A myofibrillar protein of insect muscle related to vertebrate titin connects Z band and A band: purification and molecular characterization of invertebrate mini-titin

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Summary

We show that myofibrils of insect flight and leg muscle contain a doublet of polypeptides with apparent molecular weights of 700K (K=$10^3 M_r$) (Hmp I) and 600K (Hmp II), respectively. In *Locusta migratoria* high ionic strength extraction solubilizes only Hmp II, which is readily purified in native form. It probably reflects a proteolytic derivative of the non-extractable Hmp I. On the basis of its viscosity radius and sedimentation coefficient, Hmp II has a molecular weight of 600K and seems to consist of a single polypeptide chain. The highly asymmetric structure of the molecule is confirmed by rotary shadowing. The flexible rods have a uniform diameter of 3–4 nm and an average length of 260 nm. Polyclonal antibodies show cross-reactivity between Hmp II and its putative precursor Hmp I. We discuss the similarities and differences between the larger titin I/titin II of vertebrate sarcomeric muscle and the smaller Hmp I/Hmp II of invertebrate muscle and conclude that the latter may reflect a mini-titin. In line with the smaller length, immunoelectron microscopy locates the insect mini-titin to the I band and a very short portion of the A band only, while vertebrate titin is known to connect the Z band to the M band. Mini-titin has also been purified from several other insects including *Drosophila*. Immunofluorescence microscopy on frozen sections shows that mini-titin is present in the sarcomeric muscles of various species from different invertebrate phyla. These include Annelida, Nematomorpha, Plathelmintha, Nemertea and Nematoda like *Ascaris lumbricoides* and *Caenorhabditis elegans*. This wide-spread occurrence of invertebrate mini-titin is confirmed by immunoblotting experiments.

Key words: elastic filaments, insect muscle, nematodes, sarcomere, titin.

Introduction

The ultrastructure of diverse sarcomeric muscles can differ strongly among metazoa, while the molecular mechanism of contraction seems essentially the same (reviewed, e.g., by Squire, 1981). To understand the function of sarcomeric muscle early models already invoked an elastic component in addition to the thin and thick filaments. Important evidence for elastic filaments came from electron-microscopical observations of insect flight muscles, which pointed to fine connections between the Z-band and the ends of the thick filaments (see, for instance, Ashhurst, 1977; Canadia Carnevali *et al.* 1980; Shafiq, 1963).

The concept of 'connecting' filaments originally developed for insect flight muscle was very instructive in the later analysis of a third filament system in vertebrate skeletal muscle. The early work in the laboratories of Maruyama and those of Wang led to the discovery of an extremely high molecular weight protein, usually called titin (connectin), which is thought to be a major component of the elastic filaments. Direct solubilization of vertebrate myofibrils by SDS shows a titin doublet in the molecular weight range of 2000K (K=$10^3 M_r$) upon subsequent gel electrophoresis. While the parent TI molecule has not yet been extracted under native conditions, several procedures allow the purification of the smaller TII, thought to be a proteolytic derivative of TI (for reviews, see Maruyama, 1986; Wang, 1985). Immunoelectron microscopy with a bank of 15 distinct monoclonal antibodies has delineated the course of the titin molecule through the half sarcomere from the Z-band to the M-band (Fürst *et al.* 1988, 1989*a*). Since epitopes present on TI only are restricted to the Z-line and its direct environment, the TI-specific end of the titin molecule is firmly attached to the Z band. With the most distant epitope located in the M band, titin molecules have half-sarcomere length. Earlier electron micrographs of purified TII indicating long and thin strings of rather non-homogeneous appearance (Maruyama *et al.* 1984;

Trinick *et al.* 1984; Wang *et al.* 1984) can be greatly improved on by mechanical or centrifugal orientation of the molecules before metal-shadowing (Nave *et al.* 1989). Purified TII molecules appear as long rods with a uniform diameter of 3–4 nm and a length of 0.9 μm, bearing a globular head at one end only. Immunoelectron microscopy of TII molecules indicates that the head domain corresponds to the M band anchorage region, while the free end of the molecules seems to reflect the approximate position of the N_1 line in the sarcomere. Thus the remainder of the parent TI molecule extending into the Z line is still lost upon extraction. Although the entire TII molecule seems to be elastic, incorporation into the half-sarcomere changes the situation. Only the epitopes located between the A/I junction and the N_1 line show stretch dependency (Fürst *et al.* 1988, 1989*a*; Itoh *et al.* 1988; Whiting *et al.* 1989). A further high molecular weight protein, called nebulin (apparent molecular weight 500–800K; Wang, 1985; Wang and Wright, 1988) is present only in skeletal muscle and not found in cardiac muscle (Fürst *et al.* 1988; Hu *et al.* 1986; Locker and Wild, 1986; Wang and Wright, 1988). As nebulin has not been isolated in native form its actual function in skeletal muscle sarcomeres is still open to discussion.

Some earlier reports using solely SDS–polyacrylamide gel electrophoretic criteria have suggested that titin and nebulin are also present in different invertebrate muscles (Hu *et al.* 1986; Locker and Wild, 1986; see also Wang *et al.* 1979). Although the putative invertebrate titins were reported to have a lower molecular weight, a detailed molecular characterization of the molecules has not been performed. Here we have approached the problem of elastic filaments in insect muscle using some of the same biochemical and molecular techniques that we found useful in the characterization of vertebrate titin (Nave *et al.* 1989). We describe a related polypeptide doublet with greatly reduced molecular weight (700/600K), of which only the 600K species is extracted under native conditions. The purified protein has the same diameter as titin (3–4 nm) but in line with its lower molecular weight its length is reduced to about 260 nm. This dimension fits the I band decoration pattern seen in immunoelectron microscopy using antibodies that react with both components of the doublet. Thus elastic filaments of insect muscle extend from the Z line only to the A/I junction. In consequence we call the protein doublet *mini-titin*. Immunofluorescence and immunoblotting experiments show that mini-titin is found as a myofibrillar protein of many invertebrate phyla.

