The Organization of Titin Filaments in the Half-Sarcomere Revealed by Monoclonal Antibodies in Immunoelectron Microscopy: A Map of Ten Nonrepetitive Epitopes Starting at the Z Line Extends Close to the M Line

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Abstract. mAbs specific for titin or nebulin were characterized by immunoblotting and fluorescence microscopy. Immunoelectron microscopy on relaxed chicken breast muscle revealed unique transverse striping patterns. Each of the 10 distinct titin antibodies provided a pair of delicate decoration lines per sarcomere. The position of these pairs was centrally symmetric to the M line and was antibody dependent. The results provided a linear epitope map, which starts at the Z line (antibody T20), covers five distinct positions along the I band (T21, T12, T4, T1, T11), the A–I junction (T3), and three distinct positions within the A band (T10, T22, T23). The epitope of T23 locates 0.2 μm before the M line. In immunoblots, the two antibodies decorating at or just before the Z line (T20, T21) specifically recognized the insoluble titin TI component but did not recognize TII, a proteolytic derivative. All other titin antibodies recognized TI and TII. Thus titin molecules appear as polar structures lacking over large regions repetitive epitopes. One physical end seems related to Z line anchorage, while the other may bind close to the M line. Titin epitopes influenced by the contractional state of the sarcomere locate between the N1 line and the A–I junction (T4, T1, T11). We discuss the results in relation to titin molecules having half-sarcomere lengths. The three nebulin antibodies so far characterized again give rise to distinct pairs of stripes. These locate close to the N2 line.

THE pioneering work of the laboratories of Maruyama and Wang has suggested that sarcomeric structure relies not only on the well-known thick and thin filaments with their various associated proteins but also involves an elastic component (for recent reviews see 19, 35). While the nature of the elastic filaments is still poorly understood, it seems clear that they are built from very high molecular weight polypeptides (20, 22, 38). One major component of the system is titin. Its polypeptide molecular weight is variously given as 1 or 2.8 million (21, 31, 32, 39). Titin is usually present as a doublet on low porosity gels and only the second species TII can be purified under native conditions (10, 11, 31, 40). TII is thought to arise by limited proteolysis from the nonextractable TI species (19, 35). Although electron micrographs of purified TII indicate morphological heterogeneity, they clearly document very thin and rather long threads. While the exact biophysical properties are still in dispute, length measurements range from ~0.6 to 1.2 μm (21, 31, 40). In agreement with the proposal that it plays a pivotal role in sarcomere integrity, titin is found both in skeletal and cardiac muscle, although possibly not as identical molecules (7, 9, 17). Much less is known about nebulin from skeletal muscle. Its molecular weight is ~0.5–0.8 million (21, 35, 38). As it has not been isolated in native form, its function and shape are not known. In addition, there are reports that nebulin is not expressed in cardiac muscle (9, 17).

There is distinct disagreement about the arrangement of titin within the sarcomere. Some immunocytochemical studies with polyclonal antibodies indicate that titin filaments could reach from the Z band through the A–I junction deep into the A band where they may run parallel to the thick filaments (19, 23). Other results, however, have argued for a titin disposition between the two N2 lines of the sarcomere (32, 34, 35, 37, 38). In spite of discrepancies as to the exact molecular features of the titin molecule, all results point to long threads with the ultrastructural inhomogeneity possibly arising from suboptimal conditions used during purification or specimen preparation. More recent electron micrographs have raised the possibility that titin filaments contain repetitive domains (31, 35). Here we approach the problem of titin and nebulin with a set of distinct mAbs. Immunoelectron microscopy shows that each of the 10 titin antibodies we describe detects its epitope as a single stripe per half-sarcomere. The position of these stripes starts at the Z band and ends close to the M line. The results indicate an epitope polarity most likely

related to a structural polarity of the elastic filaments. They also show that the extra mass of the titin TI form is involved in filament anchorage at the Z line.

Materials and Methods

Preparation of Nebulin and Titin

Nebulin from chicken breast muscle was isolated according to Wang and Greaser (36) with the following modifications. The myofibrillar pellet was extracted with a solution containing 0.6 M KCl, 10 mM imidazole-HCl, pH 7.0, 1 mM EDTA, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to remove most of the myosin. After three washes in 10 mM Tris-maleate, pH 6.8, 100 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM PMSF, much of the actin was removed by substituting 1 M KI for 0.6 M KCl in the myosin extraction buffer. The resulting pellet was washed with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM PMSF. The "high salt-extracted myofibrils" were subsequently solubilized by SDS and applied to a Sephacryl S-500 gel filtration column (2.5 × 100 cm) equilibrated in 1% SDS, 1% 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.3. Fractions containing nebulin were detected by gel electrophoresis. Detergent was removed by ion-pair extraction (6) and the protein pellet was stored at −20°C. The lower band of the titin doublet (titin II) was isolated under native conditions essentially as described (31).

Immunization and Monoclonal Antibodies

For the first fusion BALB/c mice were immunized with nebulin suspended in PBS by sonication. 30–50 μg was used per injection and a standard immunization protocol was used (1). Spleen cells were fused with mouse myeloma cells (line PAI) as before (1). Macroscopic colonies were visible by day 9. Supernatants were screened first by immunofluorescence microscopy on frozen sections of chicken breast muscle, and then by immunoblotting (see below). Positive colonies were subcloned by limiting dilution. Ascites fluids were elicited in female BALB/c mice primed with Pristane. This fusion yielded the mAbs to nebulin described below as well as titin antibodies T10, T11, and T12. For the second fusion, mice were immunized with native titin purified as TII by standard procedures. This fusion yielded mAbs T20, T21, T22, and T23. Three additional titin antibodies (T1, T3, and T4) were previously characterized (7). Polyclonal antibodies to native titin TII were obtained in two rabbits using multiple injections (0.2 mg/injection).

