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Identification of myosin light chains in *Rana pipiens* skeletal muscle and their expression patterns along single fibres

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Summary

Isoforms of myosin heavy chain (MHC) and myosin light chain (MLC) influence contractile kinetics of skeletal muscle. We previously showed that the four major skeletal muscle fibre types in *Rana pipiens* (type 1, type 2, type 3 and tonic; amphibian nomenclature) contain four unique MHC isoforms. In the present study we defined the MLCs expressed in each of these *R. pipiens* fibre types. The MLC composition of single MHC-typed fibres was determined from western blots using a panel of monoclonal MLC antibodies. A total of seven MLCs were identified, including four types of MLC1, two of MLC2 and a single MLC3. Twitch fibre types (types 1, 2 and 3) expressed $MLC1_f$ and $MLC2_f$, while tonic fibres contained a unique set of isoforms, $MLC1_{Ta}$, $MLC1_{Tb}$ and $MLC2_T$. MLC3 was expressed primarily in type 1, type 1-2 and type 2 fibres. Surprisingly, some frogs displayed a striking pattern of MLC expression where a unique isoform of MLC1 ($MLC1_x$) was coexpressed along with the normal MLC1 isoform(s) in all fibre types. $MLC1_x$ was either expressed in all fibres of a given frog or was completely absent. The intraspecific polymorphism in MLC1 expression is likely to have a genetic basis, but is unlikely to be caused by allelic variation. The ratio of MLC3/MLC1 increased in direct proportion to the percentage of type 1 MHC, but was only weakly correlated. The variability in MLC3/MLC1 within a fibre type was extremely large. Both the MHC isoform and MLC3/MLC1 ratio varied significantly between 1 mm segments along the length of fibres. For all segments combined, MLC3/MLC1 increased with the percentage of type 1 MHC, but the correlation between segments was weaker than between fibres.

Key words: frog, *Rana pipiens*, fibre type, skeletal muscle, electrophoresis, contractile protein, isoform.

Introduction

The motor protein myosin in skeletal muscle is a hexameric polypeptide consisting of two myosin heavy chain (MHC) and four myosin light chain (MLC) subunits. Each MHC is associated with one essential light chain (ELC) and one regulatory light chain (RLC; also called alkali light chain). All vertebrates, including humans, express a family of MHC, ELC and RLC isoforms in their skeletal muscles. In a given species, variable expression of multiple MHC and MLC isoforms results in a diverse population of muscle fibres with a wide range of shortening velocities and power capabilities. Thus, myosin isoforms have a critical impact on functional diversity in muscular systems and influence how muscles are adapted to meet the specific motor requirements of an organism.

The influence of MHC and MLC isoforms on the force–velocity relationship remains controversial and may vary among vertebrates (for reviews, see Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). Mechanical studies on single skinned fibres established that MHC isoforms influence maximal shortening velocity (V_{max}) and maximal power (Sweeney et al., 1988; Larsson and Moss, 1993; Bottinelli et al., 1994a, 1996; Hilber and Galler, 1997). In some studies, V_{max} was also affected by the ratio of $MLC3/MLC1_f$ (Moss et al., 1982; Sweeney et al., 1988; Bottinelli et al., 1994a). However, Larsson and Moss found no relationship between $MLC3/MLC1_f$ and V_{max} in human fibres (Larsson and Moss, 1993). The influence of MHC and MLC isoforms on maximal isometric tension also remains the subject of considerable debate (Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000).

Frog muscle is unique in that relatively long, single, intact 'living' fibres can be isolated that retain excellent mechanical stability and from which sarcomere length transients can readily be measured by laser diffraction. For this reason,

frog muscle offers an exceptional opportunity to study the relationship between myosin isoforms and contractile function in living single cells. However, inadequate definition of both MHC and MLC isoforms in frog muscle limits our ability to characterize this relationship.

A nomenclature for amphibian fibre types has been established based on morphological, physiological, immunohistochemical, histochemical and biochemical analysis (Smith and Lannergren, 1968; Smith and Ovalle, 1973; Rowlerson and Spurway, 1988; Lutz and Lieber, 2000). In general, anuran skeletal muscles are composed of twitch fibres (types 1, 2 and 3) and tonic fibres (Rowlerson and Spurway, 1988; Lutz and Lieber, 2000). Also, a fibre type with properties intermediate between twitch and tonic fibres (type 4) has been described in *Xenopus laevis* (Lannergren, 1979), but not in Ranid frogs (Rowlerson and Spurway, 1988). Detailed mechanical analysis of intact single fibres of *X. laevis* by Lannergren and colleagues revealed differences in V_{max} and maximal power in the order: type 1>type 2>type 3>type 4>tonic (Lannergren and Hoh, 1984; Lannergren, 1987). The differences in contractile properties were correlated with MHC isoforms, while the ratio of MLC3/MLC1$_f$ did not influence the force–velocity relationship. However, interpretation of these results was complicated by the fact that MHC content in the different fibre types was not measured directly but was estimated from a combination of isomyosin banding patterns after native polyacrylamide gel electrophoresis (PAGE) and MLC content after SDS-PAGE. In addition, the identification of MLC isoforms in the various fibre types was incomplete.

We recently characterized the MHC isoform composition of *Rana pipiens* fibre types at both the mRNA and protein levels (Lutz et al., 1998a,b, 2000; Lutz and Lieber, 2000). Four major fibre types (type 1, type 2, type 3 and tonic), were differentiated by their reactivity to a panel of monoclonal MHC antibodies. Four novel MHC mRNA transcripts were cloned from whole muscle, and using single-fibre reverse transcription-polymerase chain reaction (RT-PCR), each transcript was found to be expressed predominantly in one of the four fibre types. SDS-PAGE of single immunotyped fibres showed that the MHCs from the three twitch fibre types (type 1, type 2 and type 3) were resolved into three separate isoform bands, while tonic MHC comigrated with type 1. Coexpression of two MHC isoforms in single fibres was often observed at both mRNA and protein levels.

The purpose of this report was to identify the MLC isoforms corresponding to the various fibre types in *R. pipiens* and to determine the relationship between the expression of MLC and MHC isoforms at the subcellular level. Unequivocal identification of MLC isoforms by SDS-PAGE can be more difficult to achieve than for MHCs because of the presence of numerous other proteins that migrate at similar positions on gels. In this report we identified the MLCs in the full range of *R. pipiens* fibre types using western blot analysis with a panel of MLC monoclonal antibodies. Western blots of single fibres provided clear delineation of seven unique MLCs; four types of MLC1, two types of MLC2 and one MLC3. The detailed identification of MLC isoforms presented here, along with our previous description of MHCs, enables the precise relationship between MHCs, MLCs and contractile function in intact single muscle cells to be elucidated.

Materials and methods

Animals and muscles

Adult male frogs, *Rana pipiens*, were purchased commercially (Charles Sullivan Inc, Nashville, TN, USA) and were kept at room temperature in 90 l aquaria containing dry, dark surfaces and recirculating filtered water. Frogs were fed live crickets twice weekly. Frogs were killed by double pithing and various hindlimb muscles were removed. To ensure that examples of all fibre types were obtained, a wide variety of different muscles were harvested. Typically, these were the anterior tibialis, gracilis and tonus region of the cruralis muscle, but in some cases the gluteal, sartorius and semimembranosus were also acquired. Large muscles (gracilis, gluteal and semimembranosus) were cut into strips. All muscles and strips were pinned to parafilm-coated cork and immediately frozen in isopentane cooled in liquid N_2 then stored at –80 °C. For isolation of single fibres, frozen muscles were freeze-dried for at least 1 h and brought to room temperature under vacuum. Single fibres were teased out of freeze-dried muscles with fine forceps under a stereomicroscope and were transferred into 200 µl microfuge tubes. SDS-PAGE sample buffer consisting of dithiothreitol (DTT; 100 mmol l^{-1}), SDS (2 %), Tris-base (80 mmol l^{-1}) pH 6.8, glycerol (10 %) and Bromophenol Blue (0.012 % w/v) was added to the tubes and samples were frozen immediately in liquid N_2 and stored at –80 °C. Samples were boiled for 2 min prior to loading onto gels. Because our purpose was to obtain adequate numbers of each fibre type, no attempt was made to standardize the number of muscles obtained from each frog or the number of fibres harvested from each muscle.