Materials and methods

Purification of a high molecular weight protein Hmp II (600K) from flight muscle of Locusta migratoria

Animals were kindly provided by the Department of Zoology of the University of Goettingen. The purification scheme is a modification of the protocol developed for the isolation of titin II from chicken breast muscle (Nave *et al.* 1989). Freshly dissected flight muscle was homogenized for 15 s with a Polytron homogenizer in ice-cold low-salt buffer, LSB (100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM 2-mercaptoethanol, 1 mM NaN_3, 10 mM Tris–HCl, pH 6.8) containing 2 mM $Na_2P_2O_7$. The myofibril suspension was centrifuged for 15 min at 3000 ***g***. The pellet was washed three times with LSB. The final pellet was resuspended in ice-cold extraction solution (0.6 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM 2-mercaptoethanol, 10 mM imidazole·HCl, pH 7.0) for 5 min followed by centrifugation (20 000 ***g***, 45 min). The supernatant was extensively dialyzed against buffer T (50 mM Tris–HCl, pH 7.9, 2 mM EGTA, 1 mM 2-mercaptoethanol, 1 mM NaN_3) containing 80 mM KCl and clarified by centrifugation (100 000 ***g***, 45 min). The sample was passed through a column of DEAE-cellulose (Whatman DE-52; 1.6 cm×10 cm for 5 g muscle) equilibrated in the same buffer. The flow-through fractions were dialyzed against buffer T containing 500 mM KCl and subjected to high-resolution gel permeation chromatography (GPC). This gel filtration step was performed at room temperature at a flow rate of 12 ml h^{-1} using a TSK 6000 PW column (7.5 mm×600 mm, LKB) equilibrated in the dialysis buffer (see Results). Hmp II eluted as a narrow peak at 17.2 ml. For large-scale preparations a Superose 6 FPLC column (HR 10/30, Pharmacia) was used prior to the TSK column. Here Hmp II eluted in the void volume.

Using this protocol Hmp II was also isolated from the following muscles: *Locusta migratoria* leg muscle and *Apis mellifera* (honey bee) flight and leg muscle. The procedure can also be used to obtain Hmp II from *Drosophila melanogaster*. About 150 flies (700 mg) were homogenized in liquid nitrogen using a pestle and mortar. The resulting preparation processed through the TSK 6000 column yielded about 20 μg of pure protein (see Results). Fly Hmp II eluted at 17.6 ml from the TSK column. The only difficulty experienced with the method was observed with body muscle of the nematode *Ascaris lumbricoides*, since the preparation was still heavily contaminated by myosin. Electron-microscopical analysis of this material demonstrated typical Hmp II molecules in addition to myosin (data not shown).

Antibodies

Polyclonal antibodies to the native *Locusta* Hmp II were obtained in two rabbits using multiple injections (about 0.2 mg/injection) and standard immunization protocols. The IgG fraction was isolated using a Protein A FPLC column (HR10/2, Pharmacia).

Gel electrophoresis and immunoblotting

Electrophoresis was performed on SDS–polyacrylamide gradient gels (2% to 12% acrylamide and 0.5% bisacrylamide) without a stacking gel using the Laemmli buffer system as described for titin (Fürst *et al.* 1988). Gels were routinely stained with Coomassie Brilliant Blue. Transfer of proteins to nitrocellulose followed standard protocols. Decoration with rabbit antibodies was visualized by incubation with peroxidase-labeled mouse anti-rabbit antibody (DAKOPATTS, Copenhagen) followed by peroxidase substrates.

Immunofluorescence microscopy

Glycerinated myofibrils from muscle were prepared essentially as described (Fürst *et al.* 1988). Frozen tissue sections were cut from material previously snap frozen in isopentane at −140°C and then stored at −70°C. Sections were fixed in acetone for 8 min at −10°C. Indirect immunofluorescence staining of frozen sections and myofibrils followed standard protocols using fluorescein isothiocyanate-labeled goat anti-rabbit antibodies (Cappel Laboratories, Cochranville, PA) at a concentration of 200 μg ml^{-1}. Rabbit anti-Hmp antibodies were used at 50 μg ml^{-1}. Frozen tissue blocks of various invertebrates were

available from a previous study on invertebrate intermediate filaments (Bartnik and Weber, 1989; see there for References).

Immunoelectron microscopy

Tissue preparation and antibody labeling was performed as described (Fürst *et al.* 1988). Animals were anesthetized for about 10 min in an ether atmosphere containing the muscle relaxant mephenesin (Aldrich) and then killed by CO_2 gas. Flight and leg muscles were removed and tied to small plastic strips. Smaller muscle fibers (e.g. honey bee leg muscle) were adhered to gold grids (50 mesh) using a small drop of Histoacryl (Braun, Melsungen, FRG). Skinning, decoration with the first antibody (rabbit anti Hmp IgG at 500 $\mu g\,ml^{-1}$), washing steps, decoration with the second antibody (affinity-purified sheep anti-rabbit IgGs), washing steps, fixation and dehydration were according to Fürst *et al.* (1988). Prior to embedding in Epon, the tissue was removed from the strips or grids. Ultrathin sections were positively stained with uranyl acetate and Reynold's lead citrate and viewed on a Philips 301 electron microscope at 80 kV.

Electron microscopy of single molecules

The normal procedure involved the spraying from a 50% glycerol solution followed by metal shadowing. In addition we made use of the procedure developed to orient titin II molecules of vertebrate muscle (Nave *et al.* 1989). Briefly, molecules were layered on the mica in buffer containing 50% glycerol. The mica was stuck to a cardboard plate (at a radius of 4 cm), which was placed on top of the rotor of an Eppendorf centrifuge 3200. Centrifugation was for 15 s at top speed (12 000 revs min^{-1}). The specimens were dried at room temperature *in vacuo* in the evaporation chamber of a shadowing apparatus (improved Balzers, BAE 120). This was followed by rotary or unidirectional shadowing with tantalum/tungsten at an elevation angle of 5° and carbon at 90° using standard Balzers electron gun evaporation sources. Replicas were floated off on distilled water and collected on copper grids (400 mesh).

Miscellaneous procedures

Samples of *Locusta* Hmp II used for further analysis were collected from the central portion of the GPC peak (see above). Sedimentation coefficients were determined with a model E Beckman centrifuge equipped with Flossdorf optics and a photoelectric scanner. Centrifugation experiments were in buffer T containing 500 mM KCl at 20°C. Protein concentrations of 50–200 $\mu g\,ml^{-1}$ were used. Sedimentation coefficients were corrected to $s_{20,w}$ values.