Immunofluorescence Microscopy

Tissues were snap frozen in isopentane cooled to −140°C and stored at −70°C until use. 5-μm sections were treated with acetone for 6 min at −10°C and air dried before antibody staining. Glycerinated myofibrils from various muscles (chicken breast, pigeon breast, rabbit back, rabbit psoas, and rat psoas) were prepared essentially as described (13). Indirect immunofluorescence staining of frozen sections and myofibrils followed standard procedures (7) using commercial fluorescein-labeled second antibodies at 1:60 dilution (goat anti-mouse antibodies; Cappel Laboratories, Cochranville, PA). The polyclonal rabbit anti-α-actinin antibody was a generous gift from Dr. J. V. Small (Austrian Academy of Sciences, Salzburg, Austria). For double labeling, second antibodies (FITC-labeled goat anti-mouse and rhodamine-labeled goat anti-rabbit) were cross absorbed on unlabeled IgGs of the other species.

Gel Electrophoresis

Linear polyacrylamide gels (2–12% acrylamide and 0.5% cross-linker) without a stacking gel in the buffer system of Laemmli (14) were used in SDS-PAGE. Polypeptides were electrophoretically transferred to nitrocellulose (29). Decoration with mAbs was visualized by incubation with peroxidase-labeled rabbit anti-mouse antibody (DAKOPATTS, Copenhagen) followed by peroxidase substrates.

Electron Microscopy

Tissue preparation and antibody labeling was performed after a modification of the method of Dennis et al. (2).

Chickens anesthesized with ether were injected intravenously with the muscle relaxant Pantolax (CuraMed, Freiburg, FRG). Muscles were removed and the animal was sacrificed. Small strips (1 × 10 mm) of pectoralis major muscle were dissected. After relaxation for 1 h on ice in solution A (100 mM KCl, 4 mM ATP, 5 mM $MgCl_2$, 4 mM EGTA, 20 mM Tris-HCl, pH 7.0, 1% glucose, 0.5 mM iodoacetamide, 0.1 mM PMSF) they were tied to plastic strips. Subsequent skinning was by immersion for 6 h into ice cold solution B (10 mM phosphate buffer, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1% glucose, 1 mM iodoacetamide, 0.1 mM PMSF, 0.02% NaN_3) containing 0.2% Triton X-100. Muscle strips were incubated overnight at 4°C with 1.5 ml of the first antibodies. These were either hybridoma culture supernatants concentrated 5–10-fold or IgGs purified from ascites fluids (final concentration ~0.7 mg/ml). After several washes in solution B (four changes in 2 h), muscle strips were treated with antigen affinity-purified sheep anti-mouse antibody (0.3 mg/ml) for 6 h and washed as before. Muscles used as controls were treated only with the second antibody. Subsequently specimens were fixed with 1% glutaraldehyde in solution B for 30 min, washed three times with 0.1 M Na-cacodylate buffer, pH 7.0, postfixed in 1% OsO_4 in 0.1 M Na-cacodylate buffer, pH 7.0, for 10 min, dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were positively stained with uranyl acetate and Reynold's lead citrate and observed in a Philips 301 electron microscope operated at 80 kV.

Results

Monoclonal Antibodies to Titin and Nebulin

Myofibrils of chicken breast muscle were subjected to myosin extraction. Nebulin was then solubilized with SDS and purified by gel filtration on Sephacryl S-500. Although fractions highly enriched in nebulin still contained a 400-kD proteolytic fragment of titin as judged by immunoblotting with titin-specific antibodies, the preparation was used to immu-

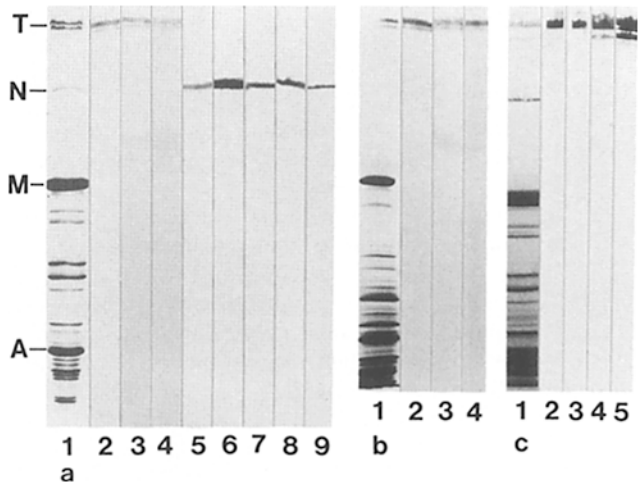


Figure 1. Immunoblot analysis of monoclonal titin and nebulin antibodies on (*a* and *c*) chicken skeletal and (*b*) chicken heart muscle. (*a*) Lane *1*, Coomassie-stained whole extract of chicken breast muscle. The positions of the titin doublet (*T*), nebulin (*N*), myosin heavy chain (*M*), and actin (*A*) are indicated. Lanes *2–9* are immunoblots with antibodies T10, T11, T12, Nb1, Nb2, Nb4, Nb5, and Nb6, respectively. (*b*) Lane *1*, Coomassie-stained whole extract of chicken heart muscle. Lanes *2–4* are immunoblots with antibodies T10, T11, and T12, respectively. (*c*) Lane *1*, Ponceau-stained blot of whole extract of chicken breast muscle. Lanes *2–5* are immunoblots with antibodies T20, T21, T22, and T23, respectively. T20 and T21 reacted only with the upper band of the titin doublet in skeletal muscle. T12 labeled both bands in skeletal, but only the upper band in heart muscle. The other titin antibodies recognized both bands in all samples. For the possible lack of nebulin in heart muscle see text. Gels in *a*, *b*, and *c* were run on different occasions.