The anterior tibialis muscle was used exclusively for experiments investigating MHC and MLC content along the length of single cells. For these experiments care was taken to insure that full-length fibres were dissected from freeze-dried muscle. Fibres were cut into 1 mm segments along their full length and segments were suspended in SDS-PAGE sample buffer.

SDS-PAGE and western blots

Single fibre samples were split; 10 % of the sample was used for analysis of MHCs and the remaining 90 % for MLCs. The MHC composition was determined for a large number of fibres to obtain sufficient numbers of each type and then MLC analysis was performed on a subset of these same fibres.

Myosin heavy chains

MHC isoforms were separated by SDS-PAGE, based on methods of Talmadge and Roy (1993) as previously described (Lutz et al., 1998a). Total acrylamide concentration was 4 %

and 8 % in the stacking and resolving gels, respectively (acrylamide:bis-acrylamide, 50:1). Gels (16 cm×22 cm, 0.75 mm thick) were run at a constant current of 10 mA until voltage reached 275 V, and thereafter at constant voltage for 21 h at 4 °C. Homogenized anterior tibialis muscle was used as the standard for identification of MHC isoform bands. The MHCs from whole anterior tibialis appear as a triplet, with type 1, type 2 and type 3 MHCs running as the middle, upper and lower bands, respectively (Lutz et al., 1998a). Tonic MHC comigrates with type 1 MHC in the middle band, but we could distinguish whether a band at the middle position was type 1 or tonic, because tonic fibres were always harvested from a part of the muscle where type 1 fibres were not present. Gels were silver stained and MHC bands were quantified by densitometry (PDI, BioRad, Hercules, CA, USA).

Myosin light chains

Western analysis was used to identify the MLCs present in the various fibre types. Single fibre samples for which MHC composition had been determined were split into two parts and subjected to identical SDS-PAGE. Total acrylamide concentration was 4 % and 14 % in the stacking and resolving gels, respectively (acrylamide:bis-acrylamide, 38:1). Gels (7.5 cm×10 cm, 0.75 mm thick) were run at a constant current of 20 mA for 2.5 h at 4 °C. One gel was silver stained and its counterpart was transferred to nitrocellulose for western analysis. Typically, two thirds of the single fibre sample was run on the silver-stained gel and the remaining one third was used for western blots. Western blots were reacted with three different anti-avian MLC monoclonal antibodies, T14, MF5 and F310. The T14 and F310 antibodies were a generous gift from Dr Frank Stockdale (Stanford University). T14 has been shown to react with $MLC1_f$, $MLC2_f$ and $MLC2_s$ in avian muscle, while F310 reacts with $MLC1_f$ and MLC3 (Crow et al., 1983). The MF5 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) reacts with $MLC2_f$ in avian muscle (Shimizu et al., 1985). Primary antibodies were applied overnight at 4 °C at dilutions of 1:5, 1:500 and 1:100 for T14, MF5 and F310, respectively. The secondary antibody (diluted 1:5000) was peroxidase-labeled, anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA). Bands were visualized using ECL western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The specific MLC reactivity of the antibodies is shown in Table 1. In most cases, blots were stripped and reprobed sequentially through the series of antibodies. Banding patterns on western blots were compared with silver-stained gels to assess the position of MLC bands relative to other bands. Apparent molecular masses were calculated for all MLCs by comparison with standards of known molecular mass.

Quantitative assessment of the MLC isoform ratios within single fibres was performed on silver-stained gels using densitometry and NIH Image software. Molar ratios were calculated from the band density and molecular mass of each isoform. For this purpose we used molecular mass derived from amino acid sequence analysis, rather than apparent molecular mass calculated from gel mobility, which are prone to significant error. This is especially true of MLC1, which is known to migrate much more slowly on gels, and hence at higher apparent molecular mass, than its actual molecular mass deduced from its amino acid composition (Frank and Weeds, 1974). We used molecular masses of 20.7, 19.0 and 16.5 kDa for $MLC1_f$, $MLC2_f$ and MLC3, based on the derived amino acid composition of cloned full-length cDNAs for each of these isoforms (G. J. Lutz and R. L. Lieber, unpublished data).

Troponins

To identify the other myofibrillar proteins present in the various fibre types in the MLC region of the gels, western blots were performed with monoclonal antibodies against troponin I (MAB1691; Chemicon International Inc., Temecula, CA, USA) and troponin C (Novocastra NCL-TROPC; Vector Laboratories, Burlingame CA, USA). Western blots were reacted with anti-TnI (diluted 1:100) and anti-TnC (diluted 1:40) as described for MLCs. MHC isoform content was determined from a sample of each fibre as described above.

Skinned fibres

To determine which bands on silver-stained gels were cytoplasmic (soluble) proteins and thus not incorporated into myofibrils (myofibrillar proteins), freeze-dried fibres were cut into two segments. One segment was skinned to remove soluble proteins and the other was placed directly in SDS-PAGE sample buffer. For skinning, fibre segments were pinned in Sylgard dishes, skinned for 30 min (1 % Triton X-100 in standard relaxing solution), washed twice with relaxing solution, transferred to dry tubes and immersed in SDS-PAGE sample buffer. Skinned and non-skinned segments of individual fibres were subjected together to SDS-PAGE.

All values are reported as mean ± S.E.M. unless otherwise indicated. Comparison of means was performed by Student's *t*-test.

Table 1. *MLC isoform reactivity to monoclonal antibodies*

	Isoform						
Antibody	$MLC1_x$	$MLC1_{Ta}$	$MLC1_f$	$MLC1_{Tb}$	$MLC2_f$	$MLC2_T$	MLC3
T14	+	+	+	+	−	+	−
MF5	−	−	−	−	+	−	−
F310	+	+	+	−	−	−	+

Results

Identification of myosin light chains

Western analysis was used to identify the MLCs in the various fibre types of *R. pipiens* skeletal muscle. Single fibres were isolated from freeze-dried muscles and fibre types were determined by SDS-PAGE analysis of MHC isoform content. Fibres representing each of the major and intermediate fibre types (types 1, 1-2, 2, 2-3, 3 and tonic), were then analyzed on western blots for their reactivity to three monoclonal MLC antibodies. A representative example of the silver-stained banding pattern and corresponding western blots is shown for a complete series of fibres obtained from one frog (Fig. 1). The specific reactivity of the antibodies is provided in Table 1. As indicated by the summary representation in Fig. 1, in this frog, a total of six different MLC bands were identified in the various fibre types by the three antibodies combined: three distinct MLC1 bands, two MLC2 bands and a single MLC3 band. All twitch fibre types (types 1, 1-2, 2, 2-3 and 3) contained only one common MLC1 and an MLC2 band, designated as $MLC1_f$ and $MLC2_f$ (where f denotes fast). A single MLC3 band was observed in all twitch fibre types except type 3, and was not present in tonic fibres.

Tonic fibres expressed two unique MLC1 bands and one unique MLC2 band not found in twitch fibres (Fig. 1). The MLCs expressed exclusively in tonic fibres are designated with the subscript T as $MLC1_{Ta}$, $MLC1_{Tb}$ and $MLC2_T$. The $MLC1_{Tb}$ and $MLC2_T$ bands in tonic fibres are clearly different from the bands designated as $MLC1_f$ or $MLC2_f$ in twitch fibres, based both on their differential gel mobility and their differential reactivity to MLC antibodies. The $MLC1_{Ta}$ band could not be differentiated from $MLC1_f$ by antibody reactivity, and appeared to comigrate with $MLC1_f$ on most gels (Figs 1, 2); however, on several gels the $MLC1_{Ta}$ band clearly migrated more slowly than $MLC1_f$, strongly suggesting they are unique MLC1 isoforms.