Results

Purification of Hmp II from Locusta *flight muscle*

In low-porosity polyacrylamide gels, SDS-solubilized muscle shows a pronounced polypeptide doublet of very high molecular weight (Fig. 1A, lane 3). Hmp I and Hmp II have apparent molecular weights of about 700K and 600K, respectively, and thus differ distinctly from the titin doublet of around 2.4×10^6 seen in vertebrate skeletal muscle (compare lanes 3 and 2 of Fig. 1A). Fig. 1A also shows that Hmp I and Hmp II have a lower electrophoretic mobility than vertebrate nebulin. In high-salt solutions (0.6 M KCl, see Materials and methods) Hmp II was readily extracted, while Hmp I remained insoluble. The following scheme allowed the purification of Hmp II. Washed myofibrils were extracted with 0.6 M KCl. Upon subsequent dialysis against 80 mM KCl (see Materials and methods) actomyosin was precipitated while Hmp II remained soluble. Anion-exchange chromatography on DE-52 provided Hmp II at 90–95% purity (Fig. 1A, lane 4). A number of smaller polypeptides present in such preparations were removed by gel filtration on Superose S6 where Hmp II eluted in the void volume. A sample of the fraction was subjected to high-resolution gel permeation chromatography (GPC) on a TSK 6000 PW column (7.5 mm×600 mm). Hmp II eluted as a narrow peak (17.2 ml) within the included volume of the column (Fig. 1C). Gel electrophoresis showed a single polypeptide chain at 600K (Fig. 1A, lane 5). When only small amounts of Hmp II were required the Superose S6 step was omitted. A sample of the material from the DE-52 column was immediately processed on the TSK column.

Flight muscle of *Locusta migratoria* is good starting material for the purification of Hmp II. Up to 200 mg of muscle can be obtained from one adult animal and 500 μg of pure Hmp II are available when 1 g of muscle is processed through the Superose gel-filtration step. The method was also used to obtain Hmp II from leg muscle of *Locusta migratoria*. In addition Hmp II was isolated from flight and leg muscles of the honey bee *(Apis mellifera)*. Interestingly, the procedure also leads to the isolation of pure Hmp II of *Drosophila melanogaster* when the flies are used *in toto* (see Materials and methods).

Calibration of the TSK 6000 PW column with known molecular weight standards (Nave *et al.* 1989; Potschka, 1987) provided hydrodynamic information on *Locusta* Hmp II. Its viscosity radius is about 30 nm. Ultracentrifuge experiments documented a single sharp boundary with an $s_{20,w}$ value of 7 S. Using the approach of Potschka (1987), the viscosity radius and the sedimentation coefficient lead to a native molecular weight of about 600K. Since essentially the same value is obtained by gel electrophoresis in the presence of SDS (Fig. 1; see above), the Hmp II molecule seems to consist of a single polypeptide chain.

Hmp is muscle-specific

Hmp II of *Locusta* flight muscle was used to raise rabbit antibodies. IgGs purified from the immune sera were used in immunoblotting and immunofluorescence microscopy. The immunoblots of Fig. 1B show that the antibodies to Hmp II detected on the whole-muscle extracts only Hmp II and the higher molecular weight form Hmp I, which is not solubilized by high salt. The same polypeptide doublet was also detected in flight and leg muscles of the honey bee and in total extracts of *Drosophila*. Interestingly, the antibodies also detected a doublet in body muscle and pharynx of the nematode *Ascaris lumbricoides*. Only a band corresponding to Hmp I was detected in body muscle of the annelid *Lumbricus terrestris*.

Hmp II antibodies were also used in immunofluorescence microscopy on frozen tissue sections (for a summary see Table 1). Here they decorated the myo-

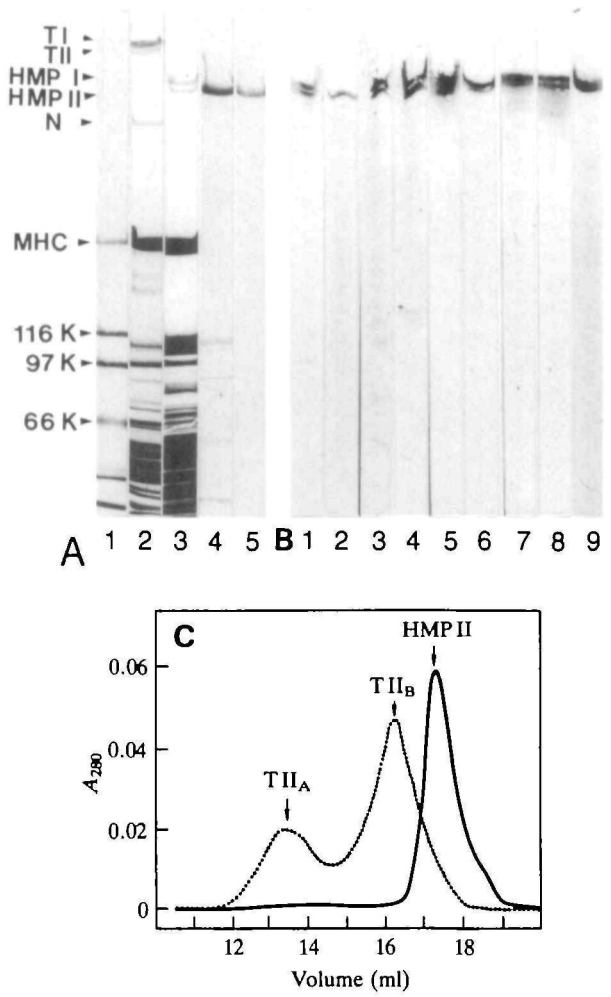

Fig. 1. Purification of Hmp II from *Lucusta* flight muscle monitored by gel electrophoresis (A) and immunoblot results (B). Molecular weight standards (myosin heavy chain MHC 200K, 116K, 97K and 66K) marked by arrows are shown in A, lane 1. A total extract of chicken breast muscle with SDS is given in A, lane 2. The positions of the titin doublet (TI, TII), nebulin (N) and myosin heavy chain (MHC) are indicated at the left. A, lane 3 presents a corresponding total extract of *Locusta* flight muscle. Note the presence of a doublet of polypeptides with apparent molecular weights of 600K and 700K (Hmp I and Hmp II) and the absence of bands related in size with titin and nebulin. Crude Hmp II obtained from the DE-52 column shows only the lower band of the doublet and a few impurities (A, lane 4). These are removed after gel filtration on the TSK 6000 PW column (A, lane 5; for elution profile see C). Rabbit antibodies to Hmp II detect on total muscle extracts (B, lane 1) only the Hmp doublet. B, lane 2 shows the positive reaction of pure Hmp II. The following seven lanes document the broad cross-species reactivity of the antibodies on total extracts of honey bee flight muscle (B, lane 3), honey bee leg muscle (B, lane 4), cockroach leg muscle (B, lane 5), *Drosophila* total animals (B, lane 6), body muscle of *Ascaris lumbricoides* (B, lane 7), pharynx of *Ascaris* (B, lane 8) and body muscle of *Lumbricus terretris* (B, lane 9). Note the specific decoration of a doublet of polypeptides corresponding in size to Hmp I and Hmp II of *Locusta* flight muscle in all these muscles. C compares the behavior of *Locusta* Hmp II and chicken titin II in gel permeation chromatography using a TSK 6000 PW column (7.5 mm×600 mm). Hmp II extracted and purified from *Locusta* flight muscle (continuous line) elutes as a narrow peak at 17.2 ml. The dotted curve shows the profile of a titin preparation from chicken breast muscle containing titin TII$_A$ and titin II$_B$ species, which elute at around 13 and 16 ml, respectively (for details see Nave *et al.* 1989). The elution positions of the titin forms and Hmp II are marked.

