. While in vertebrates phosphorylation of RLC only slightly enhances the activation initiated by calcium release, in flies IFM stretch activation and hence flight ability require RLC phosphorylation. The *Drosophila* MLC2 also has an N terminal extension of 46 amino acids that precedes the conserved myosin light chain kinase (MLCK) phosphorylation sites. In *Drosophila*, the conserved MLCK target sites are located further into the molecule, at Ser 66 and Ser 67 (corresponding to the vertebrate Thr18 and Ser 19), due to the presence of the N terminal extension in MLC2. This N terminal extension is not found in the vertebrate MLC2, but has some homology to an extension present in vertebrate ELCs expressed in skeletal and cardiac muscles (Moore et al., 2000).
The N terminal extension is thought to interact with actin and function as a passive link or weak crossbridge promoting the formation of stronger crossbridges by the myosin head during stretch activated oscillatory work and power output (Irving et al., 2001; Moore et al., 2000).

In *Drosophila*, two genes encode the ELC. *Myosin light chain 1 (Mlc1)* encodes the sarcomeric form and is located at position 98A6 of the chromosome, while *Myosin light chain-cytoplasmic (Mlc-c)* encodes the nonmuscle form located at position 5A6 of the polytene chromosome. The gene for the muscle specific form is composed of 6 exons that are alternative spliced to produce two transcript classes that differ in their pattern of expression. One transcript is IFM specific, while the other is expressed in all other muscle types (Bernstein et al., 1993; Yamashita et al., 2000).

**Actin**

*Drosophila* actins are encoded by a gene family consisting of six genes, each expressing a different isoform. Two of the genes, *Act5C* and *Act42A* encode cytoplasmic actins as they are expressed ubiquitously throughout development. The four remaining isoforms encoded by *Act57B, Act79B, Act87E* and *Act88F* are thought to be differentially expressed only in muscle. All actins are modified after translation by undergoing multiple acetylations, cleavage and methylation of specific amino acids. In addition, ACT88F is ubiquitinated to produce the protein arthrin (Ball et al., 1987). Arthrin seems to be found only in the IFM and ubiquitination of actin does not seem to occur in nonfibrillar muscle, thus this appears to be a tissue specific event. Since arthrin is only
found in the IFM it is believed to provide some of the unique properties to this tissue as it may be involved in structural or stretch activation functions.

Until recently, the Act88F isoform was recognized as the only IFM specific isoform as homozygous flies for a null mutation of Act88F (Act88FKM88), are flightless and viable and they lack actin and thin filaments in the IFM (Bernstein et al., 1993; Fyrberg et al., 1983). However Nongthomba et al. (Nongthomba et al., 2001), have shown Act88F gene expression in other muscle types (leg, uterine and abdominal muscles) and nonmuscle cell types (wing blade, bristle and epidermal cells), although at much lower levels. The defects seen in the non-IFM muscle types of the Act88FKM88 mutant are mild suggesting other actin isoforms are also expressed in these muscles. The expression of this isoform in nonmuscle cell (wing and bristles) suggests that these apparent muscle isoforms may also play a role in nonmuscle cells, however, what this function might be is still unanswered as the mutant flies show no phenotype associated with the wings or bristles.

**Tropomyosin**

Two different genes encode multiple Drosophila tropomyosin isoforms, TmI and TmII. Both genes are located adjacent to each other at position 88F of the third chromosome (Bernstein et al., 1993; Vigoreaux, 2001). Alternative splicing of transcripts occurs in both genes to produce numerous protein isoforms with tissue specific expression (Bernstein et al., 1993; Kreuz et al., 1996).

The TmI gene produces two 284 amino acid protein isoforms through alternative splicing of exon 4. The two isoforms, Scm-TmI and Ifm-TmI, differ only in their C
terminal 27 amino acids. The Scm-TmI isoform is expressed in larval, adult head and abdominal muscles while the Ifm-TmI isoform (which include the C terminal 27 amino acids) is expressed in IFM and jump muscle (Kreuz et al., 1996).

The TmII gene is comprised of 17 exons and produces 4 transcripts through alternative splicing and the use of different promoters. Three transcripts are muscle specific and one is nonmuscle specific. Two of the muscle specific isoforms are also IFM specific and are termed Troponin-H (heavy troponin): Troponin-H 33 (TnH-33) and Troponin-H 34 (TnH-34). The two TnH isoforms are naturally occurring fusion proteins in which the 27 C terminal amino acids of the muscle specific TmII isoform are replaced with a proline rich 250 amino acids extension (Clayton et al., 1998). Since Tn-H is an IFM specific component of the sarcomere and the IFM is regulated by stretch activation, it has been proposed that Tn-H may play a role in stretch activation regulation by providing a link between thin and thick filaments. However, Kreuz et al. (1996) have shown that Tn-H heterozygous mutants have normal muscle structure and only a slight reduction in power output that does not prevent flight. It is expected that a 50% reduction in the amount of a stretch activation component would elicit a more dramatic effect, but this reduced effect may be the consequence of other factors contributing to the stretch response. One possibility for such a link could be MLC-2 (see above and Tohtong et al., 1995). Clayton et al. (1998) have also shown an interaction between Tn-H and glutathione S-transferarse-2 (GST-2). GST-2 is another myofibrillar protein found only in the IFM (see below), and it has been shown to be associated with the thin filaments and possibly thick filaments as well and both are possibly positioned very close to myosin cross bridges (Clayton et al., 1998).
Troponin

All the components of vertebrate troponin have been identified in *Drosophila*. In addition, *Drosophila* has a fourth element, heavy troponin (TnH, see tropomyosin section). TroponinT (TnT) binds tropomyosin. The *tnt* gene is located at position 12A3 on the X chromosome and is composed of 11 exons producing four transcripts through alternative splicing (Benoist et al., 1998; Bernstein et al., 1993). The smallest transcript is missing exons 3, 4 and 5, and is expressed in IFM and jump muscles. The transcript expressed in larval supercontractile muscles is missing exon 4 which is a microexon consisting of only 3 nucleotides (GAA) encoding for a lysine residue. All troponin T proteins from different organisms have a C terminal extension, however, the C terminal extension found in the *Drosophila tnt* gene is the longest and is highly acidic. It has been suggested that this domain is important for Ca$^{2+}$ binding to TnT (Benoist et al., 1998).

Troponin I is the actin binding and ATPase inhibitory component of the troponin complex. The *tni* gene localizes to region 16F of the X chromosome and consists of 10 exons encoding multiple transcripts through alternative splicing. Some *tni* transcripts are expressed throughout development while others are stage specific. *tni* transcripts are expressed in the embryo, and the adult tubular muscle and IFM, but expression of exon 3 is IFM specific (Bernstein et al., 1993).

Troponin C is the Ca$^{2+}$ binding component of the troponin complex. Troponin C protein is encoded by a gene family consisting of 4 genes (Qiu et al., 2003). Two of the *tnc* genes are closely related. One is located at position 47D of the second chromosome and encodes a larval muscle isoform, while the other is located at position 73F of the
third chromosome and encodes an isoform expressed in larvae and adults (primarily leg muscles). The third tnc gene encodes a more distantly related TnC isoform that is expressed in adults and the gene maps to position 41C of the second chromosome. A fourth gene (CG12408) was found by searching the *Drosophila* genome sequence (Flybase) and was predicted to code for a TnC isoform. Qiu *et al.* (2003) have shown these last two isoforms are expressed in the IFM.

**Glutathione S-transferase-2 (GST-2)**

Glutathione S-transferase-2 (GST-2) is a 35 kDa protein which cross reacts with antibodies to the IFM specific TnH-34 tropomyosin isoform. GST-2 is also IFM specific. The gene has been mapped to position 53F on the second chromosome. The gene produces a 35 kDa protein that is associated with the thin filaments, but interaction with myosin is also necessary to maintain proper sarcomere structure, thus GST-2 may also be associated with thick filaments (Clayton *et al.*, 1998). It has been shown that GST-2 in the thin filament is stabilized by interaction with TnH (Clayton *et al.*, 1998). It has been proposed that the N terminal region of GST-2 interacts with the C-terminal region of Tn-H and both proteins are positioned very close to myosin cross bridges (Clayton *et al.*, 1998).

**α-Actinin and other Z band proteins**

α-Actinin has been localized to the Z bands (Saide *et al.*, 1989) of the sarcomere and is one of the best characterized actin-crosslinking proteins first identified in vertebrate striated muscle. Both muscle and nonmuscle isoforms have been identified.
In *Drosophila*, a single gene at position 2C on the X chromosome codes for the α-Actinin isoforms (Dubreuil & Wang, 2000; Roulier *et al*., 1992; Vigoreaux, 2001). Muscle (two) and nonmuscle (one) isoforms are generated by use of multiple promoters and alternative splicing (Dubreuil & Wang, 2000; Roulier *et al*., 1992). Mutant analysis has revealed that α-Actinin mutants affecting nonmuscle isoforms produce no nonmuscle phenotype (Fyrberg *et al*., 1990; Roulier *et al*., 1992) and rescue experiments of a α-Actinin null mutant with a muscle isoform show that the nonmuscle isoform is not essential for viability (Dubreuil & Wang, 2000). These analyses suggest it is likely that α-Actinin function is redundant in nonmuscle cells (Dubreuil & Wang, 2000; Fyrberg *et al*., 1990; Roulier *et al*., 1992). Studies also reveal that muscle phenotypes are mild, and that α-Actinin may function to stabilize and anchor the thin filaments rather than actually organizing the thin filament array during myofibril formation, and suggests that the muscle function may also be redundant (Dubreuil & Wang, 2000; Fyrberg *et al*., 1990; Roulier *et al*., 1992).

Another component of the Z bands is Z (210), renamed zetalin, found in the IFM and a subset of jump muscles (Saide, 1997; Saide *et al*., 1989; Vigoreaux, 2001). Zetalin appears to be an abundant component of the Z bands as it is present in a 2:1 ration with α-actinin (Saide, 1997; Saide *et al*., 1989).

A third component if the Z bands is Z (400/600) and is thought to be Kettin (see below) (Saide *et al*., 1989; Vigoreaux, 2001). The distribution of this protein is different from α-actinin or zetalin. Instead, the Z (400/600) protein is located along the lateral borders of the Z bands and extend into the I bands maybe helping to anchor thin filaments (Saide *et al*., 1989).
**Paramyosin and miniparamyosin**

The thick filament associated proteins paramyosin (PM) and miniparamyosin (mPM) are unique to invertebrate muscles (Liu *et al.*, 2003; Maroto *et al.*, 1995). The PM gene is located at position 66D, and by using an alternative promoter (located in an intron 8 kb downstream of the paramyosin promoter) and alternative RNA splicing, the PM gene produces a transcript encoding miniparamyosin (Arredondo *et al.*, 2001; Becker *et al.*, 1992; Liu *et al.*, 2003; Maroto *et al.*, 1995). Miniparamyosin shares its C-terminal region with PM but has a unique N-terminal domain of 114 amino acid residues that lacks homology to other proteins (Becker *et al.*, 1992; Maroto *et al.*, 1995). PM is present in embryonic and adult muscles while mPM is only present in adult musculature. In the IFM, PM is found along the A band, while mPM localizes to the M line core and both ends of the thick filaments (Liu *et al.*, 2003; Maroto *et al.*, 1996). Paramyosin is thought to facilitate thick filament assembly, as mutants show a reduction in thick filament number and is located in the filament core: it assembles into a crystalline core and myosin is then arranged on the core surface (Arredondo *et al.*, 2001; Liu *et al.*, 2003).

**Flightin**

Like paramyosin and miniparamyosin, flightin is also a protein unique to invertebrates. Flightin is an IFM specific protein which localizes to the A band of the sarcomere and is therefore associated with thick filaments (Liu *et al.*, 2003; Vigoreaux *et al.*, 1993). One gene at position 76D encodes for Flightin, however posttranslational
modifications produce multiple phosphovariants (Vigoreaux et al., 1993). Analysis of IFM structure in Flightin null flies shows an increased thick filament length and subsequent disruption of the myofibrillar lattice upon flight, suggesting that Flightin is required for thick filament assembly during muscle development and for thick filament stability after initiation of the contraction machinery (Reedy et al., 2000). The Flightin null flies also show site-specific cleavage of myosin heavy chain showing that there is interaction between these two thick filament components (Reedy et al., 2000). The interaction of Flightin with MHC has been shown to occur at the myosin rod region (the C-terminal tail region of the myosin head), as Mhc rod mutants lack Flightin in the IFM (Ayer & Vigoreaux, 2003). Also binding assays have shown that flightin binds to a recombinant fragment of the myosin rod and this binding is abolished by a single amino acid substitution mapping to the myosin rod region of the myosin head (Ayer & Vigoreaux, 2003).

Titin

A family of giant proteins with homologues in both vertebrate and invertebrate muscles exist, whose molecular mass can be in the megadalton range. In vertebrate muscle the giant protein is called titin or connectin (Trinick, 1994), in C. elegans it is twitchin (Benian et al., 1989). In Drosophila three members of the titin family have been identified: projectin, D-Titin (and Kettin) and Stretchin-Mlck. Like vertebrate titin, all proteins have kinase activity (at the C-terminal region), multiple immunoglobin (Ig) domains, fibronectin type-III (Fn3) domains and PEVK rich regions (Ayme-Southgate et al., 1991; Ayme-Southgate et al., 1995; Benian et al., 1989; Champagne et al., 2000;
Fyrberg et al., 1992; Labeit et al., 1992; Labeit et al., 1990; Tohtong et al., 1997; Zhang et al., 2000). PEVK regions (domains containing high percentages of P, proline; E, glutamic acid; V, valine and K, lysine) were first identified in vertebrate titin and are thought to be responsible for the elasticity of the protein. Thus titin and other proteins belonging to the titin-like protein superfamily are believed to be members of the connecting filaments (connecting the Z-line to the myosin A band) and are thought to confer passive tension and elastic properties to the muscles upon stretch.

**Projectin:** This giant protein is encoded by the *bent* locus at position 102 C-D of the fourth chromosome. The longest projectin transcript is 27.3 kb encoding a protein of 9120 amino acid residues with a molecular mass approaching 1 MDa (Southgate & Ayme-Southgate, 2001). Projectin is a functional kinase protein and the kinase domain is located toward the C terminal region. It also contains 39 each of Ig and Fn3 domains and a PEVK rich region toward the N-terminus (Ayme-Southgate et al., 1991; Daley et al., 1998; Southgate & Ayme-Southgate, 2001). Projectin is found to localize to the I bands (it is anchored to the Z-disks and associates with myosin at the A band edge) in asynchronous (IFM) muscle and to the A band in synchronous muscle (Daley et al., 1998; Saide et al., 1989; Southgate & Ayme-Southgate, 2001; Vigoreaux et al., 1991).

**D-Titin and Kettin:** The *Drosophila* homologue of titin is encoded by the gene *sallimus*, located at position 62 C2-4 of the third chromosome. It is a large gene of over 70 kb that is predicted to encode a protein of 17,903 amino acid residues with a predicted molecular weight of 1.9-2 MDa (Machado & Andrew, 2000; Zhang et al., 2000). The sequence includes the already characterized muscle protein kettin. Kettin appears to be the N-terminal one third of the D-Titin arising from alternative splicing (540 kDa)
D-titin has 56 repeats of the Ig domain, 6 of the Fn3 domain, 2 large PEVK domains and is predicted to have kinase activity (predicted by sequence similarity with the human titin, (FlyBase, 1998)) (Kolmerer et al., 2000; Machado & Andrew, 2000; Zhang et al., 2000). Kettin consists of 35 Ig domains separated by spacers (Hakeda et al., 2000; Kolmerer et al., 2000; Machado & Andrew, 2000; Zhang et al., 2000). Kettin has been found to associate at the N-terminus with actin in Z-disks (Kulke et al., 2001b; van Straaten et al., 1999) and with myosin at the C-terminus (Kulke et al., 2001a). This correlates with evidence that kettin is localized in the I bands and that it extends from the Z disks to the edge of the A band (Kulke et al., 2001a; Kulke et al., 2001b; van Straaten et al., 1999). Based on sequence homology of D-Titin to vertebrate titins, it is predicted that the localization of the larger D-Titin isoforms may mimic the localization of the larger vertebrate titins. The evidence that Kettin extends from the Z disks to the A band is consistent with this prediction (Machado & Andrew, 2000).