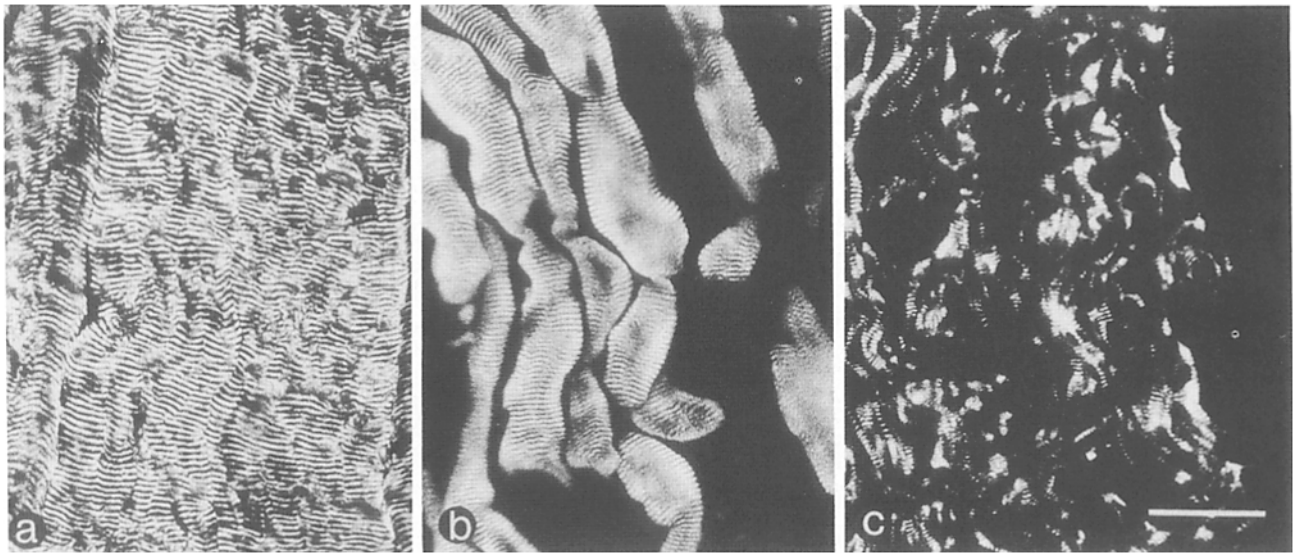


Figure 2. Immunofluorescence micrographs of frozen sections of chicken skeletal muscle (*a*), human skeletal muscle (*b*), and bovine heart Purkinje fibers (*c*), stained with antibodies against nebulin (*a* and *b*) and titin (*c*). (*a*) Chicken skeletal muscle; antibody Nb2. (*b*) Human skeletal muscle; antibody Nb6. (*c*) Bovine heart Purkinje fibers; antibody T12. Note that in *c* the titin staining covers only the Purkinje fibers and not the connective tissue. Bar, 10 μm.

nize BALB/c mice. Test sera were screened by immunofluorescence on frozen sections of breast muscle at different dilutions. Spleen cells of the mouse with the highest titer were fused with PAI myeloma cells. Hybridoma supernatants were monitored by immunofluorescence microscopy. Interesting hybridomas were cloned from single cells and grown in culture and/or as ascites. mAbs were characterized by immunoblotting on total extracts of chicken breast muscle separated on 2–12% acrylamide gradient gels. This gel type provided a better resolution than the previously described 3.2% gels (33) and clearly resolved most of the low molecular weight polypeptides (Fig. 1). Three mAbs (T10, T11, T12) specifically reacted with both bands of the titin doublet (Fig. 1). Five mAbs (Nb1, Nb2, Nb4, Nb5, Nb6) were specific for the 500-kD nebulin band.

All antibodies were further characterized by immunoglobulin type and by cross-reactivity patterns on skeletal and cardiac muscle of various vertebrates as seen by immunofluorescence microscopy and immunoblotting (see Fig. 2 and Table I). Many antibodies (e.g., T12, Nb1, and Nb2 in Table I) had a broad cross-species reactivity often extending from cold-blooded vertebrates to man (Table I). While all

Table I. Properties of Monoclonal Antibodies

Clone number	T10	T11	T12	T20	T21	T22	T23	Nb1	Nb2	Nb4	Nb5	Nb6
Immunoglobulin type	IgG2a	IgG2b	IgG1	IgG1	IgG1	IgG1	IgG1	IgG2b	IgG1	IgG2a	IgG1	IgM
Fish skeletal muscle (*Torpedo* species)	−	+	+	−	+	+	−	+	+	−	−	−
Toad skeletal muscle (*Bufo marinus*)	−	+	+					+	+	−	−	−
Salamander skeletal muscle* (*Ambystoma tigrina*)	−	+	+	−	+	+	−	+	+	+	−	
Salamander heart muscle	−	+	+					−	−	−	−	−
Alligator skeletal muscle (*Alligator mississippiensis*)	+	+	+					+	+	+	+	−
Pigeon skeletal muscle*‡	+	+	+					+	+	+	+	+
Chicken skeletal muscle*‡	+	+	+	+	+	+	+	+	+	+	+	+
Chicken heart muscle*	+	+	+	+	+	+	+	−	−	−	−	−
Chicken gizzard smooth muscle*	−	−	−	−	−	−	−	−	−	−	−	−
Mouse skeletal muscle	−	−	+	−	+	−	−	+	+	+	+	+
Rat skeletal muscle*‡	+	+	+					+	+	+	+	
Rat heart muscle	+	+	+					−	−	−	−	−
Rat rhabdomyosarcoma			+					+	+	+		
Rabbit skeletal muscle‡	+	+	+					+	+	+	+	+
Bovine heart muscle	+	+	+					−	−	−	−	−
Pig skeletal muscle*	+	−	+	−	+	−	−	+	+	+	+	−
Human skeletal muscle	+	+	+	−	+	−	−	+	+	+	+	+
Human rhabdomyosarcoma			+					+	+			

Summary of the reaction of the mAbs on various muscles. The standard test was immunofluorescence on frozen sections. The reaction on human skeletal muscle was ascertained by blotting data for antibodies T10, Nb1, Nb2, Nb3, Nb4, and Nb6.
* Additional immunoblotting experiments.
‡ Immunofluorescence was also performed on isolated myofibrils.