fibrils of several arthropod muscles (Fig. 2A–D). They reacted strongly on flight muscle of *Locusta* and honey bee (Fig. 2A and C; see also the immunoblotting in Fig. 1). A positive reaction was also obtained on the musculature of the two annelids tested (*Lumbricus terrestris* and *Hirudo medicinalis*. Fig. 2E,F,G,H). The large cells of the body musculature of the nematode *Ascaris lumbricoides* were also clearly positive (Fig. 2I,J) and so was the pharynx (not shown). In crushed samples of the small nematode *Caenorhabditis elegans* strong staining of body musculature and pharynx was observed (Fig. 2K,L). *Gordius* sp., a member of the Nematomorpha, also showed strong myofibrillar decoration (not shown). In all cases the decoration pattern was specific for sarcomeric muscles. Table 1 summarizes our results on the cross-species reactivity of the rabbit Hmp II antibodies.

Sarcomeric location of Hmp I/II in immunoelectron microscopy

Indirect immunofluorescence microscopy using freshly prepared myofibrils showed that Hmp I/II proteins were restricted to the I/Z/I area of myofibrils in *Locusta* flight and leg muscle (Fig. 3A,B). The same result was obtained on corresponding myofibrils of the honey bee (Fig. 3C,D). To study this location in more detail, myofibrils were also analyzed by immunoelectron microscopy. Use of the muscle relaxant mephenesin (see Materials and methods) helped in obtaining relaxed sarcomeres. Even very small fiber bundles could be adequately processed when they were fixed to gold grids during the first steps of the procedure (see Materials and methods). Fig. 4 summarizes some of the results. It shows very strong decoration of the entire I band with the label extending by 180 nm into the A band of the myofibrils from the flight-muscle of honey bee (Fig. 4B) and of bumble bee (Fig. 4D). In contrast, the deeper A band always remained undecorated.

Ultrastructure of Hmp II molecules

Since the hydrodynamic properties of *Locusta* Hmp II point to a very asymmetric molecule (see above) a detailed electron-microscopical analysis was performed. Standard glycerol spraying of Hmp II followed by rotary metal shadowing reveals flexible rod-shaped molecules in various arrangements (Fig. 5A). Using the specimen preparation methods developed for titin TII from vertebrate muscle (Nave *et al.* 1989; see Materials and methods) electron micrographs show a surprisingly homogeneous population of rods impressively oriented by centrifugal force. Nearly all molecules seen in the field are straightened (Fig. 5B). Thus the dimensions of the molecule could be easily calculated using myosin mol-

Table 1. *Hmp antibody reactivity on invertebrates is specific for sarcomeric muscles*

Phyla	Species	Immunofluorescence	Immunoblotting
Arthropoda			
Insecta	*Locusta migratoria*•†	+	+
	Apis mellifera•†	+	+
	Bombus sp.†	+	+
	Periplaneta americana	+	+
	Drosophila melanogaster	+	+
Crustacea	*Astacus* sp.	+	nd
	Crangon crangon	+	nd
Chelicerata	*Limulus polyphemus*	+	nd
Annelida	*Lumbricus terrestris*	+	+
	Hirudo medicinalis	+	nd
	Nereis diversicolor	+	nd
Nematomorpha	*Gordius* sp.	+	nd
Nematoda	*Ascaris lumbricoides*‡	+	+
	Caenorhabditis elegans	+	nd
Nemertina	*Lineus viridis*	+	nd
Plathelmintha	*Planaria gonocephala*	+	nd

Summary of the reaction of the rabbit antibodies to *Locusta* flight muscle Hmp II. The standard test was immunofluorescence microscopy on frozen sections. Phyla are taken from the textbook of Barnes (1980). +, positive reaction; nd, not done.

• Immunofluorescence was also performed on isolated myofibrils.

† Flight and leg muscle were tested separately and found to be strongly reactive.

‡ Pharynx was also positive in immunofluorescence and immunoblotting analysis.

ecules as internal standards. The rods have a constant diameter of 3–4 nm over their entire length. The length distribution is very narrow (230–290 nm) with a pronounced peak at 260 nm. Combination of this length and the molecular weight of 600K (see above) yields a mass per unit length value of 2.3 kilodaltons nm^{-1}, which is in excellent agreement with the value found for vertebrate titin by the same approach (Nave *et al.* 1989). Fig. 5C shows six molecules of the *Locusta* protein at higher magnification. Essentially the same molecules were seen when Hmp II was purified from honey bee flight and leg muscles (Fig. 6A,B).

Hmp II was also obtained by homogenizing fruit flies in liquid N_2 with a mortar and a pestle and then following the standard procedure. The *Drosophila* protein eluted on the TSK 6000 column at 17.6 ml and reacted with Hmp II antibodies in immunoblots (not shown; for reactivity of total animal extracts see Fig. 1B lane 6). As shown in Fig. 6C it has essentially the same ultrastructure as the more readily available muscle protein of *Locusta* (Fig. 5). Since only 20 μg of *Drosophila* protein was obtained from 700 mg of flies a more detailed analysis was postponed.

Table 2. *Comparison of insect mini-titin with vertebrate titin*

	Locusta Hmp (mini-titin)	Vertebrate titin
Apparent polypeptide size (gel electrophoresis), 6 M-guanidine-HCl (K)	~700 (Hmp I) ~600 (Hmp II)	~2800• (TI) ~2100• (TII) ~2400† (TII)
Soluble in high salt	Hmp II	TII
Viscosity radius (nm)	30	48‡
$s_{20,w}$ (S)	7	12.5‡
Native M_r (K)	~600	~2000‡ ~2400§
Subunits/molecule	1	1†
Diameter of molecule (nm)	3–4	3–4‡
Length of molecule (nm)	~260	~900‡
Mass per unit length (kilodaltons nm^{-1})	~2.3‖	2.7±0.5§ ~2.2¶
Sarcomere localization	Z to A band	Z–M (TI) N_1–M (TII)

• Maruyama (1986).