**Drosophila Stretchin-Mlck**

A gene more closely related to vertebrate myosin light chain kinases (MLCKs) was cloned in our lab and simultaneously by another group. The gene, *Stretchin-Mlck* (*Strn-Mlck*), is localized to the second chromosome in the 52D subregion (Kojima et al., 1996; Tohtong et al., 1997). The gene produces multiple transcripts ranging in size from 3.6 kb to >18 kb, through alternative promoters and RNA splicing. The smaller transcripts (3.6 and 5.2 kb) have been characterized and they encode for proteins that are more closely related to the vertebrate MLCKs. They contain a kinase domain, a
regulatory domain, multiple Ig domains and an Fn3 domain. The catalytic and regulatory regions are well conserved with the corresponding domains in the MLCKs of other species. The chicken smooth muscle/nonmuscle MLCK is 55% similar in the catalytic region and 52% similar in the regulatory region to *Drosophila* Strn-Mlck. With the rabbit skeletal muscle MLCK, Strn-Mlck shares 52% identity in the catalytic and 27% identity in the regulatory domains. Various isoforms are produced by alternative splicing of exon 32 (removal of exon 32 alters the reading frame producing an isoform with different C-terminal residues) and the use of different donor splice sites (short and long forms of exon 32). Exon 32 codes for two thirds of the regulatory region and isoforms lacking exon 32 contain a regulatory domain devoid of a Ca$^{2+}$/CaM binding site, thus this isoform is presumed to lack Ca$^{2+}$/CaM regulation. The biological relevance of such an isoform or whether it is regulated in an alternative matter is not known (Champagne *et al.*, 2000; Kojima *et al.*, 1996; Tohtong *et al.*, 1997).

*In situ* hybridization to embryos with an RNA probe corresponding to the catalytic region revealed expression in early (nonmuscle expression in precellularizing embryos) and late embryos (various muscle cells) suggesting *Strn-Mlck* codes for both muscle and nonmuscle isoforms of the protein (Kojima *et al.*, 1996; Tohtong *et al.*, 1997). Reverse Transcription-PCR analysis supports this by showing *Strn-Mlck* expression in IFM and S2 tissue culture cells (Tohtong *et al.*, 1997). Also, analysis of protein expression in embryos using antibodies generated against two peptides corresponding to the two different C-termini (one recognizes isoforms lacking exon 32 and one recognizes isoforms including the long or short form of exon 32) shows muscle and nonmuscle expression (Tesic, 2001).
After the annotation of the *Drosophila* genome (Adams *et al.*, 2000), computer analysis and predictions of the genomic structure and conceptual proteins encoded by the gene were made (Champagne *et al.*, 2000). The gene spans over 38 kb of genomic sequence and includes 33 exons predicted to encode for transcripts as large as 25 kb potentially capable of producing a protein of ~1 Mda (Champagne *et al.*, 2000). The gene is predicted to produce 7 conceptual transcripts (Figure 1.9) encoding 7 conceptual proteins (Figure 1.10). The smaller transcripts (3.6 and 5.2 kb, encoding the small MLCKs) have been shown to be more closely related to the vertebrate MLCKs. The larger transcript (25 kb, Stretchin-Mlck) is more similar to titins. Another transcript, which does not overlap with the small MLCKs (7 kb, Kettin-like), is composed mainly of Ig motifs and is thus similar to *Drosophila* Kettin (see above). It is the similarities of the predicted conceptual proteins to titin and the titin-like protein superfamily that allowed the identification of Stretchin-Mlck as a member of this family of giant proteins (Champagne *et al.*, 2000).

Figure 1.9 shows a representation of the 33 exons composing the *Strn-Mlck* transcriptional unit, the alternative splicing sites, the exons containing polyadenylation sites (exons 15, 16 and 33), the putative promoters (exons 7, 20 24 and 26) and the 7 conceptual transcripts. Five transcripts end with a polyadenylation signal in exon 33: a ~25 kb transcript (exons 1-33), a ~13 kb transcript (exons 7-33), a ~5.2 kb transcript (exons 20-33), and two ~3.2 kb transcripts (exons 24-33 and 26-33). Two other transcripts end with polyadenylation signals in exons 15 or 16: a ~19 kb transcript (exons 1-15 or 16) and a ~7 kb transcript (exons 7-15 or 16). Due to the use of alternative promoters and polyadenylation signals, the gene can function as a single transcriptional
Figure 1.9 Schematic representation of seven conceptual transcripts of Strn-Mlck. (A) The gene comprises 33 exons (numbered boxes). (B) Alternative splicing occurs at the 5’, middle and 3’ ends of the gene. (C) There are 3 polyadenylation signals (pA). (D) 5 putative promoter signals give rise to seven conceptual transcripts (E). However, the presence of the 3.5 kb small-Mlck (exons 26-33) has not been confirmed by RT-PCR analysis with multiple different exon specific primers in all stages of development (Tesic, 2001). Figure adapted from (Champagne et al., 2000).
Figure 1.10  Schematic representation of conceptual translational products of Strn-Mlck. Diagram of the seven conceptual protein products of Strn-Mlck depicted with structural elements. Details of the structural motifs are given in the text. Figure adapted from (Champagne et al., 2000).
Figure 1.10  Schematic representation of conceptual translational products of Strn-Mlck.
unit that can produce 3 sets of transcripts, 2 of which do not overlap at all (one set represented by transcripts initiated at exons 1 or 7 and terminated at exons 15 or 16, and one set represented by transcripts initiated at exons 20, 24 or 26 and terminated at exon 33) and one that is a composite of the two (represented by transcripts initiated at exons 1 or 7 and ending at exon 33) (Champagne et al., 2000). Although the existence of a transcript initiating at exon 26 was suggested by a cDNA isolated in our lab (Champagne et al., 2000; Tohtong et al., 1997), the presence of such transcript has not been confirmed by RT-PCR analysis (Tesic, 2001). Using multiple different exon specific primers to exon 26 and exons 27, 28, 29 and 30 for RT-PCR analysis, Ivan Tesic was unable to demonstrate the existence of this transcript in any stage of development. This suggests the cDNA isolated in our lab may be an incompletely spliced mRNA and not a bona fide transcript of Strn-Mlck.

Figure 1.10 is a cartoon representation of the 7 conceptual proteins encoded by the various Strn-Mlck transcripts and some of the related proteins. The predicted protein encoded by the giant 25 kb conceptual transcript, Stretchin-Mlck (Strn-Mlck) would have an estimated molecular mass of ~1 MDa. This titin-like giant kinase contains the 4 domains found in the titin family of giant proteins: a kinase domain, the Ig domain, the Fn3 domain and a PEVK rich region. Stretchin-Mlck contains 32 Ig domains and 2 Fn3 domains. Strn-Mlck also contains two regions of repeated motifs and high PEVK content, similar to the PEVK repeated motifs of vertebrate titin. They are composed of a tandem repeat of 65 amino acid residues that are highly conserved. The first PEVK region consists of 10 repeats of the consensus sequence that are >90% identical to each
other. The second region of repeated PEVK motifs consists of 12 repeats of the consensus sequence that are >84% identical to each other and contain the sequence SAIDE (S, serine; A, alanine; I, isoleucine; D, aspartic acid and E, glutamic acid). Because of the similarity of Strn-Mlck’s PEVK and SAIDE motifs to titin’s PEVK repeated motifs, it has been proposed that Strn-Mlck’s PEVK and SAIDE motifs could provide elastic properties to this giant protein (Champagne et al., 2000). Both the PEVK and SAIDE motifs are preceded by a stretch of 187 amino acid residues that are >64% identical to each other and containing a high percentage of leucine, isoleucine, lysine and glutamic acid (L, I, K and E), thus termed handle-LIKE domains. This motif shows no homology to any other motif in other proteins, and its function is not known. Strn-Mlck may play both a regulatory and a structural role as suggested by the structural features of this protein. It has an MLCK domain, which provides it with a kinase activity to regulate the phosphorylation of the regulatory light chain of myosin (both muscle and nonmuscle as suggested by the expression pattern in both cell types). Like titin and projectin, it also contains the Ig domains, the Fn3 domains and the PEVK regions, which are involved in protein-protein interaction and elastic properties of the protein. It has been proposed that since Strn-Mlck shares many of the feature of titin and projectin, that it could also play a structural role and provide elasticity to the muscle through the PEVK and SAIDE motifs (Champagne et al., 2000; Vigoreaux, 2001).

The other conceptual proteins have different combinations of the motifs found in the giant Strn-Mlck isoform and this may be a feature that may provide the isoforms with tissue specificity, but this needs to be analyzed experimentally. The conceptual translation of the ~13 kb transcript (exons 7-33) produces a 497 kDa isoform. This
Giant-Mlck isoform has 26 Ig domains and 2 Fn3 domains and a kinase domain but no PEVK or SAIDE repeats, comparable to a giant MLCK rather than a titin. The conceptual translation of the smaller transcripts (one ~5.2 kb, and two ~3.2 kb) produce three small MLCK isoforms with predicted masses of 167 kDa, 102 and 86 kDa. These contain the kinase domain, an Fn3 domain and varying numbers of Ig domains (167 kDa and 102 kDa isoforms) or no Ig domain (86 kDa isoform), and resemble the vertebrate MLCKs.

The two other conceptual transcripts are unique because they do not overlap with the transcripts initiated at exons 20, 24 and 26, and do not have a kinase domain. The conceptual translation of the ~19 kb transcript (exons 1-15 or 16) produces an isoform of 698 kDa and corresponds to the N-terminus of the Stretchin-Mlck isoform. This isoform does have the PEVK and SAIDE repeats and thus has been named “stretchin” to account for the resemblance to titin’s elastic motifs. The last conceptual transcript (~7 kb, exons 7-15 or 16), produces a protein with a predicted mass of 269 kDa. This isoform has no kinase domain, Fn3 or PEVK rich regions. It is composed of Ig domains only, resembling the Kettin isoform of D-Titin, thus it has been named “Kettin-like” (Champagne et al., 2000). Studies have shown that a muscle protein A (225) isolated from IFM is the product of this transcript (Patel & Saide, 2001; Patel & Saide, 2003; Vigoreaux, 2001). It was found a 6.7 kb transcript encodes the 225 kDa protein which runs as a doublet in SDS-PAGE gels (Patel & Saide, 2001; Patel & Saide, 2003). The A (225) protein is expressed in adult head, thorax and abdominal muscles. The protein is localized to A bands of the IFM myofibrils and thus the protein is likely associated with myosin. This has been corroborated by analyzing the accumulation of the A (225)
doublet in myosin mutants. In mutants lacking myosin in the IFM, the A (225) protein is absent. Further, the accumulation of the A (225) protein is reestablished in mutants with a recombinant form of MHC which lacks the myosin head but has the sequences corresponding to the myosin rod region. This shows that the A (225) protein associates with the myosin rod region of MHC (Patel & Saide, 2001; Patel & Saide, 2003) and since this isoform lacks kinase activity, it may have a structural role in the sarcomere, perhaps providing a link to promote cross bridge formation. However, this needs to be tested experimentally, by studying the effects in a mutant lacking this isoform.

The object of this research was to study the function of *Strn-Mlck* in *Drosophila*. At the time of the onset of this work, no mutants of the *Strn-Mlck* gene were known to exist. I generated mutants of the *Strn-Mlck* gene and the isolation of the mutants and their phenotypic and molecular characterization is described in the following chapters.
CHAPTER 2

MATERIALS AND METHODS

Fly stocks and culture conditions

Balancer chromosomes and marker alleles used in the experiments are described in Lindsley and Zimm (1992) and FlyBase (FlyBase, 1998). The deficiency stocks used: \textit{Df(2R)WMG/Gla}, \textit{Jp1/CyO}, \textit{Jp4/Cyo} and \textit{Jp5/CyO} have chromosome deficiencies in cytological region 52D corresponding to the \textit{Stretchin- Mlck} gene location. Wild type flies used were Oregon R and Canton S. Other fly stocks used were: P-element lines \textit{yw; P\{w+mc=lacW\}C8-2-17} and \textit{yw; P\{w+mc=lacW\}E8-2-17}, the homozygous viable \textit{curved} allele (\textit{c1}) and \textit{cn bw (cinnabar, brown)}. All stocks were obtained from the national \textit{Drosophila} stock center in Bloomington, IN. Flies were maintained at room temperature (22°C) and crosses were preformed at 25°C on standard cornmeal-agar-molasses medium.

Generation of \textit{Strn-Mlck} mutants

Two different approaches were used to generate flies with mutations in the 52D region where \textit{Strn-Mlck} is located, gamma ray irradiation to generate chromosomal aberrations and P element mobilization.

For the gamma ray screen, the smallest deficiency available of the 52D region, \textit{Df(2R)WMG/Gla} was used for screening. Wild type males that had the second

52
chromosome markers *cn bw* were mutagenized by exposure to 4000 rads of gamma rays using a cesium-cobalt nuclear reactor at the Nuclear Facility of The Ohio State University. These males were mated to *Df*(2R)*WMG/Gla* virgins and the progeny was tested for flight. These putative mutants were backcrossed to the deficiency chromosome to confirm the phenotype of the mutants and establish stocks.

The local P-element transposition scheme required the use of lines with the P element very close to the target site (*Strn-Mlck* gene). Double *in situ* hybridization experiments with salivary gland (Polevoy, 1997), confirmed that the P-element lines *yw*; *P*{

\[
\text{w}^\text{+mc}=\text{lacW}}\text{C8-2-17 and } \text{yw}; \text{P}^\text{w+mc}=\text{lacW}}\text{E8-2-17 are insertions in the 52D region of the second chromosome, indistinguishable from *Strn-Mlck* by this method. The P element mobilization was triggered by the transposase activity provided by the *w*; *wg*^{50} \text{1/CyO; ry}^{50} \text{Sb1 P}^\text{ry+172=\Delta 2-3}}\text{99B/TM6B, Tb+ line. Males of the P-element line were mated en masse to virgins carrying the transposase source. The P-element was mobilized in the F}^\text{1} \text{dysgenic progeny and these males carrying both the P-element and the transposase were selected by the markers Sb(Stubble), CyO(Curly of Oster) and their mottled eyes. These males were mated to *w*; *Sco/Cyo* virgins to remove the source of transposase from the F}^\text{2} \text{progeny. Putative transposition events were detected by eye color changes in the progeny. Putative mutants were mated singly to *Df*(2R)*WMG/Gla* virgins. The progeny was scored for the flightless phenotype to identify lines with a mutation in the 52D region.}
\]
**Flight Index**

Flight tests were performed using a graduated glass cylinder with the bottom cut off and with the internal surface coated in mineral oil (Benzer, 1973). The cylinder was marked with graduations ranging from 8 at the top to 0 at the bottom. 2-3 day old flies were dropped from the top of the cylinder in small groups of 3-5 flies at a time. The flies were scored according to the position they landed inside the cylinder. The better fliers landed close to the top, thus scoring higher numbers, and the poor fliers dropped to the bottom and score lower numbers. Non-fliers would just drop straight through the cylinder landing on the bench and scoring a zero. The flight index is given as the mean with the standard deviation.