A strikingly different, and very unusual pattern of MLC expression was observed across the full range of fibre types in some frogs (Fig. 2). As indicated by the schematic summary of the western blots in Fig. 2, in certain frogs a unique MLC1 band, designated as $MLC1_x$, was expressed in all fibre types. In each of the fibre types, the $MLC1_x$ band was coexpressed

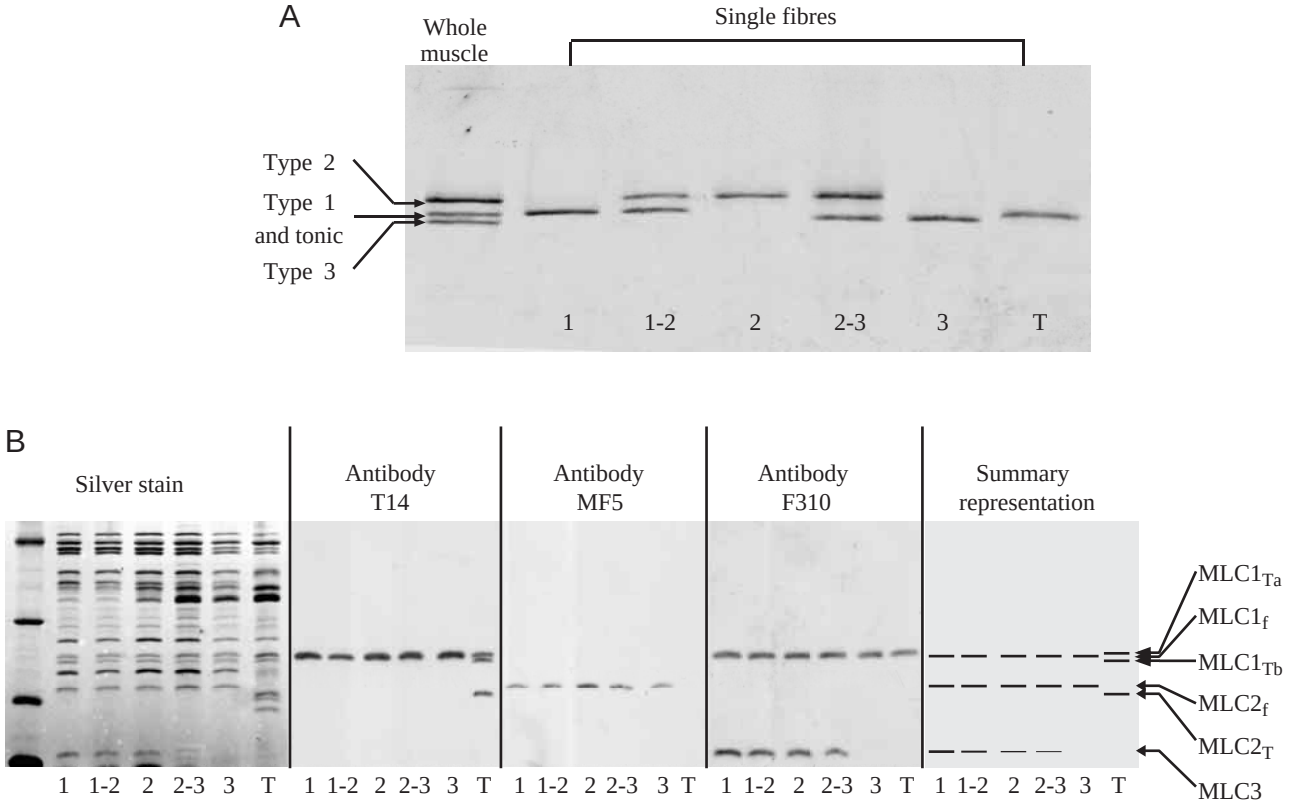

Fig. 1. Identification of MLCs in single fibres representing the full range of fibre types in *R. pipiens*. (A) MHC isoform composition of single fibres was determined by SDS-PAGE. Shown are MHC bands from single fibres representing each of the major and intermediate fibre types (types 1, 1-2, 2, 2-3, 3 and tonic T). All fibres were obtained from one frog. Fibres were typed by comparison with the known positions of MHC isoforms from a standard whole muscle homogenate (anterior tibialis muscle). Fibre types, based on MHC content, are indicated at the bottom of each gel lane. (B) Analysis of MLCs in each of the MHC-typed fibres shown in A (fibre types labeled at bottom). (Left) SDS-PAGE (silver stained) of each fibre. Gel markers in leftmost lane have molecular masses (from top to bottom) of 45, 31, 21.5 and 14.5 kDa. (Middle) Western blots of the gel on left using monoclonal MLC antibodies T14, MF5 and F310. (Right) Schematic representation based on western blots of the MLC composition of the fibre types from this frog and 23 other frogs with a similar MLC composition.

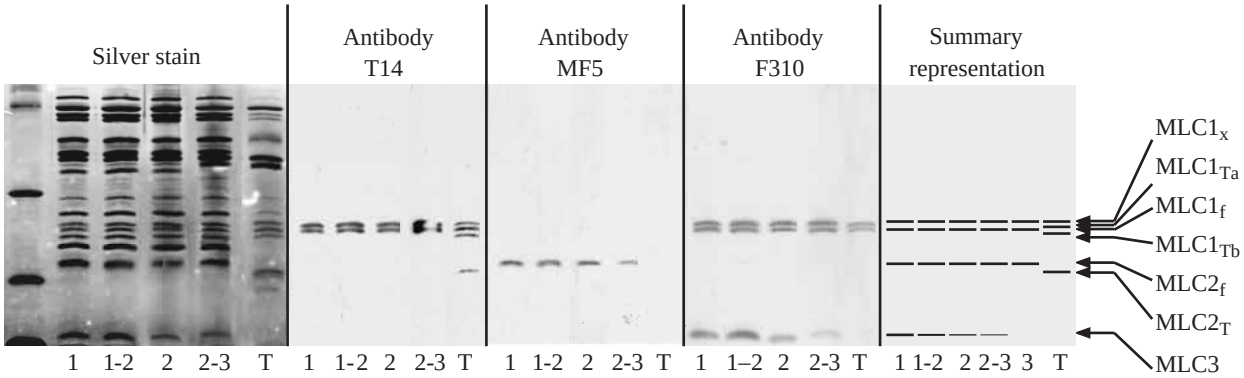

Fig. 2. In some frogs, a novel MLC, $MLC1_x$, is expressed in all fibre types. (Left) SDS-PAGE (silver stained) of single fibres representing most of the different fibre types. All fibres (fibre types labeled at bottom of gel lanes) were obtained from two frogs and were typed by their MHC banding pattern by SDS-PAGE, as in Fig. 1 (not shown). (Middle) Western blots of the gel on left using monoclonal MLC antibodies T14, MF5 and F310. (Right) Schematic representation based on western analysis of the MLC composition of the fibre types from this frog and six other frogs with similar MLC composition. A unique type of MLC1, designated as $MLC1_x$, was expressed in all fibre types, producing a strikingly different expression pattern from those seen in fibres examined in Fig. 1.

along with the usual MLC1 band (the MLC1 bands in Fig. 1). Thus, in these frogs, all twitch fibre types contained both $MLC1_f$ and $MLC1_x$, while tonic fibres contained a triplet of MLC1 bands ($MLC1_{Ta}$, $MLC1_{Tb}$ and $MLC1_x$). In qualitative terms, the only difference between the MLC expression in the frogs of Figs 1 and 2 was the presence or absence of the $MLC1_x$ band. In all other respects, the MLC expression patterns were qualitatively the same. There was no indication that the band designated as $MLC1_x$ was actually a different isoform in twitch and tonic fibres as the antibody reactivity of this band and its migration rate appeared to be identical in all fibre types (Fig. 2, Table 1).