† Kurzban and Wang (1988).

‡ Values for TII_B fraction (Nave *et al.* 1989).

§ Value for TII by STEM microscopy (Hainfeld *et al.* 1988).

¶ Calculated from the native molecular weight and the length of the TII_B molecule (Nave *et al.* 1989).

‖ Calculated as for ¶.

Discussion

We have described a convenient and fast procedure for purifying a major myofibrillar protein of high molecular weight (about 600K) from the flight and leg muscles of *Locusta* and other insects. This molecule resembles in most but not all properties the vertebrate muscle titin TII molecule (Table 2) and is therefore called mini-titin. As in vertebrate sarcomeric muscle, the insect muscles reveal a doublet of polypeptides of which only the lower molecular weight species is extracted under native conditions by buffers of high salt concentration. Again, as with titin, antibodies raised against the extractable mini-titin II crossreact in immunoblotting with the slightly larger mini-titin I species, which is tentatively assumed to be the unproteolyzed parent molecule. Electron-microscopical images show that mini-titin II molecules can be oriented by centrifugation before metal shadowing. The well-oriented molecules are thin rods with a uniform diameter of 3–4 nm and a length of 260 nm (see Table 2). Thus mini-titin II of insect and titin II of vertebrate sarcomeric muscle have the same diameter and mass per unit length and both seem built from a single polypeptide chain (for original data on titin II see also Hainfeld *et al.* 1988; Kurzban and Wang, 1988). The two proteins

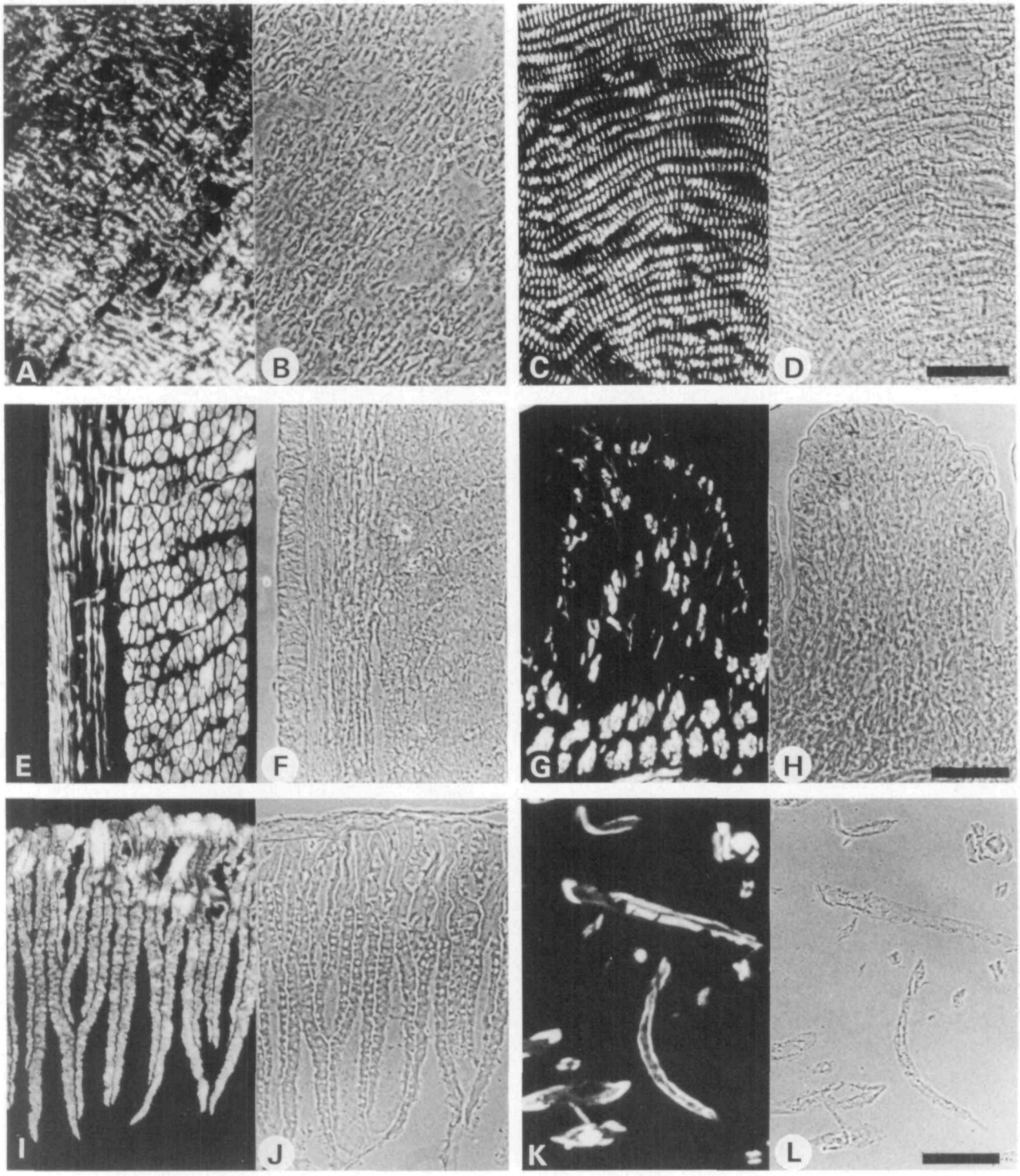

Fig. 2. Immunofluorescence and corresponding phase-contrast micrographs of frozen sections of *Locusta* flight muscle (A,B), *honey bee* flight muscle (C,D), *Lumbricus terrestris* (E,F), *Hirudo medicinalis* (G,H), *Ascaris lumbricoides* (I,J) and *C. elegans* (K,L) stained with rabbit antibodies against *Locusta* Hmp II followed by fluorescently labelled goat anti-rabbit antibodies. Note the characteristic striation pattern in the sarcomeric muscle of the arthropods (A–D). In the annelids all the muscle fibers are clearly stained (E–H). Note the specific staining of the body musculature of *Ascaris* (I,J) with epidermis and cuticle unstained. In the small nematode *C. elegans* (K,L) staining is restricted to the pharynx and the body muscle. Bars, 25 μm, A–D; 100 μm, E–I.