**Preparation of genomic DNA from *Drosophila* adults**

Genomic DNA was prepared from *Drosophila* adults following the 'single fly' method from Ashburner (1989). Briefly, 20 flies were anesthetized and placed in 1.5 ml microcentrifuge tubes at –80°C for 10 minutes. Then the tubes were taken out of the freezer and the flies were allowed to thaw before adding 200 µl of buffer A (10 mM Tris-HCl, 60 mM NaCl, 10 mM EDTA, 150 nM spermine, 150 nM spermidine, 5% sucrose; pH 7.5). Flies were homogenized manually with a pellet pestle in this buffer until no visible fly parts were seen. Buffer B (200 µl; 1.25% SDS, 0.3 M Tris-HCl, 0.1 M EDTA, 5% sucrose; pH 9.5) was added and the tube was inverted several times to mix well and then was placed at 65°C for 15-30 minutes. The tubes were allowed to cool briefly on ice and 60 µl of 8 M potassium acetate was added before incubating for 15 minutes on ice. The cellular debris and protein precipitate were removed by microcentrifugation at...
13,000 rpm at room temperature for 5 minutes. The supernatant was then precipitated with 2 volumes of absolute ethanol, and the pellet was washed with 70% EtOH twice before resuspending in 20 µl of sterile double distilled water. This way, 1 µl corresponds to a fly equivalent of genomic DNA. The tubes were kept at 4°C for restriction digestion and subsequent Southern analysis, or for genomic PCR.

**Genomic DNA-P1 clones**

Genomic DNA was also isolated from P1 clones following standard procedures (Maniatis et al., 1989). P1 clones corresponding to cytological position 52D where Strn-Mlck maps were used. The P1 clone DS08180 was selected as the genomic clone of choice as it contains the complete genomic sequence for Strn-Mlck. This clone was used as genomic control DNA for PCR reactions and as template for the generation of PCR fragments for subsequent RNA probe generation for in situ hybridizations to embryos. The P1 clones were kindly provided by Dr. Kaufman at Indiana University.

**Southern Hybridizations**

Southern hybridizations were done following standard protocols (Maniatis et al., 1989), with a few modifications. Briefly, 10 fly equivalents of genomic DNA was cut with restriction enzymes and the DNA was separated by agarose gel electrophoresis on 0.8%-1% gels in the TAE buffer system (40 mM Tris Acetate, 1 mM EDTA). The DNA was denatured by two 15 minutes washes of 1.0 M NaCl, 0.5 M NaOH followed by two neutralization washes (15 minutes each) of 3 M NaCl, 0.5 M Tris-HCl, pH 7.0. The DNA was transferred from the gel on to a nylon membrane using a Turbo Blotter system.
(Schleicher and Schuell) and 20X SSC (20X standard saline citrate, 3 M NaCl, 0.3 M Na Citrate, pH 7.0) for 4 hours to overnight. The DNA was then cross-linked to the membrane by UV irradiation. Pre-hybridization was done in 5X Denhardt’s solution, 6X SSC, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 65°C for ≥1 hour.

Random primed DNA probes were generated by Rediprime™ II Random Prime Labelling System (Amersham Biosciences). The probes were denatured at 95°C for 5 minutes and added to the pre-hybridization solution and the membrane. Hybridization was allowed to proceed overnight at 65°C. The probe was removed and the membrane was washed twice in Solution 1 (2X SSC, 0.1% SDS) for 15 minutes and twice in Solution 2 (0.1% SDS) for 10 minutes and exposed to X-ray film.

**RNA isolation from adult flies**

Total RNA from approximately 30 adult flies was prepared using TRIzol® Reagent (Invitrogen), following manufacturer's recommendations. Homogenization of the tissues was carried out manually utilizing a pellet pestle, directly in the TRIzol® reagent.

**Reverse Transcription-PCR (RT-PCR)**

First strand cDNA was synthesized using Superscript™ II RNase H Reverse Transcriptase (Invitrogen), with gene specific primers following manufacturer's recommendations. Approximately 1-2 µg of total RNA and 2 pmoles of the gene specific primer were mixed and incubated at 70°C for 10 minutes, and then cooled on ice and quickly spun down. A master mixture of 5X first-strand buffer (250 mM Tris-HCl
(pH 8.3), 375 mM KCl, 15 mM MgCl₂), supplied by the manufacturer, (final concentration of 50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl₂), 100 mM DTT (to a final concentration of 10 mM), also supplied by the manufacturer, 10 mM of each dNTP (dATP, dCTP, dGTP, dTTP, to a final concentration of 0.5 mM each) and 40 units/20 µl reaction of RNase OUT™ Recombinant Ribonuclease Inhibitor (Invitrogen) was added. The mixture was allowed to incubate for 2 minutes at 42°C, at which point 200 units/20 µl reaction of Superscript™ II Rnase H Reverse Transcriptase (Invitrogen) was added, before continuing the incubation at 42°C for 50 minutes. The RT reaction was incubated at 70°C for 15 minutes to inactivate the enzyme. To remove the RNA complementary to the cDNA, 2 units of *E. coli* Ribonuclease H (Invitrogen) was added and allowed to incubate at 37°C for 20 minutes prior to PCR. For the following PCR reactions 2 µl of the RT reaction were used as template.

**PCR amplification of genomic DNA and first strand cDNA**

Amplification was carried out with Platinum® *Taq* DNA Polymerase (Invitrogen), following standard protocols recommended by the manufacturer. The PCR reaction mix consisted of 1X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3 mM MgCl₂, provided by the manufacturer, 400 µM each dNTP (dATP, dCTP, dGTP, dTTP), 200 µM each of the forward and reverse primers, ~100ng-1µg of genomic DNA or 10% of an RT reaction (see previous section) and 2.5 units of Platinum® *Taq* DNA Polymerase (Invitrogen). The PCR reaction was overlaid with 50 µl of light mineral oil to prevent evaporation of the reaction through the high temperature cycles.
The amplification proceeded for 30 cycles. Each cycle consisted of 45 seconds at 94°C for denaturation, 45 seconds of annealing at 58°C and extension for 1 minute/kb of target at 72°C. This was followed by one last extension cycle at 72°C for 10 minutes.

For the amplification procedure, specially the RT-PCR experiments, annealing and extension conditions were primer and template dependent, therefore were adjusted accordingly to correlate with the dissociation temperature of the primer pairs being used for each experiment. Care was taken to design the primers with approximately comparable dissociation temperatures, therefore the conditions stated above were representative of the average annealing conditions used.

**DNA sequencing**

Various genomic clones and PCR fragments were purified by gel extraction according to the manufacturer’s instructions using a QIAquick Gel Extraction Kit (Quiagen) and sent to the Plant-Microbe Genomics Facility at The Ohio State University for sequencing.

**RNA in situ hybridization to *Drosophila* embryos**

Whole mount in situ hybridizations were performed on *Drosophila* embryos using digoxigenin-labeled RNA probes, and standard procedures (O'Neill & Bier, 1994). In situ hybridization was done on 24 hour collections of embryos, therefore, all stages of embryogenesis were represented. Probes tested corresponded to the catalytic region, (probe was to exons 27-29) which is shared between all transcripts with the kinase activity; exon 32, which is alternatively spliced, and transcript specific probes made to
the 5’ region of different transcripts to determine if the transcripts have tissue-specific expression patterns.

**Preparation of digoxigenin-labeled RNA probes for in situ hybridizations**

Probes were generated by *in vitro* transcription with T7 RNA polymerase (Roche). Before RNA probes could be *in vitro* transcribed, primer pairs were designed corresponding to the different transcripts. For exons 1-2, 7, 20 and 27, the reverse primers were designed to contain the T7 RNA polymerase promoter sequence at the 5’ end (TAATACGACTCACTATA), whereas the forward primers contained no additions. Table 1 lists the primers used to generate the PCR products for the different regions of *Strn-Mlck* that were tested with *in situ* hybridization. PCR was performed as described above using genomic P1 plasmid DNA (DS08018), or cDNA sequences inserted into transcription vectors as template. After PCR amplifications, the PCR products were purified from primers, nucleotides, polymerases, and salts, using QIAquick PCR Purification Kit (Quiagen) in a microcentrifuge following the manufacturer’s protocols and recommendations. For exons 24 and 32, the PCR products were subcloned into a transcriptional vector (pBluescript®II KS’). Before these products could be transcribed in *vitro*, they were linearized with Hind III restriction enzyme. T7 RNA polymerase (Roche) was used to synthesize the RNA transcript.

RNA probes were transcribed *in vitro* following the method of O’Neill and Bier (O’Neill & Bier, 1994), with modifications, using the PCR product with the T7 promoter sequence or the linearized fragments. The reactions consisted of 100-200 ng of purified
### TABLE 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Exon</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location in DS08018</th>
<th>Template</th>
</tr>
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<td>ex1b</td>
<td>1</td>
<td>GGATGTCCAATGCTGCCAG</td>
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<td></td>
<td></td>
<td>ACGTGGATCTCTGCTCCACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d11</td>
<td>7</td>
<td>GGCACCGCCCAAAAAG</td>
<td>47,569-47,584</td>
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</tr>
<tr>
<td>d17R</td>
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<td>GCGACATTAGATG</td>
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</tr>
<tr>
<td>d1</td>
<td>20</td>
<td>GAATTCGGCACGAGAG&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>cDNA, MP7</td>
</tr>
<tr>
<td>d2</td>
<td>20</td>
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<td>AACTGCTTGGCAAAGCAGTGGTGAATTCGGCACGAGAG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cDNA, MP7</td>
</tr>
<tr>
<td>B1</td>
<td>24</td>
<td>GGAATTCATTCGTTCCACGGCGACGTCCG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62,642-62,660</td>
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</tr>
<tr>
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<td>cDNA, D3, D5</td>
</tr>
</tbody>
</table>

**Table 1:** Summary of the primers and templates used in this research to make RNA probes, and their location in the DS08018 P1 plasmid.

<sup>a</sup> Sequence in bold corresponds to T7 promoter sequence added 5’ of the primer.

<sup>b</sup> Primer contains partial vector sequence (vector was pBluescript® II SK-).

<sup>c</sup> Primer contains sequence for Eco RI at the 5’ end for cloning into transcriptional vector.

<sup>d</sup> Primer contains sequence for Bam HI at the 5’ end for cloning into transcriptional vector.
PCR fragment or 2.5 µg linearized DNA, 2 µl of 10X DIG RNA Labeling Mix (10 mM ATP, 10 mM CTP, 10mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP) (Roche), 2 µl T7 RNA Polymerase (Roche), 2 µl 10X transcription buffer (included with the enzyme [Roche]), and 1 µl RNase Inhibitor (40 U/µl [Roche]) in a total reaction volume of 20µl. The reaction was allowed to incubate at 37˚C for 2 hours. At this point, 1-2 µl of the reaction was set aside to be analyzed by gel electrophoresis (1% agarose gel) to assess the quality and quantity of the transcribed RNA. The rest of the transcription reaction was precipitated by adding 2 µl of 0.2 M EDTA (ethylene diamine tetra-acetic acid) (pH 8.0), 2.5 µl of 4M LiCl and 75 µl chilled absolute ethanol, and placing the reaction at –20˚C between 1 hour to overnight. It was unnecessary to hydrolyze the RNA with 2X carbonate buffer as described by O’Neill and Bier (1994) as the primers were designed to give PCR products of less than 400 nucleotides. The concentrated labeled RNA transcripts were pelleted by microcentrifugation at 13,000xg for 15 minutes and a temperature of 4˚C. The RNA pellet was then washed twice in chilled 70% ethanol made with diethylpyrocarbonate (DEPC)-treated water and air dried for 5-10 minutes. The RNA probes were dissolved in 75 µl of hybridization buffer (50% formamide (Fluka), 5X SSC, 100 µg/ml heparin, 0.1% Tween® 20, 100 µg/ml sonicated and denatured salmon sperm DNA) and stored at –80˚C. DIG-RNA probes were diluted 1:100 or 1:200 in hybridization buffer before proceeding with in situ hybridization to embryos. Probes were utilized numerous times as increased usage typically decreases background. The probes were stored at –80˚C between uses.
Embryo collection and fixation

Adult *Drosophila* wild-type (Canton S) flies were put into small egg collection cages made from tissue culture flasks that were modified by making a hole on the top of the flask, just a little smaller than the diameter of a small Petri dish. The screw-cap opening was plugged with a cotton ball to allow air to flow into the cages. Grape juice-agar plates, smeared with a live yeast paste (Wieschaus & Nusslein-Volhard, 1986) were placed on top of the fly cage and attached in place with rubber bands. The grape juice-agar plates were made by mixing 50 ml concentrated grape juice, 100 ml water and 4 g agar, and boiling until the agar was well dissolved. The mixture was allowed to cool down at room temperature to approximately 60°C before adding 1 ml of 95% ethanol and 1 ml of glacial acetic acid, and then poured into small Petri dishes.

Timed collections were made by placing the cages at 25°C and allowing the flies to lay eggs on the grape juice-agar-yeast plates for a period of 21-22 hours. This allowed harvesting of embryos of all stages, as embryogenesis takes 22 hours at 25°C from egg fertilization to first instar larva hatching from the egg (Ashburner, 1989).

Fixation of the embryos was done according to O’Neill and Bier (1994). The embryos were flushed with distilled water to loosen them from the grape juice-agar plate and to clean them from the yeast paste. They were transferred to a small sieve made with a fine nylon mesh using a transfer pipette, and washed thoroughly with distilled water. The removal of the chorions from the embryos and consequent fixation followed the procedure described by O’Neill and Bier (1994).
**In situ hybridization**

The *in situ* hybridization protocol was according to O’Neill and Bier (1994), following only the steps corresponding to the digoxygenin (DIG) segment of the protocol with some minor adjustments. All steps were carried out at room temperature, unless otherwise specified, and in 1 ml volumes with rocking in a nutator, with the exception of the hybridization step, which was carried out in a 56˚C water bath overnight. The incubation with the anti-DIG-AP antibody (Roche) was carried out as mentioned in the protocol (O’Neill & Bier, 1994), at a 1:2000 final dilution in 500 µl of PBT (PBS + 0.1% Tween® 20), for 1 hr at room temperature or, alternatively, was performed at 4˚C overnight. PBS (phosphate-buffered saline) was 140 mM sodium chloride (NaCl), 7 mM disodium phosphate (Na₂HPO₄), and 3 mM potassium phosphate (KH₂PO₄), pH 7.0. Also, the last of the 3 washes with the alkaline phosphatase staining buffer (APB, 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris-HCl, pH 9.5 and 0.1% Tween® 20), was extended to up to an hour to increase sensitivity of staining. The staining was carried out in the dark. The coloring reaction was stopped according to the standard procedure (O’Neill & Bier, 1994), and samples were kept at 4˚C in 100% ethanol (EtOH). Embryos were either mounted in Aqua-Poly/Mount (Polysciences, Inc.), or cleared with xylene for 5 minutes and mounted in Permount (Fisher Scientific).