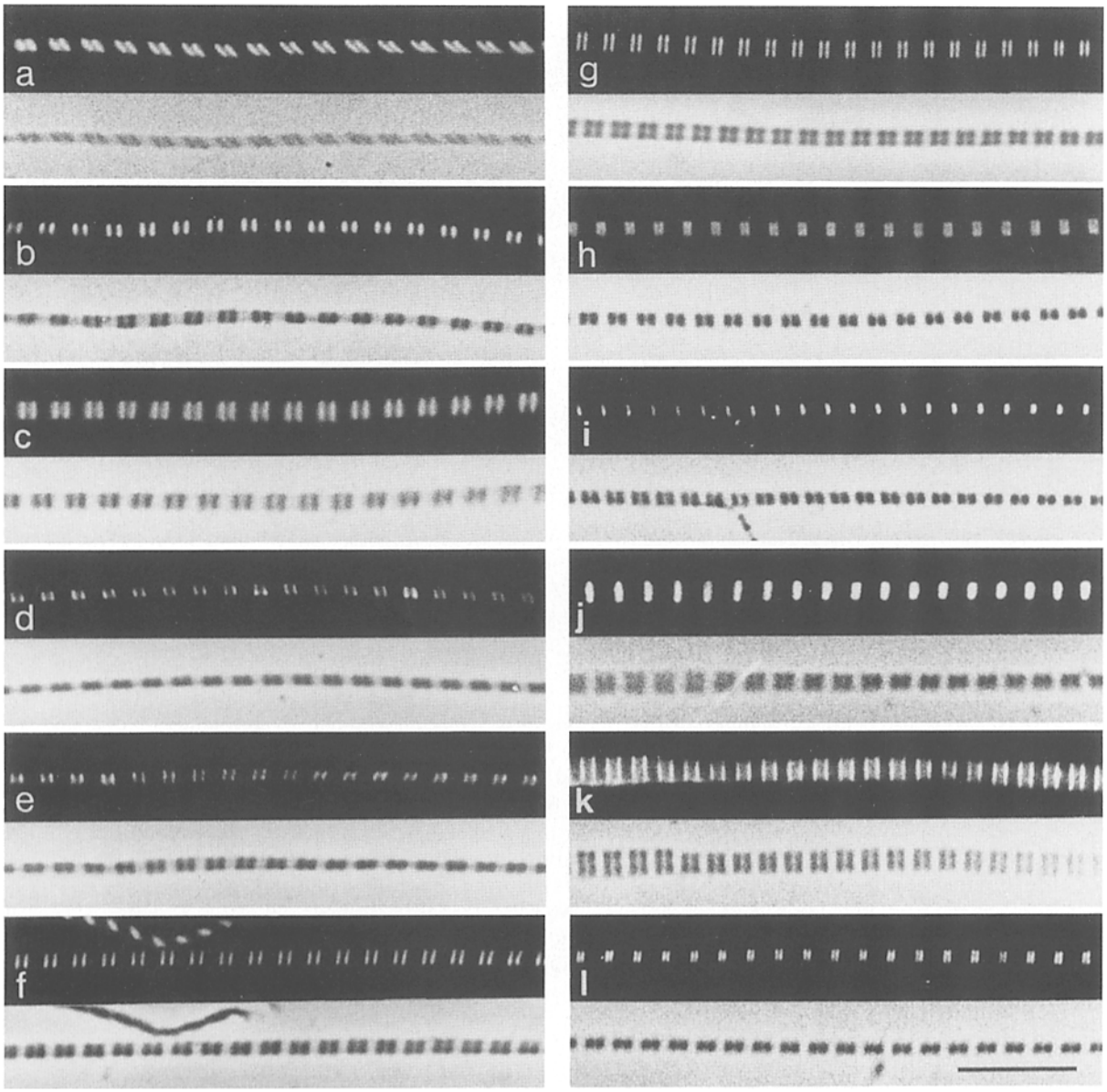
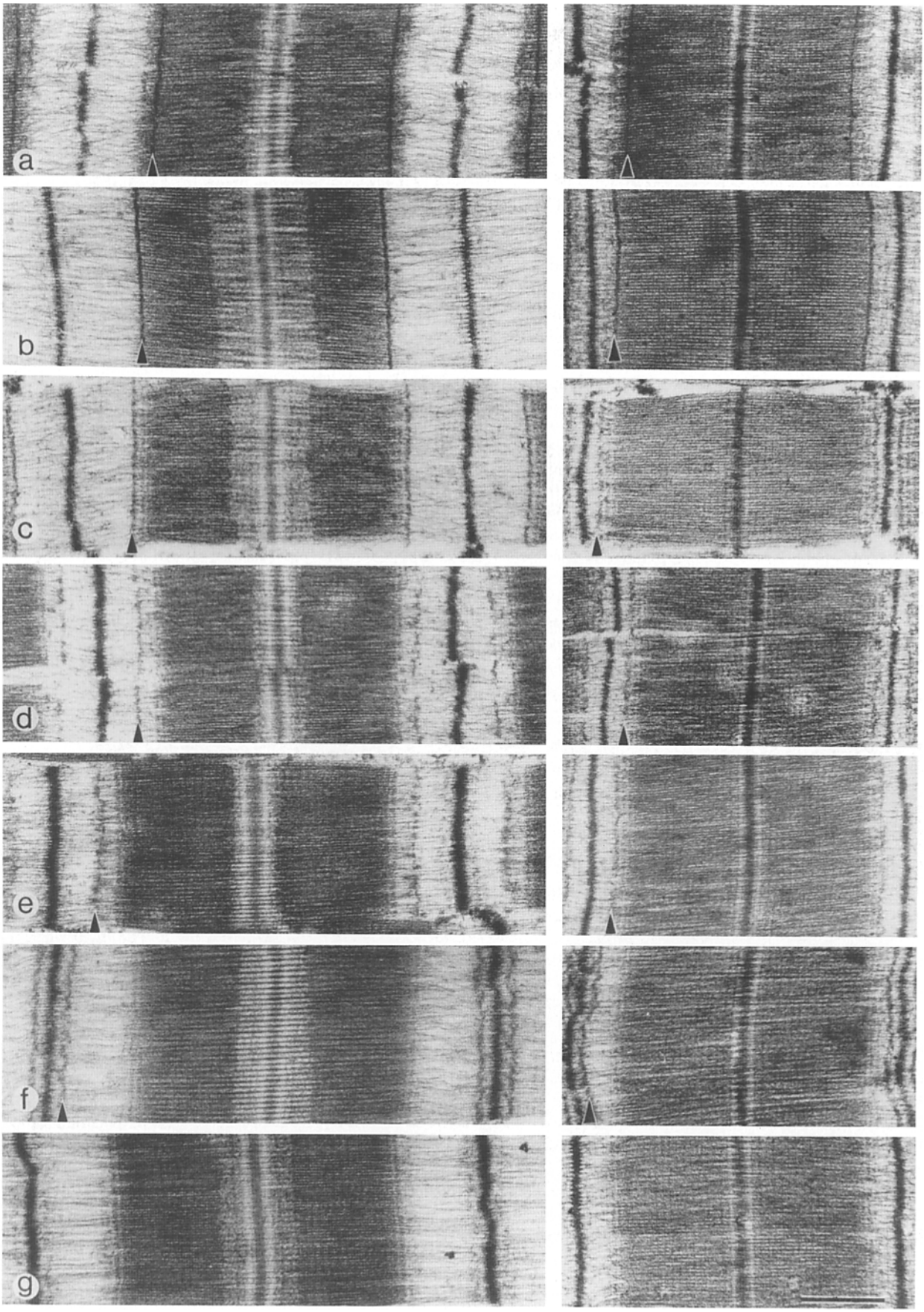