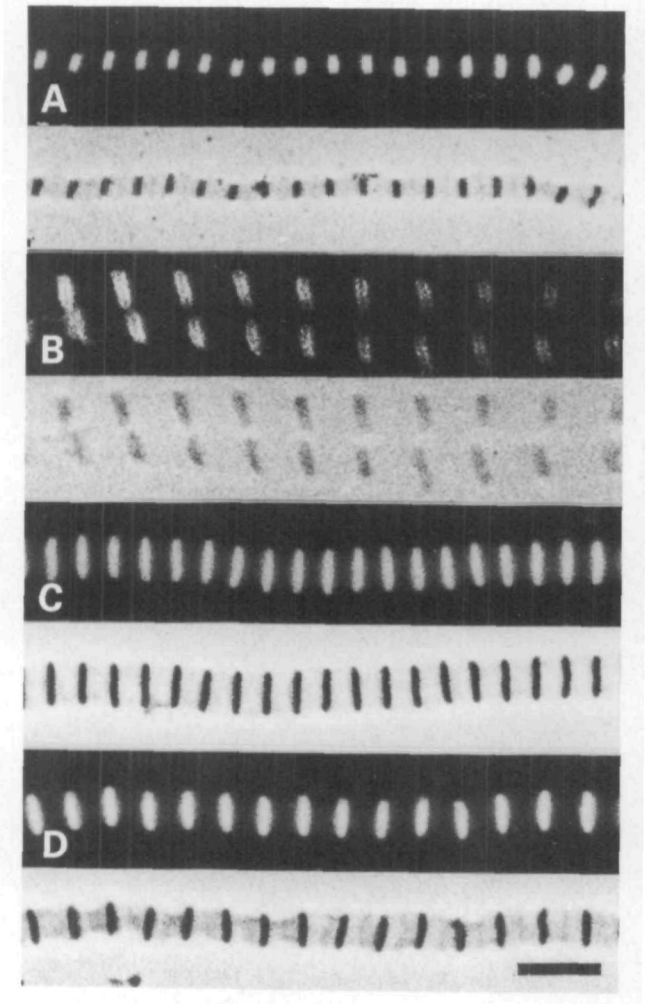

Fig. 3. Immunofluorescence and corresponding phase-contrast micrographs of myofibrils decorated with rabbit Hmp antibodies followed by a fluorescent second antibody. The upper images show the fluorescence pattern and the lower images give the corresponding phase-contrast picture. The following myofibril preparations were used: A, *Locusta* flight muscle; B, *Locusta* leg muscle; C, honey bee flight muscle; and D, honey bee leg muscle. The phase-dense areas in the phase-contrast micrographs represent the Z band of the sarcomere. Note that in each case the I–Z–I area is heavily labeled. Bar, 5 μm.

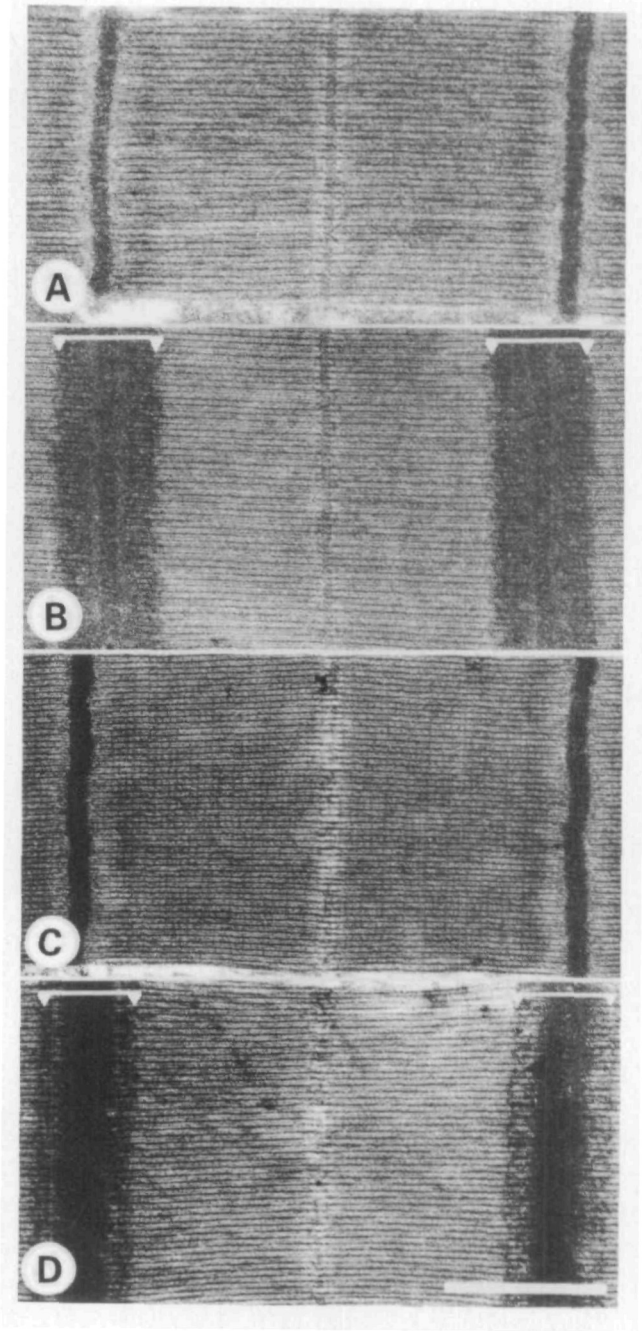

Fig. 4. Immunoelectron microscopical localization of Hmp antigens in flight muscle of honey bee (A,B) and bumble bee (C,D). Muscle fiber bundles were processed with rabbit antibodies to Hmp II followed by sheep anti-rabbit antibodies (B,D), while the corresponding controls (A,C) were treated with anti-rabbit antibodies only. Note the heavy labelling of the I–Z–I area and the absence of decoration in the deeper A band (B and D). Positions of Hmp labelling are marked by brackets. Bar, 1 μm.

differ, however, in molecular weight and molecular length. The much larger titin II molecule reaches 900 nm and has a molecular weight of approximately 2200K, while mini-titin II with a molecular weight of 600K is only 260 nm long. This difference in molecular length is reflected in the myofibrillar location of the molecules. Titin molecules are known to connect the Z and M lines of vertebrate sarcomeric muscles (Fürst *et al.* 1988, 1989*a*; see also Itoh *et al.* 1988; Whiting *et al.* 1989), while rabbit antibodies to mini-titin decorate only the I band, in immunofluorescence and immunoelectron microscopy. Thus in line with their shorter length mini-titin molecules seem to connect the Z lines with the ends of the thick filaments (for a summary see Fig. 7).