The expression of $MLC1_x$ was clearly 'all or none' as it was either expressed in all fibres of a given frog or it was completely absent. A total of 7 of 31 frogs studied contained the $MLC1_x$ band. From these 7 frogs, 52 fibres were analyzed, and each contained the $MLC1_x$ band. The 52 fibres included all fibre types as follows: type 1 (8), type 1-2 (9), type 2 (7), type 2-3 (8), type 3 (5) and tonic (15). Conversely, $MLC1_x$ was absent from each of 206 fibres obtained from the remaining 24 frogs. The 206 fibres were distributed among all fibre types as follows: type 1 (39), type 1-2 (63), type 2 (34), type 2-3 (16), type 3 (11) and tonic (43).

Overall, bands reacting positively to MLC antibodies could clearly be categorized as MLC1, MLC2 or MLC3 based on a comparison of their apparent molecular mass with those of other species. The apparent molecular mass of each of the identified MLC bands was as follows (in kDa): $MLC1_x$ (26.8), $MLC1_{Ta}$ (26.2), $MLC1_f$ (26.1), $MLC1_{Tb}$ (25.5), $MLC2_f$ (22.8), $MLC2_T$ (21.7), and MLC3 (15.0). The apparent molecular masses for both MLC2 isoforms were slightly larger than reported in previous studies of frog myosin (18–20 kDa) (Giambalvo and Dreizen, 1978). These relatively small differences (5–10 %) could easily be due to species differences, as previous studies did not include *R. pipiens*. The differences could also result from subtle differences in the SDS–PAGE system. Also, most previous studies of frog muscle have reported molecular masses of MLCs purified from whole muscle where proteolytic degradation could have been a complicating factor.

Identification of other myofibrillar proteins

To avoid potential ambiguities in qualitative and quantitative analysis of the MLCs, it was important to identify

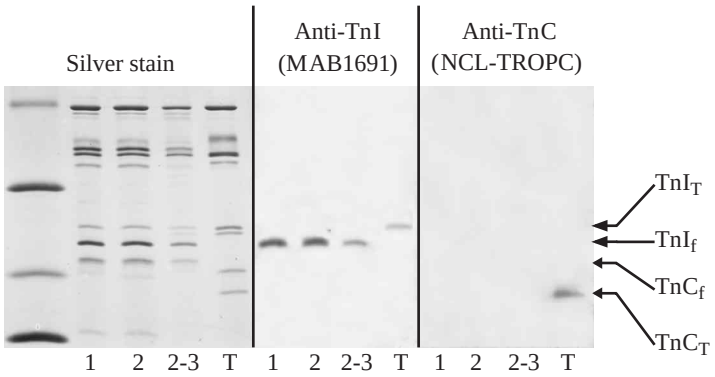

Fig. 3. Identification of troponin I (TnI) and troponin C (TnC) isoforms in the various *R. pipiens* fibre types. (Left) SDS-PAGE (silver stained) banding pattern of single skinned fibres representing most of the different fibre types. All fibres (fibre types labeled at bottom of gel lanes) were obtained from one frog and were typed by MHC banding pattern after SDS-PAGE as in Fig. 1 (not shown). (Right) Western blots of the gel on left using anti-TnI and anti-TnC antibodies. Anti-TnI labeled one isoform in fast twitch fibres (TnI_f) and a unique isoform in tonic fibres (TnI_T). Anti-TnC labeled an isoform in tonic fibres (TnC_T) that was not expressed in any other fibre type, but did not react with the TnC isoform present in fast twitch fibres (TnC_f). The position of TnC_f was deduced from indirect evidence (see Results for details). The lightly stained band below $MLC2_f$ in twitch fibres is an unidentified contaminant that is not typically present (see Figs 1, 2 and 4). Note, this frog did not contain the $MLC1_x$ isoform in any of its fibres.

the other bands that migrated in the MLC region of the gels. The only myofibrillar proteins known to migrate in the MLC region under these conditions are troponin I (TnI) and troponin C (TnC). We performed western blots on the various fibre types with monoclonal antibodies against TnI and TnC (Fig. 3). Anti-TnI and anti-TnC western blots were performed on skinned fibres (soluble proteins removed; Fig. 3) and non-skinned fibres (not shown). Anti-TnI labeled a single band (designated TnI_f) in all fast twitch fibres (types 1, 1-2, 2, 2-3 and 3), at a position between $MLC1_f$ and $MLC2_f$. Anti-TnI also labeled a unique isoform in tonic fibres (designated TnI_T) at a position slightly above TnI_f. The TnI_T band comigrated with the $MLC1_{Ta}$ isoform band. The apparent molecular masses of TnI_f and TnI_T (24.7 and 26.2 kDa, respectively) are in reasonable agreement with published values for skeletal TnI. Because we did not perform any quantitative analysis of MLCs in tonic fibres, comigration of $MLC1_{Ta}$ and TnI_T did not influence the conclusions in this study.

The anti-TnC antibody labeled a single band in tonic fibres (designated TnC_T; apparent molecular mass 20.6 kDa) that migrated just ahead of $MLC2_T$ (Fig. 3). Anti-TnC did not react with TnC in fast twitch fibres. Thus, we could not identify a putative fast TnC (TnC_f) with western blots. However, it appears that TnC_f comigrated with $MLC2_f$ on the gels in Figs 1–3, based on two observations. First, on all gels of this type the $MLC2_f$ band appeared diffuse, and on some gels it appeared as two bands (a tight doublet), composed of a darker upper and lighter lower portion. The apparent molecular mass of this band (22.8 kDa) and the fact that it was lightly stained are consistent with its identity as TnC_f (TnC is known to stain much lighter per unit mass compared to other proteins). Second, when soluble proteins were removed by exposing fibres to membrane permeabilization (skinning), the only remaining candidate band near $MLC2_f$ (where TnC_f should be located) was the band identified as TnI_f by western blot (molecular mass = 24.7 kDa). This molecular mass was higher than was expected for TnC (approximately 18–20 kDa). Importantly, none of the bands identified as MLC, TnC or TnI isoforms was removed by skinning. Overall, this identification of bands in the MLC region of gels confirmed that our identification of MLCs was accurate and that our quantitative densitometry of MLC3/MLC1 ratios in type 1, type 1-2 and type 2 fibres (see below) was not influenced by proteins that comigrated with the MLCs.

One unresolved question is the identity in twitch fibres of the band that migrated between $MLC1_f$ and TnI_f. Although skinning removed this band completely from type 1, type 1-2 and type 2 fibres, indicating that it was a soluble protein, it was not removed from type 2-3 or type 3 fibres (Fig. 3). Because this band did not react with any of the MLC or troponin antibodies we cannot conclusively identify it. It is not likely to be an MLC isoform, however, as it was not labeled by the T14 antibody, which strongly labeled all other MLC1 isoforms.

A summary of the MLC and troponin isoforms expressed in twitch and tonic fibres is shown in Fig. 4. To allow for a direct comparison, skinned and non-skinned segments of the same fibres were run in adjacent lanes of the gel. This gel clearly illustrates the bands that were removed by skinning and the major differences in myofibrillar protein expression patterns between twitch and tonic fibres.