Figure 3. Immunofluorescence and corresponding phase-contrast micrographs of chicken breast muscle myofibrils decorated with nebulin and titin antibodies. In each pair of micrographs, the upper panel shows the fluorescence pattern while the lower panel shows the corresponding phase-contrast picture. Antibodies used were as follows: (*a*) Nb1, (*b*) Nb2, (*c*) Nb4, (*d*) Nb5, (*e*) Nb6, (*f*) T10, (*g*) T11, (*h*) T12, (*i*) T20, (*j*) T21, (*k*) T22, and (*l*) T23. Bar, 10 μm.

three titin antibodies cross reacted on skeletal and cardiac muscle (Table I), the five nebulin antibodies reacted only on skeletal muscle and not on heart. In agreement with earlier reports we were unable to detect a nebulin polypeptide in cardiac muscle by gel electrophoresis even when protease inhibitors were included in the buffer to prepare the extract (7, 15). Since none of the nebulin antibodies gave a positive reaction by immunoblotting of total cardiac muscle extract, we also studied dissected Purkinje fibers, the specialized impulse-conducting system of the heart. As judged by immunofluorescence (Fig. 2 *c*) and blotting experiments, bovine Purkinje fibers displayed only titin but not nebulin. Im-

Figure 4. Immunoelectron microscopical localization of titin in chicken pectoralis muscle with six different mAbs. Extracted fiber bundles were incubated with monoclonal titin antibodies and sheep anti-mouse antibody and then processed for electron microscopy as described in Materials and Methods. Results are presented in pairs. The micrographs on the left show the result on relaxed sarcomeres while the micrographs on the right give the result in contracted sarcomeres. Positions of antibody label are indicated by arrowheads. Note that each antibody gives only one specific decoration line per half-sarcomere. To avoid overburdening the figure, arrowheads are only used in one half-sarcomere. Antibodies are (*a*) T10, (*b*) T3, (*c*) T11, (*d*) T1, (*e*) T4, and (*f*) T12. *g* shows control muscle treated only with the second antibody (sheep anti-mouse). Bar, 500 nm.


a
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g

munofluorescence and immunoblotting experiments were also performed on other tissues. No reactivity of nebulin and titin antibodies was found on smooth muscle or on different nonmuscle tissues and cultured cells. We note, however, a positive reaction of titin and nebulin antibodies on rhabdomyosarcoma (Table I), a tumor of skeletal muscle lineage (see also reference 27 for titin). The combined results document the absence of a cross-reactivity between titin and nebulin. They also suggest that most of the antibodies should detect different epitopes on their corresponding polypeptide antigens.

Immunofluorescence Microscopy on Isolated Myofibrils

To begin to understand the display of titin and nebulin within the sarcomere, freshly prepared glycerinated myofibrils of chicken breast muscle were decorated with the various mAbs (Fig. 3). Indirect immunofluorescence microscopy showed that T10 and T11 stained around the region of the A–I junction (Fig. 3), while T12 provided a Z line–type staining, which seemed to be wider than the staining pattern seen with α-actinin. Therefore, double immunofluorescence microscopy was performed with the murine monoclonal T12 and a rabbit antibody to α-actinin. T12 decoration was two to three times broader than the α-actinin pattern (result not shown). In optimal specimens the T12 stain separated into two very closely spaced lines (Fig. 3 *h*) which we tentatively assume to reflect the so-called N1 lines. These are closely located to both sides of the Z band (16).

All five nebulin mAbs stained within the I band. The location agreed with previous results using polyclonal antibodies which indicate N2 line disposition of nebulin (38). To obtain more precise details on the different titin and nebulin antibodies, we decided to perform an immunoelectron microscopical study. This included also the titin mAbs T1, T3, and T4, which had earlier been mapped by immunofluorescence microscopy to the A–I junction (7).

Immunoelectron Microscopy Provides a Linear Epitope Map for Titin

Electron microscopy allowed a much better analysis of the antibody decoration patterns than immunofluorescence. Fiber bundles of chicken pectorialis major muscle tied on plastic strips after incubation in a relaxing buffer were extracted with Triton X-100 (see Materials and Methods) and incubated first with the different murine mAbs to titin and then with a second antibody (sheep anti–mouse immunoglobulins) to amplify the signal. Specimens were fixed with glutaraldehyde, postfixed with osmium, and embedded in plastic. Ultrathin sections were examined by electron microscopy (Fig. 4). The ultrastructural appearance of control preparations exposed only to the sheep anti–mouse antibody (Fig. 4 *g*) shows that the I band is devoid of any indication of an N2 line structure, which could be confused with the antibody decoration patterns described below. By searching through muscles prepared in this way, occasional contracted myofibrils could be found. These are also shown in Fig. 4.