Whether there is a relation between mini-titin and a protein called projectin is unclear. Using isolated Z-discs of honey bee flight muscle, Saide (1981) observed a high molecular weight protein using a urea/SDS-containing gel system. Owing to the entirely different gel system and the absence of suitable markers, the reported value of 360K for projectin cannot readily be compared with that of mini-titin. However, antibodies raised by gel slices

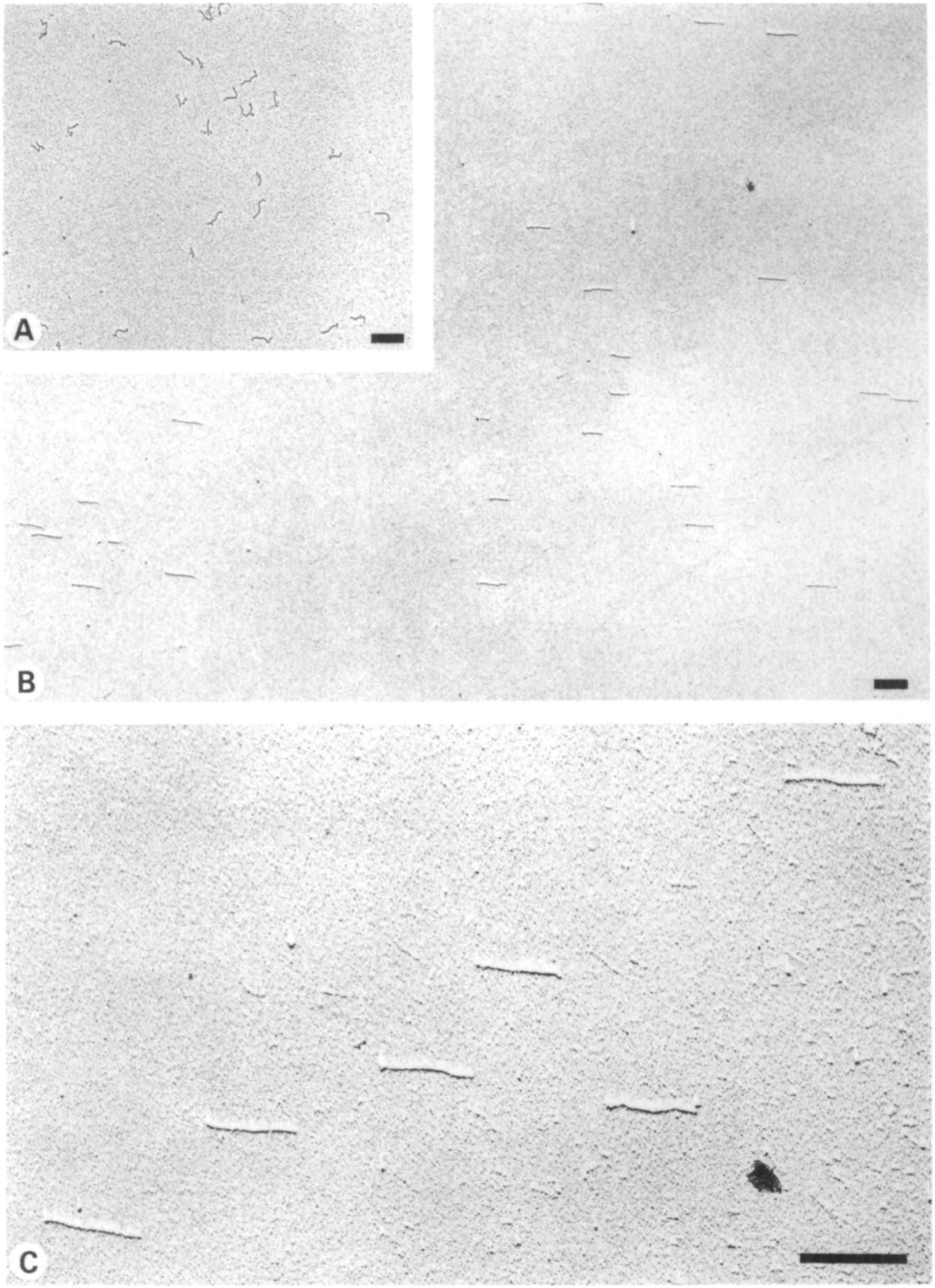

Fig. 5. Electron-microscopic appearance of purified *Locusta* Hmp II molecules after metal shadowing. The same protein preparation was used in all experiments. A. Appearance of Hmp II molecules after spraying from 50% glycerol solution and rotary shadowing. Flexibility of the rod-like molecules is clearly demonstrated but the appearance is non-homogeneous. B. Molecules layered on mica in 50% glycerol were oriented by centrifugation prior to unidirectional shadowing (for details see Materials and methods). Note the uniform orientation of all molecules in the field and their homogeneous appearance. C. Higher magnification of six molecules processed as in B. Note the constant length and diameter of the rods. Bars, 300 nm.

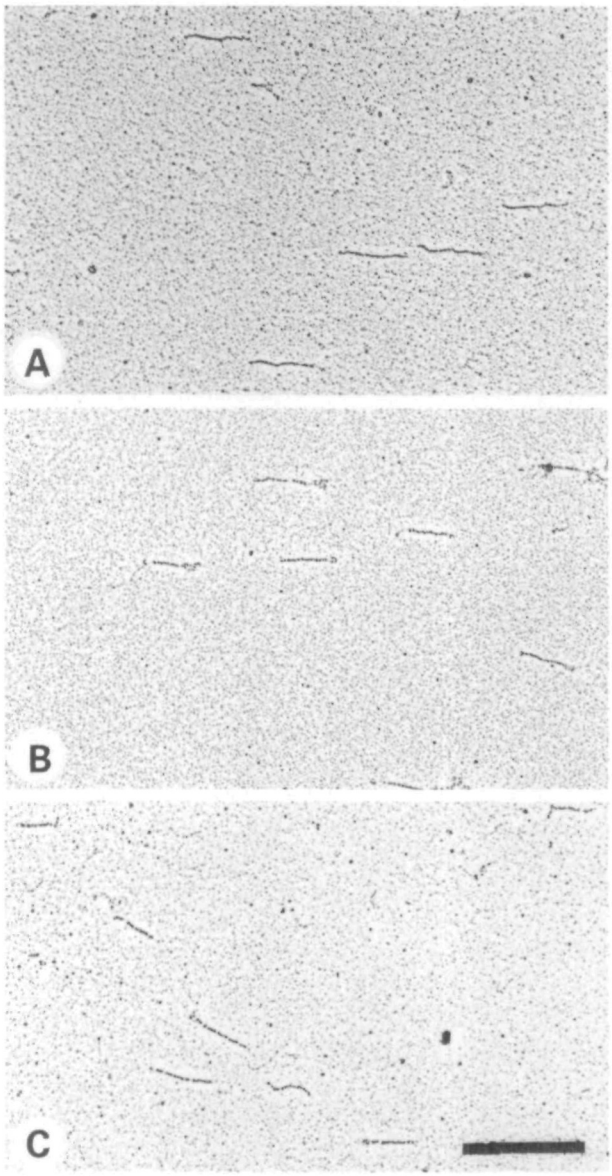

Fig. 6. Electron microscopic appearance of Hmp II molecules (mini-titin II) purified from honey bee flight muscle (A), honey bee leg muscle (B) and *Drosophila* (C) after rotary shadowing. Molecules were oriented by centrifugation as in Fig. 5B. Note the similar morphology of the mini-titin molecules. Bar, 0.5 μm.