**Electron microscopy**

Thoraces were dissected from 2-3 day old flies by pulling off the head (making sure the digestive tract came off with the head too), the abdomen, and legs using fine
point forceps. The ventral portion of the thorax where the legs are attached was also removed by cutting the unneeded musculature with super fine micro scissors (Biomedical Research Instruments, Inc.) and taking care not to cut too high up as to compromise the condition of the thorax and the myofibers inside it. Removing the unneeded musculature allows the tissue to be exposed later to the fixation solutions. The thoraces were placed in abundant amounts (1 ml per thorax) of fixative (3% paraformaldehyde, 3% glutaraldehyde, 100 mM sucrose, 100 mM sodium phosphate, pH 7.2, 2 mM EGTA) and allowed to incubate overnight at room temperature with sporadic agitation of the tissue. Following the primary fix, thoraces were passed through 3 changes of wash buffer (100 mM sucrose, 100 mM sodium phosphate, pH 7.2) for 10 minutes each. A secondary fix was done consisting of 1% osmium tetraoxide (Polysciences, Inc.) (a 4% stock solution in water was made and kept at 4°C), 100 mM sodium phosphate, pH 7.2, for 1.5 hours at room temperature. At this point, the thoraces turn black or brown due to the osmium tetraoxide and this served as a visual confirmation that the tissues had been fixed properly. Following the secondary fix with osmium tetraoxide, samples were rinsed as before with 3 changes of wash buffer, and then were dehydrated by passing through a graded acetone series of 30%, 50%, 70%, 80%, and 85% for 10 minutes each, then 2 changes of 10 minutes each of 95% and 2 final changes in 100% acetone for 10 minutes each. The thoraces were then allowed to incubate in a 1:1 dilution of acetone: Spurr’s embedding media (provided by the Campus Microscopy and Imaging Facility), overnight at room temperature with gentle agitation. Samples were allowed to incubate twice in 100% Spurr’s embedding media for 2 hours, before being transferred to embedding molds in 100% Spurr’s media (taking care to place the thoraces in the proper orientation
to obtain either longitudinal or cross sections), and allowed to polymerize at 60°C overnight. Embedded samples were sent to the Campus Microscopy and Imaging Facility to be sectioned and stained. Sections were viewed and photographed in a Phillips CM-12 transmission electron microscope.
CHAPTER 3

GENERATION AND CHARACTERIZATION OF STRETCH-IN-MLCK MUTANTS IN DROSOPHILA MELANOGASTER: EFFECTS IN FLIGHT ABILITY BUT NOT IN MUSCLE STRUCTURE

Introduction

Myosin light chain kinases (MLCKs) are Ca\(^{2+}\)/calmodulin dependent serine/threonine kinases responsible for the phosphorylation of myosin regulatory light chains (RLC), called MLC2 in Drosophila. MLCK is highly specific and myosin RLC is its only known physiological substrate. The kinase phosphorylates specific sites on the N terminus of myosin RLC. In vertebrates these conserved sites have been identified as Ser-19 and Thr-18. \textit{In vitro} studies demonstrated that a rabbit skeletal muscle MLCK was able to phosphorylate a recombinant version of the \textit{Drosophila} muscle regulatory light chain, MLC2 at Ser 66 and Ser 67 (Graham, 1992). In \textit{Drosophila} MLC2, these sites correspond to the conserved vertebrate MLCK phosphorylation sites.

Tohtong \textit{et al.} (1995) investigated the \textit{in vivo} role of the MLCK target phosphorylation sites (Ser 66, Ser 67) of MLC2. Using site directed mutagenesis, they substituted unphosphorylatable alanines at the conserved MLCK phosphorylation sites
(mutants denoted $Mlc2^{S66A, S67A}$), and introduced these mutant $Mlc2$ genes into $Mlc2$ null mutant flies. These $Mlc2^{S66A, S67A}$ mutants, whose only source of MLC2 cannot be phosphorylated at these sites, developed into recessive flightless flies. The muscle structure of the mutants was unaffected and the flightless phenotype was found to be due to a reduction in power output and a reduced response to stretch activation (Tohtong et al., 1995).

In order to further characterize the function of MLCK, the gene was cloned in our lab (Tohtong et al., 1997) and simultaneously in another lab (Kojima et al., 1996). Additionally, Champagne et al. (2000) made predictions about the gene structure and intron/exon boundaries using computer analysis of the published Drosophila genome sequence (Adams et al., 2000). Ivan Tesic, a former graduate student in the lab, further studied the gene structure experimentally (Tesic, 2001). Together the data suggest that the mlck gene (renamed Stretchin-Mlck, Strn-Mlck by Champagne et al. (2000)) is large, and has a complex structure with multiple transcripts that arise through alternative splicing and the use of different promoters.

A powerful way to study gene function is to analyze the phenotype in a mutant for the gene. As no mutants of the Strn-Mlck gene were known to be in existence at the onset of this work, I designed a screen to isolate such mutants. Generating mutants of a gene of interest in Drosophila can be facilitated by using a deficiency chromosome, which lacks the gene of interest, in order to screen for new mutations that map in the region. For this study I used a deficiency known to remove the region including 52A-52D where Strn-Mlck maps.
I based the prediction of the mutant phenotype on the characterization of the mutants in the muscle myosin regulatory light chain (Mlc2^{S66A,S67A}). Since altering the Strn-Mlck target residues in muscle MLC2 produced mutants that were recessive flightless, it was expected that mutations affecting a muscle specific transcript of Strn-Mlck should also produce recessive flightless flies. With this premise, I conducted a screen to isolate flightless mutants in the 52D region following treatment with two different mutagens.

In this chapter, I describe the isolation of three Strn-Mlck mutants. Two of these mutants are deletions in the catalytic region of the gene and are therefore kinase null. I also demonstrate through genetic complementation analysis and Southern analysis, that the previously described curved (c^1) mutant (Lindsley & Zimm, 1992) is an allele of the Strn-Mlck gene. Finally, I show that the mutants have a recessive flightless phenotype. However the indirect flight muscle (IFM) appears normal (as was the case with the MLC2 double serine mutants) and these mutants are likely to be flightless because of a wing shape and position phenotype.
Results

To characterize Strn-Mlck function, a screen was conducted to isolate Strn-Mlck mutants. Since altering the Strn-Mlck target sites in MLC2 produced mutants which were viable and exhibited a recessive flightless phenotype (Tohtong et al., 1995), we hypothesized that mutations affecting a muscle specific transcript of Strn-Mlck would also produce viable flies with a recessive flightless phenotype.

To isolate muscle specific mutants of Strn-Mlck, I screened for recessive flightless phenotypes using two different methodologies to produce mutations. These were irradiation with gamma rays and P-element mobilization. Irradiation with gamma rays is suitable for inducing chromosome breaks and results in deletions, inversions and translocations. P element mobilization typically causes mutations by transposition of the P element into a new locus, but can also create a number of rearrangements by creating precise or imprecise excisions, deletions and rearrangements that have their breakpoints at or near the existing P element (Greenspan, 1997). Having a P element that is very close to the target site greatly enhances the chances of a transposition event into the gene of interest, as so called ‘local hopping’ occurs at a high frequency. As will be discussed below, I used both gamma irradiation and P element lines (available thought the public stock center) that were very close to Strn-Mlck to generate mutations in the gene.
Flight tests of deficiency strains in the 52D region where Strn-Mlck maps show that Strn-Mlck is not haplo-insufficient for flight.

We had previously mapped Strn-Mlck to cytological region 52D on the right arm of the second chromosome, by in situ hybridization to larval salivary gland polytene chromosomes (Tohtong et al., 1997). I obtained 4 deficiency lines from the Bloomington Stock Center with deficiencies in the 52D region. I tested the flight ability of the deficiency lines as heterozygotes, using a graduated cylinder coated internally with mineral oil (Benzer, 1973; Tohtong et al., 1997). Table 2 shows the results of these flight tests. The data show that Strn-Mlck is not haplo-insufficient for flight, as the flies have scores in the normal range when compared to wild type flies. For the F1 screen and the P element mobilization screen, I chose to use Df(2R)WMG/Gla, as this is the smallest deficiency available that uncovers Strn-Mlck with breakpoints between cytological regions 52A and 52D.

Generation of Strn-Mlck mutants by P element mobilization

The scheme used to mobilize the P elements into new chromosomal positions is shown in Figure 3.1. F1 dysgenic males were generated with the P element derived from the parental males (2 different P element lines in the region were used) and females of the genotype w; wg^{Sp-1}/CyO; ry^{506} Sb^{1} P[ry^{+t7.2=Δ2-3}]99B/TM6B, Tb providing the source of transposase. P element mobilization actively occurs in F1 dysgenic males, which can be identified by the Stubble (Sb) and Curly (Cy) markers and their mottled eyes. The transposase source is removed in the next generation to prevent any further transposition.
<table>
<thead>
<tr>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>n tested</th>
<th>Flight index&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>87</td>
<td>6.15 ± 0.87</td>
</tr>
<tr>
<td>Jp1/+</td>
<td>125</td>
<td>6.30 ± 0.85</td>
</tr>
<tr>
<td>Jp4/+</td>
<td>102</td>
<td>5.80 ± 0.86</td>
</tr>
<tr>
<td>Jp5/+</td>
<td>85</td>
<td>5.87 ± 0.83</td>
</tr>
<tr>
<td>WMG/+</td>
<td>103</td>
<td>6.54 ± 0.97</td>
</tr>
</tbody>
</table>

Table 2: Flight index of deficiency heterozygotes uncovering the *Strn-Mlek* locus.

<sup>a</sup>Wild type, Oregon R; Jp1, Df(2R)51C3; F5-9; Jp4, Df(2R)51F13; 52F8-9; Jp5, Df(2R)52A13-B3; 52F10-11; WMG, Df(2R)52A; 52D. <sup>b</sup>The flight index is given with standard deviation.
Figure 3.1 Genetic scheme to create *Strn-Mlck* mutants by P-element mediated mutagenesis. Males of a P element line (*P{lacW}C8-2-17*) were crossed to females carrying a source of transposase. The F₁ males carry both the P element and the transposase allowing the P element to be mobilized in the genome. These males were crossed to females carrying markers to allow removal of the source of transposase in the next generation. The mobilization event can be followed in the F₂ generation by eye color changes. Putative mutants were crossed to a deficiency line uncovering the 52D region where *Strn-Mlck* maps. The progeny were tested for a flightless phenotype and putative mutants were maintained as balanced stocks. *New mobilization event.*
Figure 3.1 Genetic scheme to create Strn-Mlck mutants by P-element mediated mutagenesis.
events from occurring. This is accomplished by crossing the dysgenic males to
\(w;Sco/Cyo\) virgins and selecting against the \(Sb\) marker.

About 490 individual \(F_2\) males were selected based on eye color changes,
indicating a mobilization event. These putative mutants were crossed to the deficiency
line \(Df(2R)WMG/Gla\), to determine if a mobilization event caused an insertion in the
region where \(Strn-Mlck\) maps.

The progeny was scored for a flightless phenotype to identify lines with a
mutation in the 52D region which could potentially have alterations in muscle specific
transcripts of \(Strn-Mlck\). The flies were tested in a fly cage made of Plexiglas. Further
characterization (see below) showed that only one \(Strn-Mlck\) mutant was recovered from
the 490 putative \(F_2\) males. This mutant is called p131. The mutant flies have white eyes,
suggesting the mutation was caused by excision of the P-element. The p131 mutant was
recovered from the screen with the \(yw; P\{w^{mc}=lacW\}C8-2-17\) as the parental P element
line. This mutant is homozygous viable, fertile, and shows a recessive flightless
phenotype.

**Generation of \(Strn-Mlck\) mutants by gamma ray irradiation**

Radiation induces chromosome breaks resulting in deletions, inversions and
translocations (Greenspan, 1997). In the \(F_1\) screen (Figure 3.2) wild type males were
mutagenized by exposure to 4,000 rads of gamma rays. The males used had two markers
on the second chromosome, \(cinnabar (cn)\) and \(brown (bw)\). These flies have been.
Figure 3.2  F1 screen to select for flightless mutants in the 52D region. Males carrying the markers cn bw were mutagenized by exposing them to gamma rays. The mutagenized males (cn bw*) were mated to deficiency females (Df(2R)WMG/Gla) and the progeny was tested for flight. Putative mutants were retested by crossing a second time to the deficiency chromosome before establishing as stocks.
successfully used before in screens for flightless mutants in the second chromosome without affecting the phenotype sought after (John Sparrow, personal communication). The irradiations were done at the Nuclear Facility of The Ohio State University using a cobalt nuclear reactor. The irradiated males were mated en masse to Df\(2R\)WMG/Gla virgin females and the progeny was tested for flight. 

I used a flight column, which consisted of two rings of PVC (poly vinyl chloride) plumbing plastic tubing, 4 inches in diameter, attached to a ring stand with tape. This diameter permits the recovery of the maximum number of flightless flies while allowing the recovery of only a small number of false positives (John Sparrow, personal communication). The PVC tubing rings were attached approximately eight inches apart. Two pieces of transparency sheets were taped together and lined with Tangle-Trap Insect Trap Coating (The Tanglefoot Company), and placed inside the two PVC tubing rings. Two funnels were placed on the column, one at the top of the column through which the flies are introduced, and one funnel at the bottom of the column, to collect the flies in a glass bottle or beaker. Flies with unimpaired flight ability fly immediately to the sides of the coated column and get stuck. The poor fliers and non-fliers drop through the column into the glass beaker where they are collected.

From the F\(_1\) progeny, 718 putative mutants were recovered from 275,900 flies that were tested. These 718 putative mutants were backcrossed to the deficiency chromosome Df\(2R\)WMG/Gla virgin females, and the putative mutants whose progeny exhibited the same flightless phenotype were established as stocks. Further characterization (see below) confirmed 3 of the candidates as Strn-Mlck mutants (\(\gamma_{112}, \gamma_{128}\) and \(\gamma_{39}\)). As with the mutant recovered from the transposon mobilization scheme, these mutants were
also viable and fertile as homozygotes, and exhibited a recessive flightless phenotype as expected for a putative Strn-Mlck muscle mutant.

**Complementation analysis of putative mutants**

The putative mutants were crossed *inter se* to determine if they were alleles of the same gene. The results showed noncomplementation in each combination; all the transheterozygotes were flightless. This demonstrates the mutations are in the same gene.

**Primary molecular characterization of the Strn-Mlck mutants**

Having putative Strn-Mlck mutants in hand, the next step was to investigate the gene structure of these mutants using genomic Southern hybridization analysis. Molecular data showing that the lesions in the mutants map to the Strn-Mlck gene provided definitive proof the mutants are alleles of the *Strn-Mlck* gene.

**Genomic characterization of the *Strn-Mlck* mutant**

As described above, p131 is the putative mutant recovered from the P element mobilization screen. The P element line used for the screen has a P{lacW} element, which is marked by the wild-type allele of the eye color gene *white* (*w*+). In this screen, putative mobilization events are identified by eye color changes in the F2 progeny. In the case of the *Strn-Mlck* mutant, the eye color of this putative mutant is white. In a P mobilization experiment, white-eyed flies can be recovered when a transposition event causes loss of the *white*+ gene because of an internal deletion. Another possibility is the imprecise excision of the P element from the original site. In some cases where there is
loss of the P element from a site, the excision event can also remove part of the genomic DNA from a site, creating a deletion in the gene flanking the P element insertion.

To investigate if the p131 putative mutant had a lesion in the Strn-Mlck gene, the Strn-Mlck locus was analyzed in these flies. Genomic DNA was cut with multiple restriction enzymes to eliminate the possibility of restriction site polymorphisms. The fragmented genomic DNA was separated by agarose gel electrophoresis and hybridized with several DNA probes that would allow the entire gene to be analyzed.