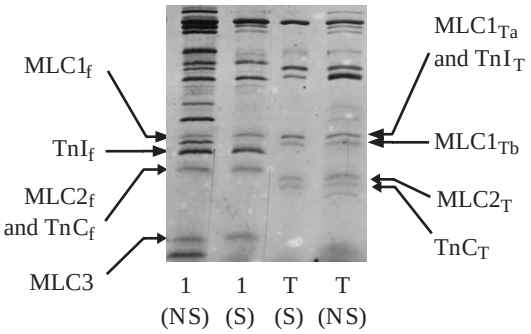

Fig. 4. Summary of the expression pattern of MLC and troponin isoforms in fast twitch and tonic fibres. This figure combines the information from Figs 1 and 3 to indicate the positions of each of the identified myofibrillar proteins (except $MLC1_x$) on a silver-stained gel after SDS-PAGE. Skinned (S) and non-skinned (NS) segments of the same fibres (fibre type indicated below each lane) are shown in adjacent lanes for visualization of the bands containing soluble proteins (i.e. the bands present in non-skinned segments but absent from skinned segments). For clarity, a type 1 fibre only was used to represent the fast twitch fibres, although significant differences in the quantitative and qualitative expression of MLC and troponin isoforms did exist among the twitch fibres. Note that skinning removed a prominent soluble protein band just above the MLC1 region in both fibre types. Skinning also removed a prominent soluble protein band between $MLC1_f$ and TnC_f, as well as a band below TnC_T in tonic fibres. Note that this frog did not contain the $MLC1_x$ isoform in any of its fibre types.

Quantification of MLCs in single fibres

In some recent studies, the molar ratio of MLC3/$MLC1_f$ significantly influenced the mechanical function of single skeletal muscle fibres (see Discussion). We therefore measured the MLC3/MLC1 ratio in *R. pipiens* fibres to determine whether any significant variability existed in this parameter among and between the various fibre types. Having established the identity of the various MLCs and their positions on SDS-PAGE gels, we quantified the relative levels of MLCs in individual fibres by densitometry of silver-stained gels. This quantification was restricted to type 1, type 1-2 and type 2 fibres. All molecular masses used in calculating molar ratios were based on derived amino acid composition, not gel mobilities (see Materials and methods). MLC1 content was taken as the sum of $MLC1_f$ and $MLC1_x$, and the same molecular mass was used for both isoforms. The ratio of MLC3/MLC1 was related to MHC isoform content in single freeze-dried type 1, type 1-2 and type 2 fibres (N=100) obtained from various muscles (Fig. 5A, Table 2). Among pure type 1 fibres (i.e. fibres that expressed only type 1 MHC), MLC3/MLC1 was extremely variable, ranging between 0.20 and 2.9 (mean=1.20±0.13; N=35). The MLC3/MLC1 ratio was significantly lower (P<0.0001) in pure type 2 fibres

(mean=0.50±0.08; N=18) compared to pure type 1 fibres. For all fibres combined, MLC3/MLC1 increased in direct proportion to the percentage of type 1 MHC (P<0.0001). However, the relationship was weak, accounting for only 24 % of the variability (Table 2). Similarly, in the anterior tibialis (AT) muscle (the only muscle from which type 1, type 1-2 and type 2 fibre types were harvested), MLC3/MLC1 ratio was weakly positively correlated with the percentage of type 1 MHC. Overall, the highest levels of MLC3/MLC1 were found in fibres obtained from the gracilis muscle. When fibres from all muscles except the gracilis were considered as a group, the MLC3/MLC1 ratio increased only slightly with the percentage of type 1 MHC and the correlation was significant but very weak (Table 2).

The slope of the relationship between MLC3/MLC1 ratio and the percentage of type 1 MHC was similar in fibres that contained $MLC1_x$ and non-$MLC1_x$ fibres (Fig. 5B, Table 2). Thus, the expression of $MLC1_x$ did not appear to affect markedly the MLC3/MLC1 ratio. Fibres with $MLC1_x$ were distributed across the full range of type 1, type 1-2 and type 2 fibres. On average, $MLC1_x$ was expressed in nearly equal molar amounts to $MLC1_f$ in type 1, type 1-2 and type 2 fibres as a group ($MLC1_x/MLC1_f$=1.10±0.07), but the range of values was substantial (0.64–1.71; Fig. 6). $MLC1_x/MLC1_f$ was slightly higher in type 2 (1.30±0.30) than in type 1 fibres (0.98±0.06; P<0.05). Regression analysis of type 1, type 1-2 and type 2 fibres showed that $MLC1_x/MLC1_f$ was inversely related to the percentage of type 1 MHC (P<0.05; Fig. 6).

To estimate the extent to which variability in the MLC3/MLC1 ratio between fibres was due to inherent inaccuracy in the quantification procedure, we measured the intrinsic variability of MLC quantification (estimated by repeated measures of samples within and between gels). To ensure adequate material for repeatability measurements, samples consisted of pairs of large fibres from the semimembranosus muscle (isolated from freeze-dried muscle). To estimate the potential influence of fibre size on variability in MLC3/MLC1 in single fibres, we included two dilutions of the repeatability standard with a fourfold difference in concentration. The dilutions encompassed the full range of sample densities in single fibres. Coefficient of variation of MLC3/MLC1 was used to express variability of the experimental method. Variability due to the quantification method itself (10 repeated measures of a single lane of one gel) was 4.3 %. Variability within gels (11 lanes on a single gel) was 15.2 %. Between gels (four lanes each of the same samples on two separate gels), mean values differed by only 2.8 %. There was no significant difference in variability between dilutions (P>0.3; four lanes each on two separate gels). These data demonstrate that while intrinsic variability was significant,

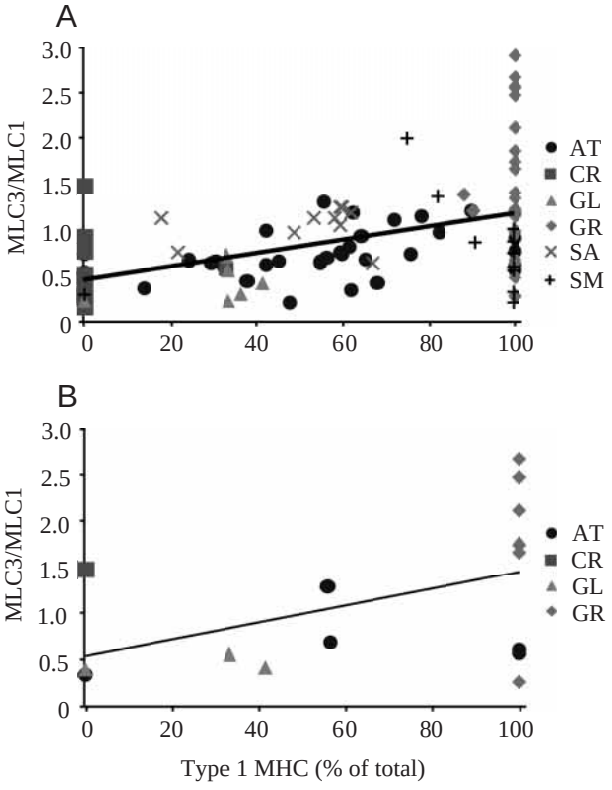

Fig. 5. The MLC3/MLC1 ratio increased in proportion to the percentage of type 1 MHC in single fibres, but was highly variable within fibre types. (A) MLC3/MLC1 ratio and MHC isoforms were measured in single type 1, type 1-2 and type 2 fibres by quantitative densitometry of gels after SDS-PAGE. Fibres (N=100) were obtained from the anterior tibialis (AT), cruralis (CR), gluteal (GL), gracilis (GR), semimembranosus (SM) and sartorius (SA) muscles. Each symbol corresponds to a different muscle. Linear regression showed that the MLC3/MLC1 ratio increased in direct proportion to the percentage of type 1 MHC. There was substantial variability in MLC3/MLC1 ratio within each fibre type, and the highest values were all found in the GR muscle. (B) MLC3/MLC1 ratio for fibres that contained $MLC1_x$ (N=16; data are subset of A). The relationship between the MLC3/MLC1 ratio and percentage of type 1 MHC was similar to A, and the full range of fibre types was represented.