As shown in Fig. 4, *a–f* (*left column*), each of the six titin antibodies provided a pair of thin decoration lines per sarcomere. The position of these pairs was centrally symmetric to the M line and changed in a characteristic fashion with each antibody. These positions are summarized schematically in Fig. 6, which shows a linear epitope map. In relaxed sarcomeres (average sarcomere length ~2.7 μm) the positions of the decoration lines were as follows. Antibody T10 located

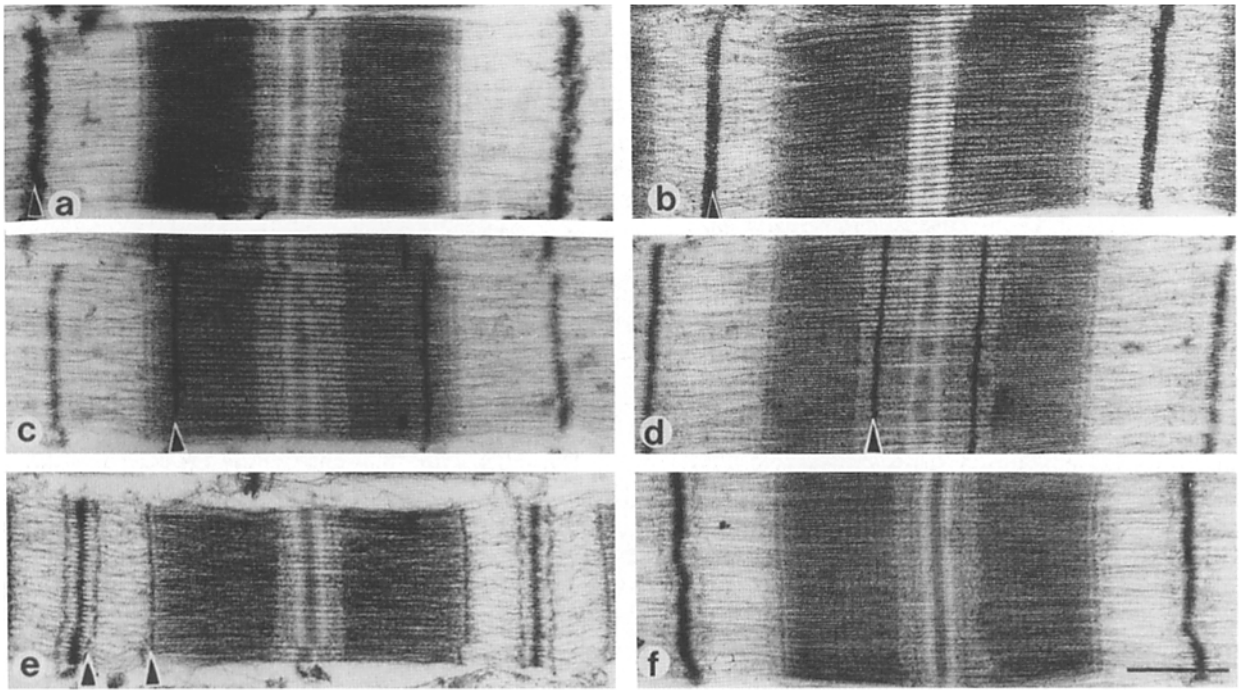

Figure 5. Electron micrographs of relaxed chicken pectoralis major muscle labeled with four monoclonal titin antibodies (*a–d*) and a reconstituted mixture of two mAbs to titin (*e*) using the procedure outlined in Fig. 4. Antibodies used are (*a*) T20, (*b*) T21, (*c*) T22, and (*d*) T23. The mixture of the two mAbs T3 and T12 (*e*) represents the sum of the two individual decoration patterns observed in Fig. 4 (*b* and *f*). (*f*) A control muscle treated only with second antibody. Bar, 500 nm.

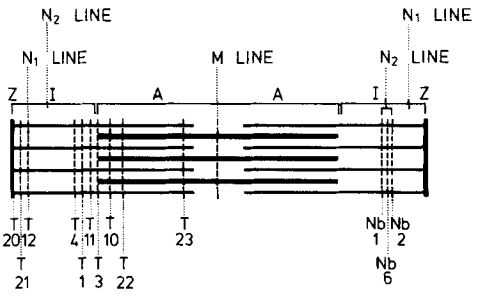

Figure 6. Schematic summary of the decoration patterns observed with 10 mAbs to titin (T1, T3, T4, T10, T11, T12, T20, T21, T22, and T23) and three mAbs to nebulin (Nb1, Nb2, and Nb6) by electron microscopy (Figs. 4, 6, and 7) on relaxed skeletal muscle. *Z*, *I*, and *A* label the known band positions. In addition, we mark the centro-symmetry given by the M line and the approximate positions of the N1 and N2 lines. Note that all mAbs label only a pair of transverse lines symmetrical to the M line. For simplicity, decoration lines labeled by titin antibodies are shown in the half-sarcomere on the left, while nebulin positions are given in the half-sarcomere on the right.

0.1 μm inside the A band (Fig. 4 *a*) while T3 outlined the end of the A band (Fig. 4 *b*). The latter result fits the antibody decoration pattern of T3 on isolated native thick filaments. Here it labeled the tips of the thick filaments (7) consistent with a myosin–titin interaction site (12, 30). The other four antibodies labeled distinct positions along the I band. T11 decorated 0.05 μm from the end of the A band while T1 and T4 gave bands 0.1 and 0.14 μm along the I band. T12 had a quite distinct location. Decoration occurred ~0.1 μm before the Z line. This position probably corresponds to the N1 line. As a further control we used reconstituted mixtures of various mAbs to titin. In each case the results coincided with the sum of the observations obtained with the individual mAbs. Fig. 5 *e*, for instance, gives the result with a combination of antibodies T3 and T12. It clearly shows the lines found for T3 and T12 with individual antibodies (Fig. 4, *b* and *f*).