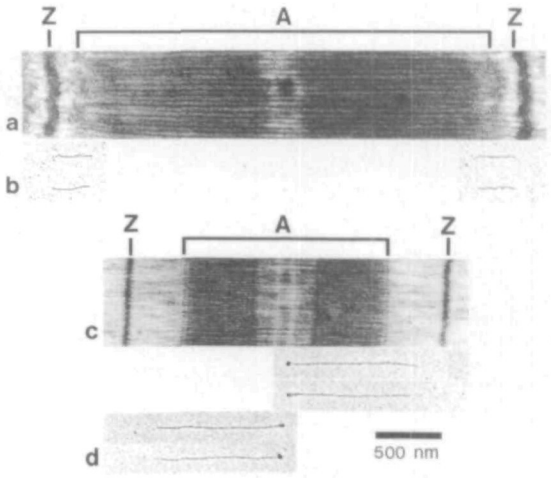

Fig. 7. Comparison of the proposed half-sarcomere localization of mini-titin (Hmp) molecules (b) in *Locusta* flight muscle (a) and titin II_B molecules (d) in the sarcomere of chicken breast muscle (c). Longitudinal muscle sections obtained after relaxation (a, c) are shown at the same magnification as the corresponding molecules observed after rotary shadowing (b, d). Note that the length of the isolated molecules corresponds to the postulated localization within the half-sarcomere. Mini-titin molecules are long enough to connect the Z lines with the end of the thick filaments. Titin II_B spans the distance from the M-band to the N_1-line. The small globular head of the titin molecules seems to reflect the M band anchoring domain (for details, see Nave *et al.* 1989). Note that in both cases the isolated molecules are proteolytic derivatives of parent molecules. While the free end of titin II locates approximately to the N_1-line, the non extractable TI continues into the Z-line (Nave *et al.* 1989). The A-band and the Z-line are indicated. Bar, 500 nm.

containing projectin showed an interesting specificity among sarcomeric muscles. Although the I/Z/I area of the honey bee flight muscle was heavily stained, no reaction was observed on leg muscle. Thus it is thought that projectin is a characteristic constituent of flight muscle only (Saide, 1981). Mini-titin is clearly different in this respect, as we have purified it under native conditions from the flight as well as the leg muscle of *Locusta* and honey bee. In addition, in all cases that we have tested, the mini-titins from the two different muscle types show very strong immunological cross-reactivity. Clearly projectin must now be purified under native conditions to establish its molecular properties.

Several laboratories have tried to explore the presence of titin-related polypeptides in various invertebrates using solely gel electrophoresis and relative abundance in their analysis of the different muscles (Locker and Wild, 1986; Hu *et al.* 1986). In addition, Wang *et al.* (1979) noted a striated staining pattern, when rabbit antibodies to vertebrate titin were used in immunofluorescence microscopy on myofibrils of the cricket. Our results confirm and extend these earlier reports. We have purified native mini-titin from the leg and flight muscle of two insects (*Locusta* and *Apis*) and shown that the same molecule is obtained when a further insect (*Drosophila*) is processed *in toto*. The rabbit antibodies raised against the flight muscle protein of *Locusta* further emphasize this point. Used in immunofluorescence microscopy, they show an unexpectedly broad cross-reactivity covering the sarcomeric muscles in various species of different invertebrate phyla (Table 1). In those cases that were further analyzed by immunoblotting (various insects, the annelid *Lumbricus terrestris* and the nematode *Ascaris lumbricoides*) a strong decoration of similar sized mini-titin polypeptides was observed. Given the strong reaction of mini-titin in *Drosophila* and *C. elegans* with the rabbit antibodies it should now be possible to approach the protein by DNA-cloning methods, which are particularly well advanced for use with these two invertebrates. Together with similar work already expected for ver-

tebrate titin it should be possible to explain the molecular and functional aspects of a different sarcomeric disposition, i.e. Z to M band linkage (vertebrate titin) and Z to A/I junction linkage (invertebrate mini-titin). Here we note that Whiting *et al.* (1989) recently suggested that titin may regulate the length of vertebrate thick filaments and Fürst *et al.* (1989*b*) have emphasized that titin could act as the integrator of I/Z/I compartments and A bands during vertebrate myofibrillogenesis. If these two aspects were correct invertebrate muscle might lack the additional M line to A/I junction linkage as the length of the thick filaments may be regulated by paramyosin (MacKenzie and Epstein, 1980; Kagawa *et al.* 1989), which is specific for invertebrates.

After submission of this report we have learned that Benian *et al.* (1989) have published the complete characterization of the *unc*-22 gene of the nematode *C. elegans*. Since mutant animals lacking the *unc-22*-encoded protein show a nearly constant twitching of the body muscles, which are unable to develop or sustain normal contraction, the gene product has been called *twitchin*. There are several reasons to suspect that the mini-titin, which we have described, and twitchin are related molecules. The molecular weight expected for twitchin is 668K and its sequence reveals characteristic motif repeats also recognized by J. Trinick and colleagues in unpublished sequences of vertebrate titin (Benian *et al.* 1989). In addition, as we have shown here, antibodies to mini-titin from *Locusta* muscle recognize a related high molecular weight polypeptide doublet in the muscle of the large nematode *Ascaris lumbricoides* and react positively in immunofluorescence microscopy on muscle of *C. elegans*. The ultrastructural relation between titin and mini-titin described by us and the sequence relation between titin and twitchin (Benian *et al.* 1989) argue for an important molecular function of these molecules in myofibrillar assembly and function.

We thank Dr D. O. Fürst for encouragement and many helpful discussions. R.N. was supported by a fellowship from the Max Planck Society.

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(Received 14 November 1989 – Accepted 8 January 1990)