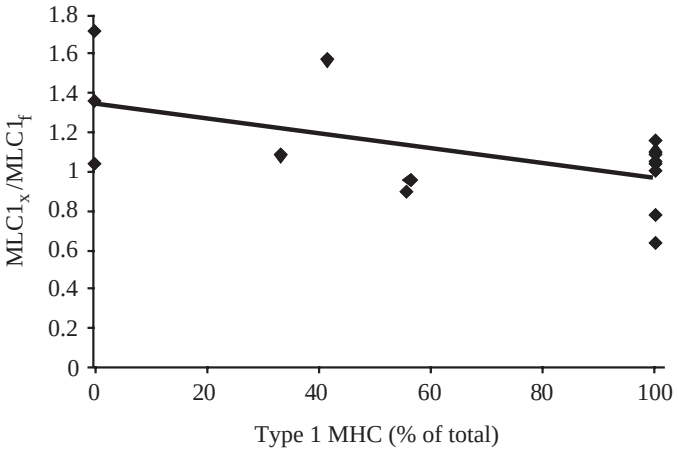

Fig. 6. $MLC1_x/MLC1_f$ was higher in type 2 than type 1 fibres. The molar ratio of $MLC1_x/MLC1_f$ was measured in single type 1, type 1-2 and type 2 fibres by quantitative densitometry of gels after SDS-PAGE. Linear regression showed that $MLC1_x/MLC1_f$ ratio decreased in direct proportion to the percentage of type 1 MHC (slope=–0.003; r^2=0.288; P=0.03).

Table 2. *Linear regression variables describing the relationship between MLC3/MLC1 and percentage of type 1 MHC*

Group	Slope	Intercept	*P*	r^2	*N*
All fibres	0.007±0.001	0.454±0.096	<0.0001	0.235	100
Non-$MLC1_x$ fibres	0.007±0.001	0.454±0.095	<0.0001	0.237	84
$MLC1_x$ fibres	0.010±0.004	0.503±0.324	0.043	0.260	16
All except GR	0.004±0.001	0.540±0.066	0.001	0.139	75
AT only	0.004±0.001	0.501±0.087	0.005	0.220	35

Variables are for linear regression equations fitted to the data in Fig. 5.
Values are means ± S.E.M.
AT, anterior tibialis; GR, gracilis muscle.

it did not account for most of the variability observed in Fig. 5, where variability in MLC3/MLC1 in type 1, type 1-2 and type 2 fibres was 63%, 43% and 64%, respectively. These data also indicate a lack of saturation of the MLC3 and MLC1 bands. All bands quantified with densitometry in this study had optical densities within this linear range.

To further characterize the accuracy of our quantification procedure for MLCs, we attempted to evaluate the molar ratio of ELCs to RLCs in single fibres. Because each MHC is known to be associated with one ELC and one RLC, the measured ratio of (MLC1+MLC3)/MLC2 should equal one. Unfortunately, because TnC_f appears to comigrate with $MLC2_f$, we could not explicitly calculate the (MLC1+MLC3)/MLC2 ratio. However, assuming that $MLC2_f$ and TnC_f comigrate, the (MLC1+MLC3)/($MLC2_f$+TnC_f) ratio was 0.823 (±0.016; *N*=100), which is reasonable considering that the lightly staining TnC_f probably makes a small contribution to the density of the $MLC2_f$-TnC_f band. Also, linear regression analysis showed that the relationship between MLC1+MLC3 and $MLC2_f$+TnC_f was highly correlated (r^2=0.98), which indicates a lack of saturation in the silver-stained gels.

Variability in MHC and MLC isoforms along the length of single fibres

Having established that MLC3/MLC1 varied substantially within fibre types, we were interested in whether this parameter also varied along the length of fibres and whether its relationship to MHC isoforms was the same as that seen at the whole fibre level. MHC isoforms and MLC3/MLC1 ratio were quantified in 1 mm segments along the length of single fibres directly from gels after SDS-PAGE. As indicated in Fig. 7, both MHC isoform content and MLC3/MLC1 varied substantially along the length of single fibres (*N*=10 fibres). In the most extreme case, the percentage of type 1 MHC changed by 40.3% between segments, while MLC3/MLC1 ranged from 2.43 to 1.03 in the same fibre (Fig. 7F). Non-parametric correlation analysis revealed no significant correlation between MLC3/MLC1 and the percentage of type 1 MHC in any individual fibre (*P*=0.17–0.93), but a significant correlation was found across all segments combined (*N*=64; *P*<0.001). Regression analysis of all segments showed that

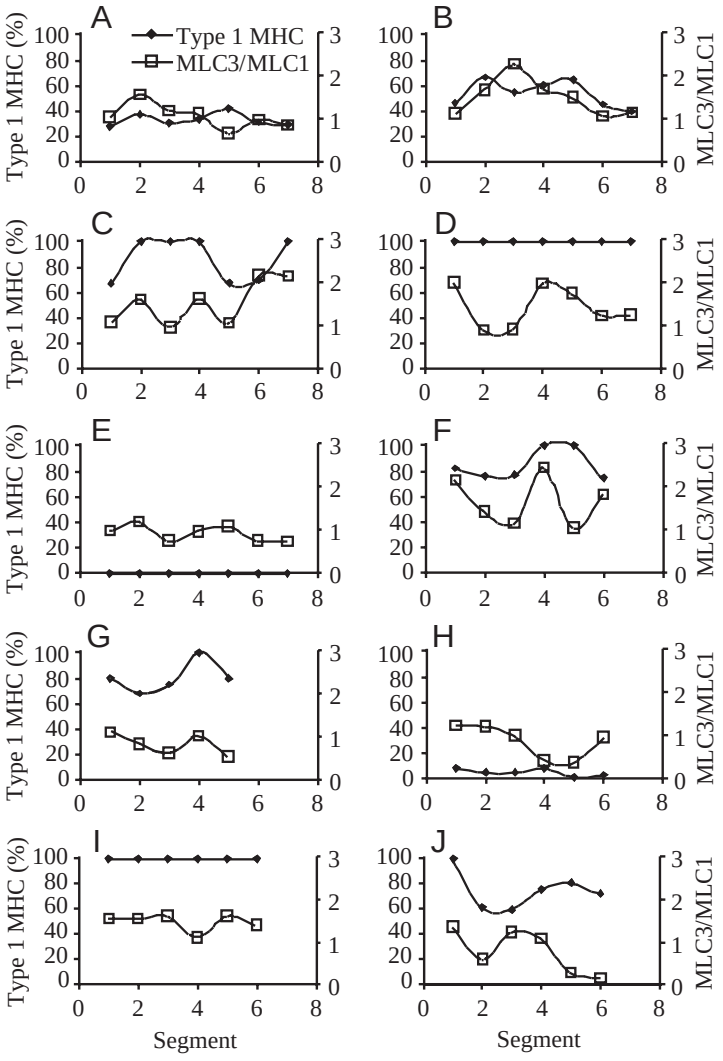

Fig. 7. MHC and MLC isoform composition varied substantially along the length of single fibres. MHC isoform content and MLC3/MLC1 ratio were determined in 1 mm segments along the full length of single fibres (*N*=10) by quantitative densitometry of gels after SDS-PAGE. All fibres were obtained from the anterior tibialis muscle. Each letter (A–J) represents a different fibre.

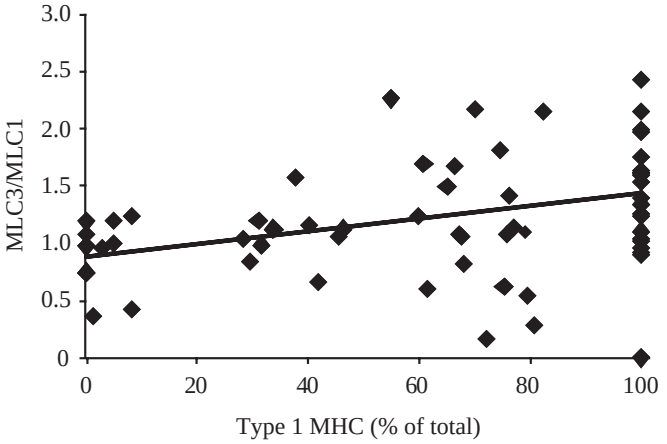

Fig. 8. The MLC3/MLC1 ratio increased in proportion to the percentage of type 1 MHC in individual 1 mm segments along the length of fibres. MLC3/MLC1 ratio plotted *versus* the percentage of type 1 MHC for all segments from each of the 10 single fibres shown in Fig. 7. Linear regression analysis showed that the MLC3/MLC1 ratio increased in direct proportion to the percentage of type 1 MHC.