Contracted sarcomeres decorated by the six mAbs are also shown in Fig. 4 (*right column*). In agreement with the results on relaxed sarcomeres, antibodies T10 and T3 located within the A band or at its end (see above and Fig. 4, *a* and *b*). They therefore kept their relative location towards the A band independent of contraction or relaxation. This was also the case with antibody T12 (Fig. 4 *f*). It kept its relative distance to the Z line at a position thought to reflect the N1 line of the sarcomere. Interestingly the decoration lines of the three other mAbs (T1, T4, T11) shown to lie before the A–I junction in relaxed muscle (see above) showed a change upon contraction. They seemed to pile up ahead of the advancing A band when contracted muscle was decorated (Fig. 4, *c–e*, *right column*).

To extend the bank of titin mAbs, a second fusion was made. The antigen used was native titin TII. Screening was again by immunofluorescence but immunoelectron microscopy was added early on as an additional screen to identify hybridomas staining new sites within the sarcomere. These were subsequently made monoclonal and characterized in detail (antibodies T20–T23). Antibodies decorating around the A–I junction were not followed up. Immunoblotting on total muscle extracts showed that T20 and T21 were specific for the T1 component of titin (Fig. 1 *c*), while the other two antibodies (T22 and T23) recognized both components of the titin doublet. The cross-species reactivity patterns of these antibodies are given in Table I, and Fig. 3 documents the myofibrillar staining pattern seen by immunofluorescence. In immunoelectron microscopy, all four antibodies detected again only one epitope per half-sarcomere (Fig. 5). Antibody T20 decorated at the Z line, while T21 located 0.04 μm before the Z line. The two new A band–specific antibodies, T22 and T23, were distinct and differed from T10. Antibody T22 labeled at 0.2 μm within the A band, while antibody T23 decorated at a distance of 0.2 μm from the M line. The labeling positions of antibodies T20 to T23 were independent of the length of the sarcomere (data not shown). The results are summarized schematically in Fig. 6.

Nebulin Epitopes Occur in Majority around the N2 Line

Using the same approach for nebulin antibodies, we concentrated on Nb1, Nb2, and Nb6. In relaxed muscle, these three antibodies, thought to correlate with the N2 line in immunofluorescence microscopy, labeled positions consistent with this interpretation. Electron micrographs show, however, that they do not decorate at identical distances between Z and A bands. Decoration lines (Fig. 7) occur at distances of 0.22 (Nb2), 0.24 (Nb6), and 0.26 μl (Nb1) from the Z lines. Although, again, the decoration patterns of the sarcomeres were symmetric with the M line, we did not observe the continuous lines (as with titin antibodies) but two obviously discontinuous stripes. The reason for this decoration pattern is unknown. A reconstituted mixture of Nb1 and Nb2 antibodies was used as control. As shown in Fig. 7 *d*, two closely spaced but separate decoration lines were obtained. The combined results are summarized schematically in Fig. 6.

Discussion

While nebulin and the upper band of titin (TI in the nomenclature of reference 40) are extractable so far only with SDS, the two components of the putative elastic system appear immunologically quite distinct. None of the mAbs used here showed cross-reactivity between titin and nebulin. Although most titin antibodies reacted on both heart and skeletal muscle (for some immunological differences see reference 7), the nebulin antibodies did not react in immunofluorescence and blotting on cardiac myofibrils. In agreement with Locker and Wild (17), we also could not detect by gel electrophoresis a polypeptide corresponding to a putative cardiac nebulin. While this difference between two sarcomeric muscle types is unsettling, the results now obtained by two laboratories seem to suggest that nebulin may not be a necessary component of all sarcomeric muscles. None of our titin and nebulin antibodies reacted with smooth muscle or nonmuscle tissues. Although it has been suggested that titin-like molecules can occur outside sarcomeric muscle (4, 5, 18), probably such molecules, if they exist, are antigenically quite distinct.

Each of the three nebulin antibodies characterized by immunoelectron microscopy on chicken breast muscle provided one pair of transverse stripes per sarcomere. Although the relative position of the stripes is antibody dependent, all of them locate around the N2 line, in support of earlier work with polyclonal antibodies used in fluorescence microscopy