The genomic Southern analysis of p131 shows this mutant has a large deletion in the 3’ end of the gene which includes the catalytic and regulatory regions (Figure 3.3 panel B). The analysis shows this mutant is also defective in the area extending to exon 20 (Figure 3.3 panel C). To investigate the length of the p131 deletion, I used standard PCR to amplify a fragment of genomic DNA, which I predicted would encompass the deletion, using gene specific primers corresponding to the areas flanking the region. I cloned the PCR product, sequenced it and found the deletion to be 7.9 kb long, starting in exon 20 and ending in the last exon (exon 33) of the gene in the 3’UTR (Figure 3.4 A). Southern and standard PCR analyses showed the deletion does not affect the neighboring gene (data not shown). This deletion completely eliminates the region encoding the 5.2 kb transcript (Tohtong et al., 1997). It also lacks the catalytic region, thus affecting all isoforms with a predicted kinase activity. As this analysis showed p131 is a mutation in Strn-Mlck, hereafter this allele is called Strn-Mlck¹.
Figure 3.3 Southern characterization of the Strn-Mlck\(^1\) mutant indicates a deletion affecting the 3’ end of the gene. DNA from wild type line Canton S (Cs), Strn-Mlck\(^1\) homozygous (mlck\(^1\)) and heterozygous (mlck\(^1\)/Gla), and +/Gla was analyzed by Southern blot. The blots were hybridized with different DNA probes spanning the MLCK region. In sum, the results indicate there is a deletion of the 3’ end of the gene, spanning exons 20-33 (also see text). In both panel B and C, the homozygote shows no signal, as these regions are completely deleted in the mutant. Analysis of heterozygotes confirms that when equal amounts of DNA are loaded, the signal is reduced when compared to wild type controls. The difference in band size seen in panel B is due to restriction enzyme polymorphism (compare to +/Gla lane). The cartoon at the top shows a schematic of the deletion. Exons are represented by numbered open boxes (not to scale). DNA probes are bold lines. The extent of the deletion is represented by a striped box. Gla-Glazed balancer chromosome.
Figure 3.3 Southern characterization of Strn-Mlck\textsuperscript{1} mutant indicates a deletion affecting the 3’ end of the gene.
Figure 3.4 Sequence analysis of Strn-Mlck$^1$ and Strn-Mlck$^3$ mutants. DNA from the mutants was amplified using PCR and the purified fragments were sequenced. (A) Sequence of the Strn-Mlck$^1$ mutants shows the break points of this deletion mutant are located in exons 20 and 33. Also, this mutant contains some inserted sequence (shaded blue) and a portion of the sequence is also duplicated (shaded in red). (B) Sequence of the Strn-Mlck$^3$ mutant shows the deletion spans from exon 27 to exon 28. This deletion causes a shift in the reading frame. In the mutant, the sequence corresponding to the catalytic region differs from the normal sequence for a span of 80 amino acids until translational stop is reached.
Genomic characterization of the *Strn-Mlck*<sup>3</sup> mutants

The γ112 and γ128 mutants were recovered from the F<sub>1</sub> screen using gamma irradiation as a mutagen. Upon Southern characterization of the genomic DNA of both mutants, I showed that the lesion in both mutants comprised a small deletion of roughly 400 bp just before the catalytic region (Figure 3.5).

I performed PCR on DNA from both mutants using gene specific primers spanning the deletion, then, I cloned and sequenced the PCR fragments. The data revealed an identical sequence for both mutants, suggesting that both γ112 and γ128 are derivatives of the same original mutation. Consistent with this, both γ112 and γ128 came from the same irradiation experiment, hence could be progeny of the same mutagenized male.

The sequence data show that the lesion is 245 bp long, starting at the end of exon 27 and ending at the beginning of exon 28, creating a frameshift in the reading frame (Figure 3.4). The alternative reading frame continues for 136 amino acids until it reaches a stop codon. This analysis showed γ112 and γ128 are the same mutation in *Strn-Mlck* and would result in products lacking kinase activity. Hereafter this mutation is called *Strn-Mlck*<sup>3</sup>.

Genomic characterization of the *Strn-Mlck*<sup>2</sup> mutant

The γ39 mutant was also recovered from the gamma irradiation screen as a recessive flightless mutant. I analyzed the *Strn-Mlck* gene in this mutant but found no
Figure 3.5 Southern characterization of Strn-Mlck3 mutants show a small deletion in the catalytic region of Strn-Mlck. Genomic DNA was extracted from Strn-Mlck3-1 (mlck3-1), Strn- Mlck3-2 (mlck3-2), and wild type Canton S (Cs) lines. Hybridization of a blot with a probe to the catalytic region (p11-p21), shows a small deletion in the catalytic region of the gene (depicted as a striped box), affecting exons 27 and 28. The cartoon at the top shows a schematic of the deletion. Exons depicted as numbered open boxes (not to scale). Sequence analysis confirms these two alleles are identical (see text).
Figure 3.5 Southern characterization of $Strn-Mlck^3$ mutants show a small deletion in the catalytic region of $Strn-Mlck$. 
Figure 3.6 Southern analysis of the Strn-Mlck\textsuperscript{2} mutant: reveals no gross change in gene structure. Genomic DNA was extracted from wild type-Canton S (Cs) and Strn-Mlck\textsuperscript{2} (mlck\textsuperscript{2}) homozygous flies. The DNA was digested with SalI, SacI, Eco RI, Bam HI, Eco RI + SacII and SacII, and hybridized with DNA probes spanning the whole region of Strn-Mlck gene. No gross aberrations were found. The cartoon at the top shows a schematic of the gene structure and restriction enzyme map. Probes used are in bold lines. Exons are represented by numbered open boxes. SII-SalI, SI-SacI, B-Bam HI, E-Eco RI, SII-SacII.
Figure 3.6 Southern analysis of the Strn-Mlck\(^{-}\) mutant: reveals no gross change in gene structure.
change detectable by genomic Southern hybridization (Figure 3.6). To extend this analysis I also used PCR to amplify regions of the \textit{Strn-Mlck} locus where the genomic fragments analyzed by Southern hybridization might have been too large to detect small changes. I used overlapping primer sets that would amplify no more than 800 bp fragments spanning the gene (data not shown). This fragment size should allow me to detect changes as small as \(~50\) bp in the DNA (data not shown). As with the genomic Southern analysis, no differences between \(\gamma 39\) and wild type could be detected using PCR. However, it is clear that \(\gamma 39\) is a \textit{Strn-Mlck} mutant because it fails to complement both \textit{Strn-Mlck}\(^{1}\) and \textit{Strn-Mlck}\(^{3}\) and it shows the same wing phenotype as the other \textit{Strn-Mlck} mutants. Hereafter this mutant is referred to as \textit{Strn-Mlck}\(^{2}\).

\textit{Strn-Mlck} mutants have wing phenotypes resembling the previously identified, spontaneous mutant \textit{curved}.  

As described above, I recovered 3 mutant alleles of \textit{Strn-Mlck} all presenting a recessive flightless phenotype. Next I characterized these mutants in order to understand the underlying defects that cause the flightless phenotype.

The wings of the \textit{Strn-Mlck} mutants are uplifted at the base and then curve downward. The wing blades are also thin textured and are warped or distorted so that they curl downward at the tips (Figure 3.7 A). Figure 3.7 A shows a comparison between a wild-type fly (left side) and a \textit{Strn-Mlck}\(^{1}\) fly (right side). It is apparent that the mutant fly is unable to hold its wings in the normal position. Complementation analysis of the \textit{Strn-Mlck} mutants demonstrate that all the putative mutants have wing position
Figure 3.7 Wing position phenotype of *Strn-Mlck* mutants. *Strn-Mlck* mutants have wings with a thin texture that are often crumpled, lifted upwards at the base and curve downward. (A) Wild type fly on the left and homozygous *Strn-Mlck* on the right. (B) Wild type fly left and homozygous *curved* fly on the right. (C) A *Strn-Mlck*/*Strn-Mlck* fly showing the wing position phenotype characteristic of all combinations of all *Strn-Mlck* mutants (representative flies seen in panel D). (D) *Strn-Mlck*/*Strn-Mlck* flies.
phenotypes as transheterozygotes. Figure 3.7 D shows a transheterozygous fly of Strn-
Mlck$^1$/Strn-Mlck$^3$.

We noted that the wing phenotype of the Strn-Mlck mutants is similar in appearance to the phenotype of the previously described mutant curved (c) (Lindsley & Zimm, 1992). As shown in figure 3.7 B, c$^1$ mutants exhibit a wing blade that is also uplifted at the base and has a curving shape throughout the length of the wing, however, the Strn-Mlck mutants seem to be more extreme as their wings are more prominently held out than flies with the c$^1$ allele.

curved maps to region 52D3-9, and therefore curved alleles should also be recovered in the screens for flightless mutants I performed. Indeed, complementation analysis between the Strn-Mlck mutants and the c$^1$ allele (obtained from the Bloomington Stock Center) shows noncomplementation (Figure 3.7 C). Therefore, the Strn-Mlck mutants isolated here are genetic alleles of the previously identified, spontaneous mutation curved. To further investigate the molecular defect responsible for the curved mutation, I performed genomic Southern analysis.

Molecular characterization of curved: c$^1$ is an insertion in the Strn-Mlck gene

Genomic DNA from a c$^1$ homozygous stock was isolated and fragmented with several restriction enzymes. The fragmented DNA was separated by agarose gel electrophoresis and hybridized with DNA probes corresponding to various regions of the Strn-Mlck gene, allowing the analysis of the entire Strn-Mlck locus. Figure 3.8 B, shows a Southern blot of a PstI digest, probed with DNA corresponding to exon 20 (probe MP7-
Figure 3.8  Analysis of the Strn-Mlck \textsuperscript{c1} mutant by genomic Southern hybridization show the mutation is caused by an insertion. Genomic DNA was extracted from the Strn-Mlck \textsuperscript{c1} mutant line (\textit{c1}) and the wild type Canton S (Cs). Genomic DNA was digested with PstI, Eco RV + PstI (RV/PstI) and Eco RV + Eco RI (RV/RI). The mutant shows a shift in band size consistent with the mutation being an insertion (B). The insertion was further mapped to a 1 Kb Eco RV/PstI fragment between exons 21 and 22 (panelA). The cartoon at the top shows a schematic of the insertion. Exons depicted as numbered open boxes (not to scale). MP7-1 and IT25-26 are DNA probes used, depicted as bold lines.
Figure 3.8 Analysis of the Strn-Mleck<sup>o</sup> mutant by genomic Southern hybridization show the mutation is caused by an insertion.
1, Figure 3.8 A). This clearly shows that $c'$ contains an insertion in this 1.8Kb PstI fragment.

I mapped the position of this insertion more precisely by performing further Southern analysis. Genomic DNA was cut with Eco RV/PstI and Eco RV/Eco RI. I first used the MP7-1 probe, corresponding to exon 20. No difference was found in the banding pattern of the $c'$ mutant compared to the wild-type (data not shown). However, when probe IT25-26 (Figure 3.8 B), which included DNA from exon 21 to exon 22 was used, there were extra bands in the $c'$ mutant, consistent with an insertion. Therefore I was able to pinpoint the position of the insertion within an EcoRV/PstI 1 Kb region of the Strn-Mlck gene to between exons 21 and 22.

$c'$ was recovered as a spontaneous mutation by Bridges and Morgan in 1919 (Lindsley & Zimm, 1992). Many spontaneous mutants are caused by insertion of transposable elements. I attempted to clone a fragment of the element using PCR but this was not successful. However, the fact that the curved allele has the same wing phenotype as the Strn-Mlck mutants, is a genetic allele of the Strn-Mlck mutants, and molecular data shows it is an insertion in the Strn-Mlck gene, allows me to conclude that curved is Strn-Mlck. With this study I have established the function of the previously identified but uncharacterized mutant curved. Hereafter I will refer to the curved allele as Strn-Mlck$^{c1}$. Although the historical precedent suggests we should rename the Strn-Mlck mutants curved, Strn-Mlck is used here because it is more informative about the function of the gene.
Examination of flight ability of Strn-Mlck mutants

Flies with unimpaired flight ability will fly if they detect they are free falling when dropped from a surface, or when they are disturbed. The Strn-Mlck mutants fall directly to the bench top when dropped and do not fly when disturbed. However they are able to jump. The flight ability of the Strn-Mlck\textsuperscript{c1} mutant is a little better than the other mutants. Although these flies fall to the bench when dropped, they are able to glide somewhat, and will perform a combination of jumping and gliding when disturbed on a surface.

To examine the differences in flight abilities of the mutants, I determined a flight index for the mutants. The flight index is a score given to the flies based on their performance in the flight test cylinder. There are a number of ways to test the flight ability of flies (Benzer, 1973; Drummond \textit{et al.}, 1991). I have performed flight tests on the mutants using a graduated cylinder coated internally with mineral oil (Benzer, 1973).

The cylinder is divided by equally distributed numbered markings, from 8 at the top to 0 at the bottom. Normal flies will fly as they are dropped into the cylinder and land near the top of the cylinder scoring a high value. Flight impaired flies will drop and land near the bottom or fall through, scoring lower values. Flight tests were performed on both homozygous and heterozygous mutants.

The flight indexes are presented in Table 3. In the flight cylinder, wild type flies score values of about six. All the Strn-Mlck mutants except Strn-Mlck\textsuperscript{c1} have a very low flight index indicating that these flies are flightless. However, the Strn-Mlck\textsuperscript{c1} mutant has a higher flight index, although not equivalent to wild-type but consistent with the ability
### TABLE 3

<table>
<thead>
<tr>
<th>Flight Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>allele/allele</th>
<th>allele/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.15 ± 0.87 (87)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Strn-Mlck&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.39 ± 0.76 (184)</td>
<td>6.66 ± 0.60 (98)</td>
</tr>
<tr>
<td>Strn-Mlck&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.73 ± 0.76 (182)</td>
<td>6.34 ± 0.76 (101)</td>
</tr>
<tr>
<td>Strn-Mlck&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.08 ± 0.62 (140)</td>
<td>6.7 ± 0.52 (180)</td>
</tr>
<tr>
<td>Strn-Mlck&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.31 ± 0.68 (117)</td>
<td>6.59 ± 0.87 (83)</td>
</tr>
</tbody>
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*Table 3: Analysis of flight ability of Strn-Mlck mutants.*

<sup>a</sup> Flight Index is given with the standard deviation.  
<sup>b</sup> Wild type is Oregon R.  
<sup>c</sup> The number in parenthesis corresponds to the number of individual flies tested.
of these flies to glide. The flight indexes of the heterozygous mutants are normal when compared to wild-type, showing that Strn-Mlck is not haplo-insufficient for flight. (This is in keeping with the results obtained with the different deficiencies of the region tested (Table 2).)

The nature of the precise DNA lesions in the mutants may affect the flight ability of the flies differently if they affect a subset of transcripts. The Strn-Mlck$^1$ and Strn-Mlck$^3$ mutants have defects in the 3' end of the gene, affecting all the isoforms with kinase activity. MLCKs have both muscle and nonmuscle functions, therefore, if Strn-Mlck is the sole MLCK in Drosophila, mutants that lack its activity would be expected to be lethal as the nonmuscle functions are essential for viability. The fact that these mutants are alive and fertile suggests that there are other genes that are able to compensate for the lack of Strn-Mlck-kinase function (see Discussion). The Strn-Mlck$^{cl}$ mutant is an insertion N terminus of the 5.2 kb message encoding a predicted 165 kDa isoform (Kojima et al., 1996; Tohtong et al., 1997). The fact that this mutant has transcripts that are unaffected (see Chapter 4) may account for the better flight ability in the mutants which allows them to glide.

**Some Strn-Mlck mutants have an extra wing vein phenotype**

Another morphological defect seen in the wings of the mutants is the presence of ectopic wing veins (Figure 3.9). This phenotype is not completely penetrant and is only seen with the Strn-Mlck$^1$ allele. I observed this phenotype in Strn-Mlck$^1$ homozygotes (Figure 3.9 C), Strn-Mlck$^1$ over a deficiency chromosome (Figure 3.9 B), and in Strn-Mlck$^1$/Strn-Mlck$^2$ transheterozygotes (Figure 3.9 D), with a penetrance of 10%, 6% and
Figure 3.9 Extra wing vein phenotype of Strn-Mlck mutants. (A) Canton S wing showing normal wing vein structure. (B) Strn-Mlck\textsuperscript{1}/Df(2R)WMG, shows an ectopic wing vein spur protruding from longitudinal vein L5. A similar phenotype is seen in wings of flies homozygous for the Strn-Mlck\textsuperscript{1} allele (C) and in Strn-Mlck\textsuperscript{1}/Strn-Mlck\textsuperscript{3} flies (D). In the most extreme cases extra vein material is seen in the posterior wing vein and vein L5. (E) Wing from Cam/+ fly, a regulator of MLCK, also shows ectopic vein material as well as a Strn-Mlck\textsuperscript{1}/Mlck-2\textsuperscript{i} fly (F). The Mlck-2\textsuperscript{i} mutant was isolated in our lab and is a mutation in a new mlck-like gene.
Figure 3.9 Extra wing vein phenotype of Strn-Mlck mutants.
8% respectively. The extra wing vein material is usually seen extending from the posterior cross vein or longitudinal vein L5 in the most extreme cases. In the milder cases, extra vein material in the intervein region between L5 and the posterior wing margin is observed.