MLC3/MLC1 increased slightly with the percentage of type 1 MHC (Fig. 8; $P<0.001$). The slope and correlation coefficient for all segments was similar to that found in whole anterior tibialis fibres (Fig. 5, Table 2). Coefficient of variation of MLC3/MLC1 in individual fibres (Fig. 7) ranged from 13.2 % to 64.2 %, which was much greater than inherent variability in the quantification procedure (see above).

Discussion

The ability to identify and quantify MLCs accurately in various fibre types of a given species is a critical prerequisite to establishing their influence on contractile function. Unequivocal identification of MLC isoforms after SDS-PAGE can be difficult because of the presence of other myofibrillar proteins that have similar molecular masses. Typically, the position of MLC bands relative to other proteins after SDS-PAGE is determined by analysis of the banding pattern of myosin purified from whole muscle. However, contamination of the myosin fraction by thin filament proteins, the presence of myosin degradation products and inadequate detection of MLCs from uncommon fibre types limits this approach. Although western analysis can potentially resolve these problems, it has often been ignored as a primary tool for identification of a family of MLCs. In the present report we identified the MLCs in the full range of *R. pipiens* fibre types using western analysis with a panel of MLC monoclonal antibodies. Western blots of single fibres, typed by MHC isoform content, clearly delineated a total of seven unique MLCs: four types of MLC1, two of MLC2 and a single MLC3 (Figs 1, 2, Table 1). Although the antibodies used were raised against avian myosins, they provided excellent cross-reactivity with frog MLCs and partial selectivity between MLCs. The detailed identification of MLCs allowed for the most complete description to date of MHC and MLC isoform expression patterns in single fibres from frog.

To reduce potential complications in quantification of MLCs by SDS-PAGE, we attempted to define the remaining myofibrillar protein bands in the MLC region of gels using western analysis with antibodies against TnI and TnC (Fig. 3). The western blots were performed on skinned fibres, to reduce contamination from soluble proteins. Two unique isoforms of TnI were identified; one in twitch fibres (TnI_f) and one in tonic fibres (TnI_T). A single TnC isoform was identified with anti-TnC in tonic fibres. A synopsis of the gel migration of all identified MLC, TnI and TnC isoforms in twitch and tonic fibres is shown in Fig. 4.

The MLC family in amphibian skeletal muscle has not been as well characterized as in other vertebrates. Only a single isoform of MLC1, MLC2 and MLC3 have been detected in myosin fractions purified from whole frog muscles (Kendrick-Jones et al., 1976; Giambalvo and Dreizen, 1978; Chanoine and Gallien, 1989) and from single fibres in *Rana esculenta* (Focant and Reznik, 1980). The fibre type expression pattern of MLCs in *Xenopus* has received the most critical scrutiny among amphibians (Lannergren, 1987). Single fibres were typed by native myosin bands on pyrophosphate gels and segments of the same fibres were analyzed by SDS-PAGE for MLC content (Lannergren, 1987). It was concluded that fast twitch type 1 and type 2 fibres of *Xenopus* contained $MLC1_f$, $MLC2_f$ and MLC3, while slow twitch type 3 fibres expressed the slow isoform $MLC1_s$, and did not contain MLC3. It was later reported that type 3, type 4 and type 5 (tonic) fibres all expressed slow isoforms $MLC1_s$ and $MLC2_s$ (Lannergren, 1992). However, it is difficult to justify these conclusions from the gels presented. Specifically, $MLC2_f$ and $MLC2_s$ appear to comigrate, and an unidentified band in the MLC1 region complicates the identification of $MLC1_f$ and $MLC1_s$ (Lannergren, 1987). Further, no MLC gels were shown for type 4 or type 5 fibres. Thus, the fibre type expression pattern of MLCs in *Xenopus* remains unclear. However, if the interpretation of MLC expression patterns in *Xenopus* is correct, it differs significantly from the present findings in *R. pipiens*. There was no evidence of slow MLCs in any of the *R. pipiens* twitch fibres, whereas in *Xenopus* the fast twitch fibres (types 1 and 2) contained a different set of MLCs than the slow twitch fibres (type 3). Also, only two types of MLC1 were found in *Xenopus*, while four types of MLC_1 were present in *R. pipiens*.

A most remarkable feature of the MLC expression in *R. pipiens* was the 'all or none' expression pattern of $MLC1_x$, which was either expressed in all fibres of a given frog or was completely absent (Fig. 2). $MLC1_x$ was always coexpressed along with the isoforms of MLC1 and MLC2 found in frogs that did not contain $MLC1_x$. Interestingly, the quantitative relationship between MLC3/MLC1 ratio and percentage of type 1 MHC was maintained in frogs that had $MLC1_x$ (Fig. 5B). Thus, apart from the expression of $MLC1_x$, the regulatory control of MLC content in fibre types was conserved

in both groups of frogs. No differences were noted in any other myofibrillar proteins between frogs with $MLC1_x$ and non-$MLC1_x$ frogs.

Intraspecific variability in $MLC1_f$ expression has been documented in fish (Martinez et al., 1990; Crockford et al., 1991, 1995), and avian (Rushbrook and Somes, 1985) muscle. In fish, breeding experiments and genetic analysis clearly showed that intraspecific variability of two $MLC1_f$ isoforms was the result of allelic variation (Crockford et al., 1995). In agreement with our data from *R. pipiens*, the MLC3/MLC1 ratio was not affected by MLC1 isoform content and no other differences in other MLCs or other myofibrillar proteins were found.

The intraspecific variability in MLC1 isoforms in *R. pipiens* does not seem to be due to allelic variation. In 31 frogs examined, $MLC1_x$ was never the sole MLC1 isoform, yet 24 frogs did not have $MLC1_x$, and seven coexpressed $MLC1_x$ with other isoforms of MLC1. If the MLC1 isoforms resulted from allelic variants, $MLC1_x$ homozygotes would have been observed in about 25 % of the 31 frogs. Further, allelic variants are likely to be expressed with typical fibre type specificity, rather than across all fibre types. Finally, $MLC1_x$ expression did not appear to be related to environmental conditions, as both $MLC1_x$ and non-$MLC1_x$ frogs were found among frogs that were held in the laboratory for the same length of time (as long as 3 months) and were observed throughout the year. Together, these data suggest that the intraspecific polymorphism observed in MLC1 expression is probably due to an as yet undefined genetic polymorphism that is not allelic in nature. The genetic basis of MLC1 isoform expression not withstanding, the ubiquitous expression pattern of $MLC1_x$ across the full range of fibre types appears to be unprecedented for MLCs or any other myofibrillar protein. This ubiquitous fibre type expression pattern seems difficult to reconcile with a functional role for variation in MLC1 isoforms.

Functional implications of MLC expression patterns in R. pipiens

There is substantial evidence from several experimental models that MLC isoforms are an important determinant of contractile kinetics in skeletal muscle, but the extent and nature of their influence remains controversial. Using an *in vitro* motility assay, Lowey and colleagues found that actin filament velocity was higher when myosin contained MLC3 rather than MLC1 (Lowey et al., 1993). Mechanical analysis of single fibres from controlled breeding populations of fishes with allelic variation in $MLC1_f$ isoforms showed that V_{max} was significantly different between homozygotes for one or the other $MLC1_f$ isoform, while heterozygotes had intermediate properties (Crockford et al., 1995). Both of these models were uniquely powerful, as they allowed for experimental control of MLC content independent of other variables.