Interestingly, Calmodulin (Cam) mutants, (Calmodulin is a regulator of MLCK) also show a similar phenotype (Figure 3.9 E)(Nelson et al., 1997). In Cam mutants, this extra wing vein phenotype also shows incomplete penetrance and is allele dependent. It is seen in some Cam alleles as heterozygous and in transheterozygous combinations. I tested the wings of flies heterozygous for Strn-Mlck and some of the Cam alleles (kindly provided by Kathy Beckingham), but did not observed wing defects.

Mlck-2 is another mlck-like gene in the Drosophila genome that has been characterized in our lab. Ivan Tesic isolated a Mlck-2 insertion mutant (Mlck-2') (Tesic, 2001). I tested for genetic interaction between Strn-Mlck and Mlck-2. I found ectopic vein material in the wings of Strn-Mlck'/Mlck-2' transheterozygous flies (Figure 3.9 F). The ectopic vein material protrudes from vein L5 and this phenotype is 6% penetrant also (see Discussion).

**Examination of the indirect flight muscle ultrastructure of the Strn-Mlck mutants**

The Strn-Mlck mutants have a wing shape and position defect that is very likely responsible for the flightless phenotype. However, I wanted to examine the muscle ultrastructure of the mutants to verify the flight impairment is not due to, or does not also involve, defects of the flight musculature. Normal structure would be in keeping with previous work done in our lab that showed that the IFM of MLC2 mutant flies with serine
to alanine substitutions at the MLCK phosphorylation target sites was normal. These flies had no functional MLC-2 except for the Ala substituted Ser66 and Ser67, thus showing that phosphorylation of MLC-2 is not necessary for myofibrillogenesis (Tohtong et al., 1995).

The ultrastructure of the IFM of the Strn-Mlck mutants was examined by electron microscopy. Transverse and longitudinal sections of the dorsal ventral muscles (DVM) and the dorsal longitudinal flight muscles (DLM) in wild-type (Canton S) and the Strn-Mlck mutants were examined. Several individuals of each line were analyzed and representative electron micrographs of these muscles are shown in Figures 3.10-3.14.

Electron micrographs of longitudinal and transverse sections of wild-type Canton S flies are displayed in Figure 3.10. The myofibrils are well organized with interconnecting thick and thin filaments running parallel to the longitudinal axis of the myofiber. The Z lines, which are the most electron dense structures seen, define adjacent sarcomeres. The Z lines and M lines are straight and extend over the entire width of the myofibril (Figure 3.10 A). Myofibers have a cylindrical shape with similar diameters as observed in cross sections (Figure 3.10 B). The myofilaments are arranged with six thin filaments surrounding each thick filament in a hexagonal pattern. The thick filaments are also arranged in a hexagonal lattice, giving the myofilament a rigid double hexagonal appearance.

Longitudinal and transverse sections of the IFM in the Strn-Mlck mutants (Figures 3.11-3.14), show the overall appearance of the muscles is normal. In cross sections, the myofilaments are arranged in the well organized double hexagonal pattern
Figure 3.10  Ultrastructure of the IFM of Canton S adults.  (A) Electron micrograph of a longitudinal section of the IFM of a wild type fly.  Five adjacent myofibrils, which are the contractile elements of the muscle cell are shown.  The myofilaments that make up the myofibrils are intact and run parallel to the longitudinal axis of the myofiber, and are made up of interconnecting thick and thin filaments.  The myofilaments are arranged in repeating units called sarcomeres that give the myofibrils their striated appearance.  The sarcomeres are defined by the Z lines, the most electron dense structures in the sarcomere.  The Z lines and M lines appear straight and span the entire width of the myofibril.  (B) Electron micrograph of a cross section of the IFM in wild type flies.  The myofibrils are cylindrical structures with similar diameters.  The myofilaments are arranged in a double hexagonal pattern, with 6 thin filaments surrounding each thick filament, which in turn are also arranged in a hexagonal pattern.
Figure 3.10 Ultrastructure of the IFM of Canton S adults.
Figure 3.11  Ultrastructure of the IFM of Strn-Mleck^4 adults. (A) Longitudinal
section. The overall appearance of the myofibrils is comparable to wild type. The Z
lines span the entire width of the myofibril, although there is a slight deviation at the
periphery of the sarcomere. (B) Cross section. The myofibrils appear normal as the
myofilaments are arranged in a double hexagonal pattern.
Figure 3.11 Ultrastructure of the IFM of Strn-Mlck^I adults.
Figure 3.12 Ultrastructure of the IFM of Strn-Mlck3 adults. (A) Longitudinal section. The overall appearance of the myofibrils is comparable to wild type. The Z lines span the entire width of the myofibril, although there is a slight deviation at the periphery of the sarcomere. (B) Cross section. The myofibrils appear normal as the myofilaments are arranged in a double hexagonal pattern.
Figure 3.12 Ultrastructure of the IFM of \textit{Strn-Mlck}^3 adults.
Figure 3.13  Ultrastructure of the IFM of Strn-Mlck$^{-1}$ adults. (A) Longitudinal section. The overall appearance of the myofibrils is comparable to wild type. The Z lines span the entire width of the myofibril, although there is a slight deviation at the periphery of the sarcomere. (B) Cross section. The myofibrils appear normal as the myofilaments are arranged in a double hexagonal pattern.
Figure 3.13 Ultrastructure of the IFM of \textit{Strn-Mllck}^{at} adults.
Figure 3.14  Ultrastructure of the IFM of Strn-Mlck\(^2\) adults. (A) Longitudinal section. The overall appearance of the myofibrils is comparable to wild type. The Z lines span the entire width of the myofibril, although there is a slight deviation at the periphery of the sarcomere. (B) Cross section. The myofibrils appear normal as the myofilaments are arranged in a double hexagonal pattern.
Figure 3.14  Ultrastructure of the IFM of *Strn-Mlck*^2^ adults.
(Figures 3.11-3.14 B). In longitudinal sections, the overall appearance of the myofibrils is comparable to wild-type. The Z lines span the width of the myofibril, although in some cases there is a slight deviation with the Z lines appearing somewhat wavy at the periphery of the sarcomere.

In summary, *Strn-Mlck* mutations appear to have no effect on muscle structure. This is in keeping with similar results found earlier in our lab with the MLC2 mutant flies that had mutations at the MLCK target sites (Tohtong *et al.*, 1995). However, despite normal muscle structure, flies mutant for Strn-Mlck show flight anomalies. This flightless phenotype is most likely due to the wing positioning/shape defect observed in the flies.
Discussion

This chapter describes a successful screen in which I isolated mutant alleles of the \textit{Strn-Mlck} gene in \textit{Drosophila}. Our lab had shown that MLC2 double mutants with serine to alanine substitutions at the MLCK target sites were viable and recessive flightless, but their muscle structure was normal. Thus we reasoned \textit{Strn-Mlck} mutants affecting muscle specific transcripts would also be recessive flightless flies, mimicking the effect seen with the serine to alanine MLC2 double mutants. Three putative mutants were isolated and confirmation that these were \textit{bona fide} \textit{Strn-Mlck} mutants came from genomic Southern analysis and sequencing data. Two of the mutants are deletions in the catalytic region of the gene (\textit{Strn-Mlck}^1 and \textit{Strn-Mlck}^3). I was unable to map the aberration underlining the \textit{Strn-Mlck}^2 mutant, although it is a genetic allele of \textit{Strn-Mlck}. Likewise, I was able to show through genetic complementation analysis that \textit{curved} is an allele of the \textit{Strn-Mlck} gene and Southern analysis verified that \textit{c}^l is an insertion in the gene.

As with the double serine MLC2^{S66A,S67A} mutants, the \textit{Strn-Mlck} mutant flies are flightless and, likewise, this behavior is most likely not due to structural muscle defects. The ultrastructure of the IFM appears normal as determined by electron microscopy, except for an occasional defect in the Z lines at the periphery of the myofibrils in the mutants. The mechanical properties of the IFM in the \textit{Strn-Mlck} mutants were examined by a former graduate student in the lab (Tesic, 2001). These studies, which were done in the \textit{Strn-Mlck}^2 and \textit{Strn-Mlck}^{c1} mutants, show no difference in the muscle mechanics as compared to wild-type. Therefore, the flight impairment in the mutants is most likely due to
to the wing position effect, and not to a reduction in MLC2 phosphorylation affecting the muscle mechanics. However, it is important to note these mechanical studies were not performed on the mutants known to completely lack catalytic function and further studies should be conducted to assess the mechanical properties of mutants which lack the kinase function of Strn-Mlck.

It should also be noted that we do not have a true null in hand that affects the entire gene and takes out the function of all the transcripts (see Chapter 4). Recent computer gene analysis of the *Drosophila* genome sequence has predicted that *Strn-Mlck* encodes several conceptual proteins, including a large Stretchin-Mlck and other isoforms (Stretchin and Kettin-like) that could play a role in muscle structure (Champagne *et al.*, 2000; Vigoreaux, 2001). Evidence that this is a possibility comes from studies in the localization of the A(225) protein which has been shown to be the protein product of the predicted ~7 kb Kettin-like transcript. Patel and Saide (2001) demonstrated this protein is localized on the A band in IFM. The Kettin-like isoform consists of tandem repeats of Ig domains, which are motifs found in many myosin associated proteins. The A(225) protein has been shown to be a myosin associated protein and it specifically interacts with the myosin rod region of the myosin heavy chain (Patel & Saide, 2003), therefore this isoform could be implicated as a structural component of the IFM.

Another screen to isolate mutants affecting these transcripts alone and all the transcripts (a true null) should be undertaken. A gamma irradiation screen similar to the one used in this study to generate the *Strn-Mlck* mutants can be done with some modifications. We could irradiate wildtype males as before and cross them to the *Strn-Mlck* mutants and screen the progeny not only for a flightless phenotype, but also for an
extra vein phenotype or a lethal phenotype as we can not predict the phenotype of knocking out the 7 kb Kettin-like and 19 kb Stretchin isoforms or a complete null of the gene would be. It would be interesting to see the effect of such a mutant not only on the mechanical properties of the muscle, but also in the muscle structure of the IFM. A mutant affecting the 19 kb Stretchin, 7 kb Kettin-like or the large 25 kb Stretchin-Mlck transcript showing muscle defects will confirm a structural role for Strn-Mlck.

Dickinson et al. (1997) suggest that only 25% of the native MLC2 protein is phosphorylated by MLCK as there is only a slight accumulation of unphosphorylated MLC2 and reduction of the phosphorylated variants in the double serine MLC2\(^{S66A,S67A}\) mutants (Dickinson et al., 1997; Tohtong et al., 1995). However, a Strn-Mlck mutant completely lacking the catalytic function has a normal MLC2 phosphorylation pattern (Tesic, 2001). However, the most significant result is that the Strn-Mlck mutants lacking the kinase activity are viable. From the analysis of the Mlc2\(^{S66A,S67A}\) mutants, we expected that a muscle specific Strn-Mlck mutant would be viable and fertile (Tohtong et al., 1995). Studies on the role of nonmuscle MLC2 phosphorylation show this is critical for a number of processes during oogenesis (Jordan & Karess, 1997). We have shown that Strn-Mlck is expressed in both muscle and nonmuscle cells and therefore Strn-Mlck may function to phosphorylate both muscle and nonmuscle isoforms of MLC2. If Strn-Mlck is the only MLCK in Drosophila, finding viable mutants that lack the kinase activity is surprising. These mutants would be expected to be lethal as the nonmuscle functions are essential for viability. The fact that the Strn-Mlck mutants are alive and fertile suggests two possibilities. One possibility is that Strn-Mlck is not responsible for MLC2 phosphorylation at the predicted MLCK dependent phosphorylation sites (Ser66 and
Ser67). Another possibility is that there might be redundancy of its function, and there
could be other genes that could provide the kinase function to these mutants and
compensate for the lack of Strn-Mlck-kinase activity.

Indeed, with the completion of the *Drosophila* genome sequence it became clear
that a second gene encoded an additional MLCK. Ivan Tesic characterized the gene as
part of his thesis (Tesic, 2001). The gene has 51% homology to Strn-Mlck in the
catalytic region (he named this new gene *Mlck*-2). Interestingly, I found double
heterozygous flies for *Strn-Mlck*¹ and *Mlck*-2¹ show an ectopic wing vein phenotype
similar to *Strn-Mlck*¹ homozygotes and Cam mutants (Figure 3.9 F), suggesting they may
be functionally related. As the Strn-Mlck phosphorylation function may be redundant for
regulating the role of MLC2 in muscle contraction, a study of the effect of knocking out
the function of both *Strn-Mlck* and *Mlck*-2 in the fly may be necessary to further elucidate
the role phosphorylation of MLC2 plays in muscle contraction.
CHAPTER 4

TRANSCRIPT CHARACTERIZATION OF STRN-MLCK MUTANTS

Introduction

Characterization of the Strn-Mlck gene demonstrates it has a complex structure, with the potential to encode multiple transcripts that give rise to many different protein isoforms. To begin to understand the function of Strn-Mlck in Drosophila, it is important to determine if the transcripts are expressed in spatially and/or temporally specific patterns. Furthermore, in order to interpret the phenotype of the Strn-Mlck mutants generated in this work (Chapter 3), it is important to determine which transcripts are disrupted in a given allele. In this chapter I present and discuss analysis of the transcript specific expression pattern in wild type embryos and the transcripts produced by the Strn-Mlck mutants.

Northern blot and cDNA analysis of the Strn-Mlck demonstrated that the gene produces multiple messages, ranging in size from a transcript of 3.6 kb to transcripts greater than 18 kb in length (Kojima et al., 1996; Tohtong et al., 1997). Utilizing the Drosophila genome sequence (Adams et al., 2000) and computer analysis, Champagne et al. (Champagne et al., 2000) were able to make predictions about the gene structure of
Strn-Mlck. The predicted gene consists of 33 exons spanning a total of 38 kb of genomic DNA. Multiple conceptual transcripts, which range in size from 3.6 kb to 25 kb, are predicted to be generated through the use of alternative splicing at the 5’(exons 11, 12) and 3’(exon 32, long form, short form and no exon 32) ends of the gene, the use of alternative promoters (exon 7, 20a, 24, 26) and alternative polyadenylation signals (exon 15a, 16). (The use of exon 26 as an alternative promoter is questionable as no evidence for the use of this promoter was found by reverse transcription (RT) and PCR analysis by Ivan Tesic in our lab (Tesic, 2001)). The predicted gene structure suggests that Strn-Mlck produces 3 classes of transcripts, 2 of which share no sequence and one class that lacks a kinase domain (Figure 1.9).

The smallest transcripts (3.6 and 5.2 kb, encoding the so called small MLCKs) are generated by using internal promoters (exon 24 and 20a respectively) in the 3’ end of the gene and use the polyadenylation signal at exon 33. The other transcripts that use the polyadenylation signal at exon 33 encode the Giant-Mlck (13 kb transcript), and Stretchin-Mlck (25 kb transcripts), which are initiated at promoters in exons 7 and 1, respectively. The other set of transcripts produced terminate at one of two polyadenylation signals in exons 15a and 16. The Stretchin transcript (exons1-16) has a predicted size of 19 kb while the Kettin-like transcript (exons 7-16) has a predicted size of ~7 kb. This set of transcripts lack kinase function and share no sequence with the transcripts initiated at exons 20a and 24.

Given that the Strn-Mlck gene has a complex structure and the potential to produce messages of different sizes encoding different isoforms, it was suspected that some of these transcripts may be tissue specific. In this chapter I describe the spatial
expression pattern of various transcripts by *in situ* hybridization to embryos using specific RNA probes. Given the putative diversity of the gene, it was important to determine the pattern of expression in the *Strn-Mlck* mutants. I also examine the transcripts made in the *Strn-Mlck* mutants by reverse transcription (RT) and PCR.
Results

Expression of Strn-Mlck transcripts in embryos

In situ hybridization with digoxigenin-labelled RNA probes was performed in wild type (Canton S) embryos following standard procedures (see material and methods) (O'Neill & Bier, 1994). Previous work done in our lab detected the expression of Strn-Mlck in embryos using a probe to the catalytic region (Figure 4.1), (Tohtong et al., 1997). This probe is common to 4 of the conceptual transcripts encoded by Strn-Mlck. In this work, we demonstrated that the probe from the catalytic region recognizes transcripts in both muscle and nonmuscle cells. We observed high levels of expression in cellularizing embryos and even greater levels of expression in muscles of late embryos (Tohtong et al., 1997). During dorsal closure (stage 14 embryos), there is intense expression in the segmental muscles of the body wall and the pharyngeal muscles (Figure 4.1). The expression seen in young embryos where there are no muscle cells present and the expression seen in late embryos, demonstrate Strn-Mlck is expressed in both muscle and nonmuscle cells and therefore the gene is likely to encode both the cytoplasmic and muscle isoforms of the enzyme.

Many proteins involved in muscle contraction in the fly have already been shown to encode tissue specific transcripts (see review by Bernstein et al., 1993), and it is therefore possible that this occurs with Strn-Mlck. In order to determine if the Strn-Mlck transcripts have tissue specific expression, I made transcript specific digoxigenin-labelled RNA probes corresponding to the 5’ ends of the messages (exon 1, 7, 20a and 24) and used them for in situ hybridization to embryos (Figure 4.1). In addition, the expression
Figure 4.1 Exon specific $Strn-Mlck$ mRNA expression pattern in wild type embryos. *In situ* hybridizations were performed in wild type (Canton S) embryos with exon specific digoxigenin labelled RNA probes. Images show the expression pattern in stage 14 embryos. Embryos hybridized with RNA probes corresponding to Exons 1, 7, 20 and the catalytic region (CAT) show a muscle specific pattern. There is abundant expression in the segmental muscles of the body wall and in the presumptive pharyngeal muscles. Embryos hybridized with probes corresponding to exons 24 and 32 show high levels of nonmuscle specific expression. The transcripts that incorporate these exons may be involved in the nonmuscle function of Strn-Mlck. Images are lateral views, oriented anterior to the left and dorsal at the top.
Figure 4.1 Exon specific Strn-Mlec mRNA expression pattern in wild type embryos.
pattern of exon 32 was analyzed as this exon is alternatively spliced and also exists in short and long forms through the use of different splice sites (Kojima et al., 1996; Tohtong et al., 1997). Exon 32 codes for two-thirds of the regulatory region. The regulatory region is involved in a mechanism whereby MLCK is inhibited in the absence of its substrate, MLC2. Isoforms lacking the regulatory region raise the possibility of constitutively active forms of Strn-Mlck in Drosophila.

**Expression pattern of exons 1, 7 and 20a in embryos**

Transcripts containing exons 1, 7 and 20a show a muscle specific pattern in late embryos (Figure 4.1). High levels of expression are seen in the pharyngeal muscles and the body wall muscles. In situ hybridization of early embryos with these probes also shows expression in the cellularizing embryo (data not shown). This demonstrates that transcripts containing these exons are expressed both in muscle and nonmuscle cells.

**Expression pattern of exon 24 in embryos**

A probe corresponding to exon 24 shows no specific hybridization to muscle cells in late embryos and transcripts containing this exon seem to be ubiquitously expressed (Figure 4.1). Expression was also seen in early embryos (data not shown). Therefore, the transcript containing exon 24 (3.6 kb small-Mlck) appears to be a nonmuscle specific transcript.
Expression pattern of the alternatively spliced exon 32 in embryos

The expression pattern of the alternatively spliced exon 32 also appears to be nonmuscle specific, as the signal is present throughout the late stage embryo (Figure 4.1) and in early stage embryos (data not shown).

Transcripts expressed by the Strn-Mlck mutants

To investigate the transcripts expressed by the mutants, reverse transcription (RT)-PCR reactions were performed. RNA was extracted from the various Strn-Mlck mutants and wild-type Canton S adult flies. cDNA pools were generated using different reverse primers for the reverse transcription reactions, and exon specific forward and reverse primers for the PCR reactions.

Transcripts containing the catalytic region are affected in some Strn-Mlck mutants

Figure 4.2 shows the RT-PCR products of cDNA pools generated with an RT reverse primer specific to exon 33. Two different PCR reactions were performed. Both PCR reactions used a reverse primer to exon 29 but different forward primers. Figure 4.2 A, shows a PCR reaction with a forward primer specific to exon 28. All mutants except for Strn-Mlck3 produce transcripts containing the catalytic region.

The deletion in the Strn-Mlck3 mutant maps close to this region (see Chapter 3). Therefore, I used a second set of primers and the same RT cDNA pool to analyze the transcripts further. Using the same reverse primer as before and a forward primer
Figure 4.2  RT-PCR characterization of the transcripts containing the catalytic region in the *Strn-Mlck* mutants. RNA from wild-type Canton S (Cs) and the *Strn-Mlck* mutants was subjected to reverse transcription with a primer in exon 33 (green arrow). This was followed by 2 different PCR reactions. Both PCR reactions shared the same gene specific reverse primer within exon 29 (red reverse arrow), but had different gene specific forward primers either to exon 28 (red forward arrow, panel a), or exon 27 (blue forward arrow, panel b). Transcripts containing the catalytic region are made in the mutants except for *Strn-Mlck*\[^1\] in which transcripts containing this region are completely absent and the *Strn-Mlck*\[^3\] mutant which has a smaller band corresponding to the deletion of exon 27 and 28. Panel c shows a control RT-PCR reaction with gene specific primers to *guf*. Genomic DNA- P1 clone DS08018. *mlck*\(^1\)-*Strn-Mlck*\(^1\), *mlck*\(^2\)-*Strn-Mlck*\(^2\), *mlck*\(^3\)-*Strn-Mlck*\(^3\), *mlck*\(^c\)-*Strn-Mlck*\(^c\).
Figure 4.2 RT-PCR characterization of the transcripts containing the catalytic region in the *Strn-Mleck* mutants.
specific to exon 27, I found that the $\text{Strn-Mlck}^3$ mutant produces a truncated (Figure 4.2 B) transcript, which correlates with the small deletion in the mutant.

It also appeared that the level of transcripts containing the catalytic region in the $\text{Strn-Mlck}^{c1}$ and $\text{Strn-Mlck}^2$ mutants is somewhat reduced (Figure 4.2 C). The insertion in the $\text{Strn-Mlck}^{c1}$ mutant maps to exons 21 and 22, corresponding to the 5’ region of the 5.2 kb transcript. It is possible that the $\text{Strn-Mlck}^{c1}$ insertion affects transcription of the gene, and this reduced transcription accounts for the phenotype. However, a quantitative analysis has not been done.

**Transcripts initiating in exon 1 made by the $\text{Strn-Mlck}$ mutants**

Figure 4.3 shows the products of RT-PCR reactions designed to analyze the transcripts containing exon 1 in the $\text{Strn-Mlck}$ mutants. Since there are two different classes of transcripts that are predicted to contain exon 1 (Figure 1.9), two sets of cDNA pools were generated with RT reverse primers specific to exon 9 (Figure 4.3 A) and exon 16 (Figure 4.3 B). The PCR reaction was done utilizing a gene specific forward primer in exon 1 and a gene specific reverse primer in exon 3. This analysis tested whether the mutants produce transcripts containing exon 1, which is predicted to be included in the 25 kb Stretchin-MLCK and the 19 kb Stretchin transcripts. The RT generated with exon 16 specific primers should be specific for the 19 kb Stretchin transcripts, as exon 16 is predicted to be spliced out of the bigger 25 kb Stretchin-Mlck transcripts (Champagne et al., 2000). The results show all mutants produce transcripts containing this region, although the levels are somewhat reduced in the $\text{Strn-Mlck}^3$ and $\text{Strn-Mlck}^2$ mutants.
Figure 4.3 Transcripts containing exon 1 made by the *Strn-Mlck* mutants. RNA from wild-type Canton S (Cs) and the *Strn-Mlck* mutants was subjected to reverse transcription with a reverse primer in exon 9 (a, green arrow), and in exon 16 (b, blue arrow). The PCR reaction was done utilizing a gene specific forward primer in exon 1 and a gene specific reverse primer in exon 3. This analysis tested whether the mutants produce transcripts containing exon 1 which is included in the 25 Kb Stretchin-Mlck and the 19 Kb Stretchin transcripts. All mutants produced transcripts with this region. Panel c shows a control RT-PCR reaction with gene specific primers to RP49. Genomic DNA-P1 clone DS08018. *mlck*<sup>1</sup> - *Strn-Mlck*<sup>1</sup>, *mlck*<sup>2</sup> - *Strn-Mlck*<sup>2</sup>, *mlck*<sup>3</sup> - *Strn-Mlck*<sup>3</sup>, *mlck*<sup>c</sup> - *Strn-Mlck*<sup>c</sup>.
Figure 4.3 Transcripts containing exon 1 made by the *Strn-Mlck* mutants.
Transcripts initiating in exon 7 made by the *Strn-Mlck* mutants

Figure 4.4 shows the RT-PCR reactions designed to analyze transcripts containing exon 7 in the *Strn-Mlck* mutants. The same two sets of cDNA pools generated for the analysis with exon 1 (see section above) were used, with RT reverse primers specific to exon 9 (Figure 4.4 A) and exon 16 (Figure 4.4 B). The PCR reaction was performed utilizing a gene specific forward primer in exon 7 and a gene specific reverse primer in exon 8. This tested whether the mutants produce transcripts containing exon 7, which is included in the predicted 13 kb Giant-Mlck and the 7 kb Kettin-like transcripts. The cDNA generated with an exon 16 specific primer is specific for the 7 kb Kettin-like transcript, as exon 16 is spliced out of the bigger 25 kb Stretchin-Mlck transcript (Champagne *et al.*, 2000). The results show all mutants produce transcripts containing exons 7 and 8.

Transcripts initiating in exon 20 made by the *Strn-Mlck* mutants

The same reverse transcription reactions utilizing a reverse primer to exon 33, generated for the analysis of the catalytic region, were used for this analysis. Figure 4.5 shows the RT-PCR results of the PCR reactions utilizing a gene specific forward primer in exon 20 and a gene specific reverse primer in exon 22. This tests the ability of the mutants to make the 5.2 kb transcript (Champagne *et al.*, 2000), as exon 20 is specific for this transcript. The results show this transcript is missing in the *Strn-Mlck*\(^1\) and *Strn-Mlck*\(^c1\) mutants. As discussed in Chapter 3, *Strn-Mlck*\(^1\) contains a deletion in the region, and therefore the transcript is expected to be absent. The *Strn-Mlck*\(^c1\) mutant contains an insertion in this region. The missing transcript could be due to the insertion interfering
with transcription. Another possible explanation is that a bigger transcript is made but it is undetected by our PCR conditions. All other Strn-Mlck mutants are able to produce this transcript.
Figure 4.4  Transcripts containing exon 7 made by the Strn-Mlck mutants. RNA from wild-type Canton S (Cs) and the Strn-Mlck mutants was subjected to reverse transcription with a reverse primer in exon 16 (a, blue arrow), and in exon 9 (b, green arrow). The PCR reaction was performed utilizing a gene specific forward primer in exon 7 and a gene specific reverse primer in exon 8. This analysis tested whether the mutants produce transcripts containing exon 7, which is included in the 13 Kb Giant-Mlck and the 7 Kb Kettin-like transcripts. All mutants produced transcripts with this region. Panel c shows a control RT-PCR reaction with gene specific primers to RP49. Genomic DNA-P1 clone DS08018. \textit{mlck}^1 - Strn-Mlck^1, \textit{mlck}^2 - Strn-Mlck^2, \textit{mlck}^3 - Strn-Mlck^3, \textit{mlck}^c - Strn-Mlck^c.
Figure 4.4 Transcripts containing exon 7 made by the *Strn-Mlck* mutants.
Figure 4.5 Transcripts containing exon 20 made by the Strn-Mlck mutants. RNA from wild-type Canton S (Cs) and the Strn-Mlck mutants was subjected to reverse transcription with a reverse primer in exon 33 (green arrow). The PCR reaction was performed utilizing a gene specific forward primer in exon 20 and a gene specific reverse primer in exon 22 (panel a). This analysis tested whether the mutants produce transcripts containing exon 20, which is included the 5.2 Kb small-Mlck transcript. All mutants produced this transcript with the exception of Strn-Mlck1, which has a deletion in the region and Strn-Mlckc1, which has an insertion in the region. Panel c shows a control RT-PCR reaction with gene specific primers to guf. Genomic DNA- P1 clone DS08018. 

mlck1 - Strn-Mlck1, mlck2 - Strn-Mlck2, mlck3 - Strn-Mlck3, mlckc1 - Strn-Mlckc1.
Figure 4.5 Transcripts containing exon 20 made by the *Strn-Mlck* mutants.
Discussion

Different strategies can be used by an organism to produce tissue specific transcripts. In *Drosophila*, there are a number of mechanisms that allow the production of the different tissue specific protein isoforms and this has been reviewed by Bernstein *et al.* for muscle genes (Bernstein *et al.*, 1993). One is the tissue specific transcription of a member/s of a multigene family, as is the case in the muscle specific isoforms of the actin gene (Fyrberg *et al.*, 1980). The other mechanism is through alternative RNA splicing, where a single gene gives rise to multiple transcripts by including or excluding an exon/exons, as is the case for the myosin heavy chain gene (Bernstein *et al.*, 1983). Also, a gene may have multiple promoters, which may encode different primary transcripts, as is the case in a tropomyosin gene (Bautch *et al.*, 1982; Hanke & Storti, 1988).

*Strn-Mlck* is a large and complex gene, which produces many transcripts through alternative splicing of exons and differential use of several polyadenylation sites and promoter regions. The possibility exists that some of these transcripts are tissue specific. Therefore, I analyzed the transcript specific expression pattern in wild type embryos by *in situ* hybridization. A probe corresponding to regions of the catalytic domain shows high levels of expression in cellularizing embryos, where there are no muscle cells, and therefore corresponds to expression of a nonmuscle transcript. Very high levels of expression are also seen in muscles of the late embryo, after the onset of muscle development (Bate, 1990; Bate *et al.*, 1991). As the *Strn-Mlck* likely codes for the muscle and nonmuscle transcripts, the expression seen in late embryos is likely due to a
higher level of expression of Strn-Mlck in muscle cells. However, this does not exclude the possibility of expression in nonmuscle cells.

By utilizing transcript specific probes, I have shown that transcripts containing exons 1, 7 and 20a are expressed in early embryos, in which there are no muscle cells present, and in muscle tissues in late embryos. Exon 1 and exon 7 are included in a number of Strn-Mlck transcripts. Exon 1 is the first exon in the 25 kb Strn-MLCK transcript and in the 19 kb Stretchin transcript. Both have similar N-termini but the smaller 19 kb Stretchin transcript uses an internal polyA site and lacks exons encoding the kinase domain. In a similar manner, exon 7 is included in both the 13 kb Giant-Mlck and the 7 kb Kettin-like transcript, with the latter lacking the kinase function.

The 5.2 kb small-Mlck transcript contains exon 20a, which is specific to this transcript. The transcript also has kinase function, and as with the probe derived from the catalytic region, this transcript is expressed in muscle and nonmuscle cells.