A correlation between MLC3 content and V_{max} of single skinned fibres has been demonstrated in a variety of vertebrates (Moss et al., 1982; Sweeney et al., 1988; Greaser et al., 1988; Bottinelli et al., 1994a; Li and Larsson, 1996) (for reviews, see Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). In rodent muscle, MLC3/$MLC2_f$ content varied significantly among a population of fibres that expressed only the IIB MHC isoform and greater values of MLC3/$MLC2_f$ were associated with increased V_{max} (Bottinelli et al., 1994a). Interestingly, MLC3/$MLC2_f$ did not appear to influence velocities at loads above zero, maximal power production or specific tension (Bottinelli et al., 1994b). In contrast, MLC3/MLC1 did not appear to influence V_{max} in human skinned fibres (Larsson and Moss, 1993).

Only one study has attempted to determine the influence of MLC isoforms on contractile kinetics in intact fibres (Lannergren, 1987). In that study of *Xenopus laevis* fibre types, MLC3/$MLC1_f$ did not appear to influence shortening velocity at half isometric tension, but complications in the identity of MLCs may weaken this conclusion (see above) (Lutz and Lieber, 2000). In accordance with this conclusion, preliminary analysis of intact *R. pipiens* single fibres revealed no differences in V_{max} among fibres that had substantial variability in MLC3/MLC1 but nearly constant MHC isoform content (G. J. Lutz and R. L. Lieber; unpublished data). Unfortunately, most studies have focused on the influence of MLCs on V_{max}, while ignoring the more physiologically relevant parts of the force–velocity and power–velocity relationships (Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). However, the preponderance of evidence to date suggests that MHCs have a greater influence on shortening velocity than MLCs, especially at velocities below V_{max}.

The variability in MLC3/MLC1 ratio within *R. pipiens* type 1, type 1-2 and type 2 fibre types and absolute levels (range 0.2–2.9; Fig. 5) were larger overall than previously reported for avian and mammalian fibres (Sweeney et al., 1986, 1988; Salviati et al., 1982; Greaser et al., 1988; Wada and Pette, 1993; Bottinelli et al., 1994a). For example, in rodent IIB fibres, MLC3 ranged between 0 and 0.5 of the total ELC content (Bottinelli et al., 1994a). Despite the relatively low magnitude and low variability in MLC3 molar content, a large range was observed in V_{max} among the same fibres. It could be argued that the comparatively high magnitude of MLC3/MLC1 in *R. pipiens* will produce an even larger influence on mechanical function than in mammalian muscle. Alternatively, the weak correlation between MLC3/MLC1 ratio and type 1 MHC, and the substantial variability in MLC3/MLC1 ratio within a given fibre type, may indicate a lack of tight regulation and hence a lack of functional significance.

Subcellular variability in myosin isoform composition

We quantified MHC and MLC isoform content in discrete segments along the full length of single fibres and found that both MHC isoform and MLC3/MLC1 ratio varied significantly between segments (Fig. 7). The magnitude of the differences among segments was as high as 40 % for MHC isoforms and even larger for the MLC3/MLC1 ratio. The relationship

between MLC3/MLC1 ratio and MHC isoform content was the same whether compared between segments or whole fibres. Previously, coexpression of two MHC isoforms in single fibres has been shown to occur in a significant portion and even a majority of the fibres in a given muscle (Larsson and Moss, 1993; DeNardi et al., 1993; Peuker and Pette, 1997; Lutz et al., 1998b). It is apparent that this coexpression is a common feature of adult muscle in steady-state conditions, and not simply present during fibre type transitions (Peuker and Pette, 1997). The variability in myosin isoform content along the length of fibres also appears to be a feature of adult muscle under steady-state conditions. Only one previous study has documented changes in MHC isoform content along the length of single fibres using direct methods, but quantitative differences were not reported (Peuker and Pette, 1997). Single cell myosin-ATPase and immunohistochemistry suggest that myosin isoform composition may change along the length of *Rana temporaria* single fibres (Edman et al., 1988), but this assay for myosin isoform content was indirect and only semi-quantitative. There appear to be no previous studies of variability in MLC isoforms along the length of fibres. Thus, to our knowledge, this is the first study to quantify directly the MHC and MLC isoforms along the length of the same individual cells.

The cellular and molecular mechanisms that regulate the distribution of myosin isoforms along the length of multinucleated muscle cells are not known. Since frog muscle fibres often contain more than one motor endplate, the observed variability in myosin isoforms along fibres could be associated with the position of motor endplates. In *R. pipiens*, the sartorius muscle averaged 2.5–3 endplates per fibre (Weakly, 1980). The anterior tibialis (AT) muscle has similar fibre type composition to the sartorius, but the fibres are two- to fourfold shorter. This indicates the AT fibres will average at most 2–3 endplates per fibre. In our study, we measured MHC and MLC isoforms in 6–7 contiguous segments along the full length of fibres (Fig. 7). In some cases the variability in MHC and MLC isoform content was random along the fibre length, while in other cases there was a clear gradient from one end to the other. Also, in some fibres MLC3/MLC1 was independent of MHC isoform content, while in other fibres they were more closely correlated. It seems unlikely that these patterns of variability in myosin isoform expression in AT fibres were correlated with the location of endplates.

Interestingly, it has previously been shown in single intact amphibian fibres that mechanical properties are not uniform along the length of single fibres (Edman et al., 1985). Whether this non-uniformity in mechanical behavior is related to differences in myosin isoforms remains to be determined. Edman and colleagues reported a weak correlation along the length of individual frog muscle fibres between V_{max} and indirect measures of myosin isoform composition (myosinATPase staining and MHC antibody reactivity) (Edman et al., 1988). They found that variability in V_{max} along the length of fibres was weakly correlated with changes in apparent MHC composition. These studies were limited, however, in that MHC isoforms were not identified and MLCs were not included in the analysis.

Comparison of frog MLC expression with mammals

The fibre type expression pattern of MLCs in *R. pipiens* showed a striking similarity to those of mammals. In *R. pipiens*, the MHCs from twitch fibres (MHC1, MHC2 and MHC3) were associated with a different set of MLCs compared to the MHC in tonic fibres (MHCT). These four MHCs in *R. pipiens* were cloned and their evolutionary relationships were established (Lutz et al., 1998a, 2000; Lutz and Lieber, 2000). Homology analysis showed MHC1, MHC2 and MHC3 were all much more similar to each other than to MHCT. Thus, the MLC isoform expression pattern mirrored the evolutionary relatedness between MHCs. A similar relationship occurs in mammalian muscle, where fast and slow MLCs are associated with the most evolutionarily divergent MHCs.

There was a notable difference in the MLC expression pattern between frogs and mammals that may have functional consequences. Twitch fibres in *R. pipiens*, which make up over 95 % of the muscle volume in the hindlimb (Lutz et al., 1998b), did not contain a slow MLC isoform. In contrast, slow isoforms of MLC1 and MLC2 in mammals are expressed in twitch fibres that make up a much larger fraction of some muscles (Salviati et al., 1982). The lack of a slow MLC in *R. pipiens* twitch fibres is consistent with the lack of a type 4 fibre such as is found in *X. laevis* muscle, indicating that the expression patterns may be different within anurans.

Conclusions

In this study, the MLC family in *R. pipiens* skeletal muscle was defined using western blot analysis of single fibre proteins. This approach yielded a more complete definition of MLCs in the full range of mechanically divergent fibre types than was possible using traditional purification methods. A total of seven unique MLCs were identified, including four types of MLC1, two types of MLC2 and a single MLC3. Our analysis included the first ever quantitative measurements of MHC and MLC isoform content along the length of fibres. Both MHC isoform and MLC3/MLC1 ratio varied substantially along the length of cells. MLC3/MLC1 was also highly variable within and between fibre types, and was not tightly coupled to MHC isoform expression. This study also provided the first example of the all-or-none expression of an MLC isoform across all fibre types, and it was shown that this MLC is subject to intraspecific variability. The precise definition of MLCs in this study provides the foundation for establishing the influence of MHC and MLC isoforms on mechanical properties of intact 'living' single fibres.

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