On the contrary, no apparent signal was observed with the probe specific to exon 24 in any muscle cells. Exon 24 is unique to the 3.6 kb small-Mlck transcript, which is expressed throughout development (Kojima et al., 1996; Tohtong et al., 1997). This was confirmed by Ivan Tesic (2001) using RT-PCR. The expression analysis in embryos I have performed, suggests that this is a cytoplasmic specific transcript as it is ubiquitously expressed. Champagne et al. (2000) suggest this might be the closest functional homologue of the vertebrate smooth muscle/non-muscle MLCK (sm/nm MLCK).

Expression of the transcripts containing exon 32 (the alternatively spliced exon which can also generate transcripts with a long or short form of exon 32) is also ubiquitous. Exon 32 encodes part of the regulatory region and transcripts lacking exon
32 are presumed to be regulated in a Ca\textsuperscript{2+}/CaM independent fashion. In keeping with the results, exon 32 is likely to be included in transcripts encoding primarily cytoplasmic Strn-MLCKs, while transcripts lacking exon 32 encode primarily Ca\textsuperscript{2+}/CaM independent Strn-MLCKs in muscle cells. The expression of proteins in embryos supports this idea. Using antibodies generated against the C-terminal region of the Strn-Mlck isoforms, it was demonstrated that isoforms lacking exon 32 are expressed in more muscle types (gut, pharyngeal, body wall) than the isoform containing exon 32 (only hindgut) (Tesic, 2001).

Figure 4.6 is a summary of the data on the Strn-Mlck transcripts affected in the mutants. The results of the RT-PCR analysis of the mutants correlate with the genomic Southern analysis. Three main points have emerged from this study. First, none of the mutants lack transcripts corresponding to the 7 kb Kettin-like or 19 kb Stretchin transcripts (see also below). Second, two mutants lack transcripts encoding isoforms with a kinase domain. Third, the Strn-Mlck\textsuperscript{c1} allele is likely to be the mildest; the mutant produces transcripts encoding isoforms with a kinase domain and the flies are able to glide evidenced by a slightly better flight ability over the other alleles. These points are expanded on in the next section.

I have described the isolation of the first Strn-Mlck mutants in Drosophila. The mutants are recessive flightless and the muscle structure of the IFM is unaffected,
Figure 4.6 Summary of Strn-Mlck mutant data and transcripts affected in the mutants. (A) Representation the Strn-Mlck mutants mapped so far and their position relative to the putative promoters (numbered open boxes), and the catalytic region (CAT). (B) Six conceptual transcripts that are the result of alternative splicing events and the use of 3 polyadenylation signals and 4 putative promoters throughout the region. Figure adapted from (Champagne et al., 2000). (C) Table representing the conceptual transcripts affected in the mutants.
A) Promoters

B) Conceptual transcripts

C) Conceptual transcripts affected in mutants

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Figure 4.6 Summary of Strn-Mlck mutant data and transcripts affected in the mutants.
corroborating our previous finding that MLCK mediated phosphorylation of MLC2 is not required for myogenesis. However, after the annotation of the *Drosophila* genome and the analysis of the *Strn-Mlck* genetic locus, we can conclude these mutants are not complete nulls of *Strn-Mlck*. The biggest deletion mutant available (*Strn-Mlck*) does not affect a set of transcripts that are potentially encoded by the gene. The possibility still exists that Strn-Mlck plays a structural role in muscle assembly or stability, as there are other conceptual transcripts the gene can encode which would produce kinase independent isoforms. Our antibodies do not react with these isoforms, and these transcripts are unaffected in our mutants. One of these isoforms is rich in PEVK and SAIDE motifs, which have been implicated in muscle connectivity as structural components of the IFM. PEVK domains were first identified in vertebrate titin and later in other members of the titin family (D-Titin, projectin and sm/nm MLCK), and have been associated with the elasticity of the protein but also have been implicated in a structural role by possibly supporting specific interactions with other myofibrillar proteins.

The other isoform lacking kinase activity (7 kb Kettin-like) consists of tandem repeats of Ig domains, which are motifs found in many myosin associated proteins, and this isoform could be a structural component of the IFM as it likely associates with myosin. Indeed, evidence that this is a possibility comes from studies in the localization of the A (225) protein. Studies have shown this is the protein product of the predicted ~7 kb Kettin-like transcript (Patel & Saide, 2001; Patel & Saide, 2003). These authors demonstrated the protein is localized in the A band in IFM. They also found mutants lacking IFM myosin show no accumulation of the A (225) protein. Interestingly, Bloor
and Kiehart (Bloor & Kiehart, 2001) show that nonmuscle myosin II is found in the Z-lines of larval muscles and that it may play a role in sarcomere formation. This suggests the possibility that the nonmuscle isoform of Strn-Mlck may also play a role here as the larger PEVK and SAIDE containing isoforms may be able to associate with proteins in the Z-lines (as is the case with the larger titins). As our Strn-Mlck mutants do not affect all transcripts, it will be necessary to isolate complete nulls of Strn-Mlck as a mutant affecting all protein isoforms may show disrupted IFM structure thus confirming a structural role for Strn-Mlck in myofibrillogenesis.

The Strn-Mlck\(^2\) mutant was generated using gamma irradiation, which tends to produce gross chromosomal lesions. However, Southern hybridization and PCR analysis of the Strn-Mlck locus in this mutant failed to identify an aberration, suggesting a lesion involving a small change. The transcript analysis utilizing RT-PCR shows that although the transcripts produced in this mutant are present, the levels appear to be somewhat reduced when primers corresponding to the catalytic region, exon 1 and exon 7 are used. A possibility exists that the lesion in this mutant is one that creates a premature stop codon and causes the transcripts to be degraded by nonsense mediated decay (NMD). NMD is a surveillance mechanism that is thought to eliminate any defective mRNAs that possess a premature termination codon (Alonso & Akam, 2003; Gatfield et al., 2003; Harries et al., 2004). However, a quantitative analysis of transcript levels in Strn-Mlck\(^2\) has not been done and would be necessary to precisely quantify the transcripts levels.

The mutants include two, which completely lack the kinase activity (Strn-Mlck\(^1\) and Strn-Mlck\(^3\)), and yet these flies are viable and fertile as homozygotes. This suggests redundancy of the kinase function. A first line of evidence that other kinases are able to
phosphorylate MLC2 at the predicted MLCK-dependent phosphorylation sites comes from the double serine MLC2$^{S66A,S67A}$ mutants generated in our lab (Dickinson et al., 1997; Tohtong et al., 1995). These mutants have only a slight increase in accumulation of the unphosphorylated variants of MLC2, and a reduction of the phosphorylated variants (Dickinson et al., 1997; Tohtong et al., 1995). Dickinson et al. (1997) suggest that only 25% of the native MLC2 protein is phosphorylated by MLCK and 70% is phosphorylated by other as yet unknown kinases.

More direct evidence for the redundancy of kinase function of Strn-Mlck comes from the analysis of the MLC2 isovariant accumulation in the IFM of the Strn-Mlck mutants (Tesic, 2001). This analysis shows the phosphorylation pattern is normal even in a mutant completely lacking kinase activity, suggesting either Strn-Mlck is not responsible for MLC2 phosphorylation at the predicted MLCK dependent phosphorylation sites (Ser66 and Ser67) or that there might be redundancy of its function, with another Strn-Mlck-like gene providing a similar catalytic function. In the Drosophila genome there is a second gene encoding an additional MLCK-like protein. Ivan Tesic isolated and characterized a mutant for the gene. I have shown this mutant interacts genetically with Strn-Mlck and may possibly be involved in a similar process as Strn-Mlck (Chapter 3). Inhibition of this second MLCK-like gene, also known as CG1776, using RNAi was discovered to cause morphological defects in tissue culture cells (Kiger et al., 2003) showing a function in nonmuscle cells. Further studies need to be undertaken to elucidate the possible role this new kinase or other possible kinases may play in MLC2 phosphorylation. As the kinases may be functioning concurrently with Strn-Mlck to provide the flies with a constant supply of kinase function, it may be
necessary to inhibit the function of both genes simultaneously to be able to see an effect in the flies.
Concluding Remarks

The focus of this study was to understand the role of Stretchin-Myosin light chain kinase (Strn-Mlck) phosphorylation of the regulatory light chain (MLC2) in muscle function and structure in *Drosophila*. We had previously shown that phosphorylation of MLC2 at the predicted Strn-Mlck target sites was required for the stretch activation response which powers flight, but not for myogenesis (Tohtong *et al.*, 1995). Flies with mutations at the Strn-Mlck target serines that could not be phosphorylated, were flightless. This inability to fly was not due to altered muscle structure, as the IFM appeared normal, but rather to a reduction in power output. In order to learn more about the role of Strn-Mlck phosphorylation, our lab and another lab cloned the *Drosophila* *Strn-Mlck* gene (Kohama *et al.*, 1996; Tohtong *et al.*, 1997). Analysis showed that the *Strn-Mlck* gene encoded both cytoplasmic and muscle isoforms of the enzyme.

At the time I initiated my thesis work, there were no mutants of the gene available. This was a significant deficit as mutants could provide valuable insight into the function of *Strn-Mlck*. Functional analysis in other systems using MLCK inhibitors in vertebrates and the MLCK-A mutants of *Dictyostelium* provided strong evidence for the importance of myosin regulatory light chain phosphorylation by MLCK in a variety of cellular functions. A role in cell proliferation and cell shape changes was shown by Shoemaker *et al.* (Shoemaker *et al.*, 1990), by using an antisense oligonucleotide for MLCK to inhibit MLCK function in cultured fibroblasts. Kishi *et al.*, (Kishi *et al.*, 2000) inhibited function of MLCK in vascular smooth muscle cells by using antisense RNA and pharmacological inhibitors and found a role for MLCK in cell migration and membrane
ruffling. Endothelial cell retraction as well as RLC phosphorylation was blocked by both removal and inhibition of MLCK (Wysolmerski & Lagunoff, 1990; Wysolmerski & Lagunoff, 1991). Additionally, the use of pharmacological agents to specifically inhibit MLCK has shown a role for MLCK function in other cellular processes such as platelet aggregation and contraction (Mamiya et al., 1989), mast cell secretion (Choi et al., 1994) and cytoskeletal arrangements that affect ion exchange at the plasma membrane (Shrode et al., 1995; Szaszi et al., 2000).

In Dictyostelium discoideum myosin II is regulated through the phosphorylation of the regulatory light chain (RLC) and the myosin heavy chain (MHC). Phosphorylation of RLC occurs at Ser-13. Cells that have a mutant RLC in which Ser-13 has been changed to unphosphorylatable alanine appear normal in all aspects of growth and development (Ostrow et al., 1994), suggesting that RLC phosphorylation is not required for myosin function in vivo. Dictyostelium has an unconventional MLCK (MLCK-A), which is Ca-calmodulin independent, in contrast to vertebrate MLCK that require Ca²⁺/calmodulin for activity (Tan & Spudich, 1990; Tan & Spudich, 1991). Genetic studies with MLCK-A null cells suggest a role for MLCK-A in cytokinesis as these cells are often multinucleated (Smith et al., 1996). However, the levels of phosphorylated RLC are not completely abolished in these MLCK-A null cells (Smith et al., 1996). This suggests redundancy of function with additional kinases in Dictyostelium that are capable of phosphorylating RLC.

As mentioned above, the role of RLC phosphorylation has also been studied in Drosophila muscle using mutant RLC constructs with alanines substituting at the MLCK phosphorylation sites (Tohtong et al., 1995). The function of nonmuscle RLC has also
been examined by Roger Karess’s group. The nonmuscle RLC is encoded by *spaghetti squash* (*sqh*) (Karess *et al.*, 1991). Altering the Strn-Mlck phosphorylation sites in *sqh* to unphosphorylatable alanines shows a phenotype similar to a *sqh* null, and causes defects in proliferation and cytokinesis (Jordan & Karess, 1997). This suggest that phosphorylation of the RLC of nonmuscle myosin is required for proper myosin function during oogenesis and embryogenesis (Jordan & Karess, 1997).

The major contribution of my thesis work to this field of study is the isolation of *Strn-Mlck* mutants in *Drosophila*. Characterization of the mutants has confirmed the spontaneous mutant *curved* as a mutant of *Strn-Mlck*. Significantly, the *Strn-Mlck* mutants completely lacking kinase activity are viable. Expression analysis suggests that Strn-Mlck may function to phosphorylate both muscle and nonmuscle isoforms of MLC2. Therefore, we expected that if Strn-Mlck is the only MLCK in *Drosophila*, the mutants would be lethal as the nonmuscle functions are essential for viability. The fact that the *Strn-Mlck* mutants are alive and fertile suggests redundancy of its function. This idea is also supported by the normal MLC2 phosphorylation pattern of the *Strn-Mlck* mutants (Tesic, 2001). The mutants show a recessive flightless phenotype, however, this is not due to defects in the flight musculature but more likely a wing blade defect, a nonmuscle rather than muscle cell type, suggesting a role in epithelial morphogenesis. Nonmuscle myosin II has been shown to play a role in cell shape changes. In *Drosophila*, imaginal disc morphogenesis depends on cell shape changes (Young *et al.*, 1993). Nonmuscle myosin II and nonmuscle RLC have been implicated in disc morphogenesis as several mutant alleles of these genes produce a malformed phenotype (in wings, legs and eyes) (Edwards & Kiehart, 1996; Gotwals & Fristrom, 1991; Halsell & Kiehart, 1998; Young
et al., 1991). Malformation of the adult appendages suggests improper cell shape changes driven by myosin contraction during disc morphogenesis. Thus, the malformed wing blade phenotype of the Strn-Mlck mutants implicates Strn-Mlck as a cytoskeletal component in the nonmuscle myosin driven contraction that regulates morphogenesis.

It appears that a general theme of redundancy is emerging. The normal MLC2 phosphorylation pattern of Strn-Mlck mutants which suggest redundancy of function, parallels the residual RLC phosphorylation seen in Dictyostelium MLCK-A null mutants. The idea of redundancy of function has also been suggested in the vertebrate system, specifically in the role of RLC phosphorylation in cytokinesis. It has been shown that in addition to MLCK phosphorylation, there are other kinases capable of phosphorylating RLC at the sites that are phosphorylated by MLCK (Ser 19 and Thr18) (reviewed by Matsumura et al., 2001). These are Rho-kinase (Amano et al., 1996; Kureishi et al., 1997), p21 activated kinase (PAK, Chew et al., 1998; Zeng et al., 2000) and citron kinase (Matsumura et al., 2001). However, it is not yet clear whether the kinases are truly redundant in function during cell division acting simultaneously on the same substrate or if different regulatory systems utilizing these different kinases control specific aspects of cell division.

It is certainly likely that other kinases regulate RLC function in Drosophila and indeed Ivan Tesic characterized a second Mlck-like gene, Mlck-2, as part of his thesis (Tesic, 2001). Kiger et al., (2003) have shown that Mlck-2, also known as CG1776, has a role in nonmuscle cell morphogenesis by using RNAi to silence the gene in S2 tissue culture cells. I have demonstrated a genetic interaction between Mlck-2 and Strn-Mlck suggesting they may be functionally related and involved in the same process.
Furthermore as in vertebrates, there could be many more kinases capable of phosphorylating MLC2 that are not in the MLCK family. With the sequencing of the Drosophila genome, and following the methods of Kieger et al., an RNAi screen could be done to screen the genome for other kinases that may be involved in nonmuscle RLC phosphorylation (Kiger et al., 2003).

It will also be important to analyze Mlck-2 function in more depth. Genetic screens should be undertaken to isolate additional mutant alleles of the Mlck-2 gene, followed by their complete characterization. Examining the mutant alleles at the genetic, molecular and biochemical levels would determine possible roles Mlck-2 may play in RLC phosphorylation. It will then be important to generate double mutants of Mlck-2 and Strn-Mlck to examine if there is any change in the phosphorylation pattern of MLC2, or maybe in the mechanics of flight as suggested by the MLC2 double Ser mutants, or if there are defects in the musculature. Inhibiting both genes simultaneously may be required to reveal the full consequence of loss of MLCK function.
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