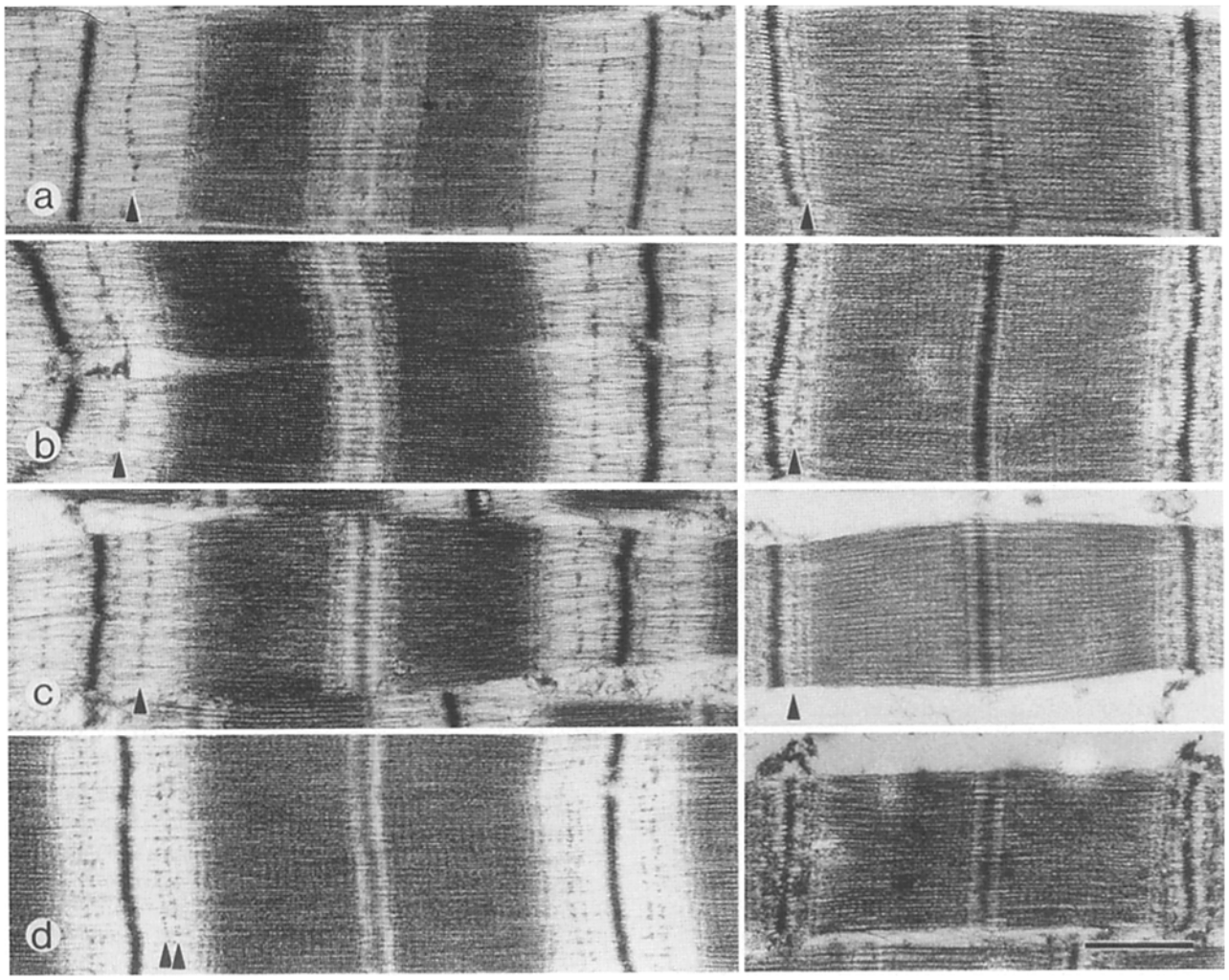

Figure 7. Electron micrographs of chicken pectoralis major muscle labeled with monoclonal nebulin antibodies using the procedure outlined in Fig. 4. In each pair, the micrograph on the left shows the label in relaxed sarcomeres, while the one on the right provides the pattern in contracted sarcomeres. Antibodies used are (*a*) Nb1, (*b*) Nb6, and (*c*) Nb2. The mixture of antibodies Nb1 and Nb2 (*d*) reveals two separate decoration lines in appropriate positions. Positions of decoration lines are indicated with arrowheads. Bar, 500 nm.

(38). Speculations on the organization of the nebulin molecules in the half-sarcomere have to be postponed until some structural properties of the native nebulin molecule are established. Because of their broad cross-species reactivity, we have used the nebulin antibodies (Nb1, Nb2, Nb3, Nb4, Nb6) to show (3) that contrary to the predictions of Wood et al. (42) this protein is not the product of the human gene affected in Duchenne muscular dystrophy (25). Our results are in line with the recent report of Hoffman et al. (8) who have identified a novel protein (dystrophin) as the target of Duchenne muscular dystrophy.

The 10 mAbs to titin establish a linear epitope map (Fig. 6). Immunoelectron microscopy of muscle showed that each antibody gave rise to only one pair of transverse bands per sarcomere. The position of these bands was symmetric to the M line and was unique for each antibody.

Before discussing the implications of these results we connect our data with previous localization studies performed with polyclonal antibodies in immunofluorescence microscopy and, in one instance, in electron microscopy. Maruyama's laboratory favors the view that titin (connectin) filaments run parallel to the thick filaments. Starting at a position ~0.15 μm from the center of the A band, they are thought to continue to the Z line (19, 24). In contrast, Wang's laboratory proposes from light microscopical data that titin filaments are continuous between the two N2 lines of the sarcomere, while the gap between the N2 line and the Z line is filled by nebulin. Thus the elastic filament system is viewed as "two distinct and nonoverlapping" domains provided by titin and nebulin, respectively (32, 34, 35). Our linear map of titin epitopes starts at the Z line (T20 antibody) and contains two additional epitopes (T21 and T12 antibodies) before the N2 line. Therefore titin filaments clearly extend from the A band beyond the N2 line and contact the Z line. As this anchorage involves the extra mass specific for titin I vs. titin II, an interesting problem arises. Some laboratories have purified from sarcomeric muscle higher molecular weight proteins of unusual insolubility. These are thought by immunofluorescence criteria to be Z line constituents (26, 28). Our results open the possibility that such proteins could also arise as proteolytic degradation products of titin.

The immunoelectron micrographs obtained in our study

indicate that neighboring titin filaments are arranged in register. Per half-sarcomere, the titin filaments are polar structures essentially without epitope repeats. Consistent with this view, it was also possible to identify the location of one of the two physical ends of the molecules. mAbs decorating at or close to the Z line (T20 and T21) were specific for TI in immunoblots of total muscle extracts, while all other eight antibodies reacted with both components of the titin doublet (TI and TII). Thus the Z line end of titin seems provided by the extra mass specific for TI. As TII is thought to arise by limited proteolysis of TI (19, 35), it is not immediately obvious how we succeeded in obtaining TI-specific mAbs using a TII preparation as antigen. As polyclonal rabbit anti-titin antibodies raised against titin II provided no stripes close to the Z line (our unpublished results), the following explanation seems possible. The particular mAbs arose from a small but particularly antigenic contamination of TI-like molecules in the standard TII preparation.

The decoration patterns observed also clarify some recently posed questions of titin organization. Trinick et al. (31) raised the idea of repetitive structural domains along the filament. While this possibility still remains, the epitope map based on 10 mAbs argues against extended regions of truly identical domains. Locker has favored the idea that titin filaments provide an interior core of the thick filaments (15). In contrast, our immunoelectron microscopical data and those of Murayama's laboratory (24) show that titin antibodies decorate defined positions of the A band before any destructive influences of fixation. In addition, in crude preparations of native thick filaments titin-like strings running parallel to the isolated A band have been observed (30). Thus the titin filaments lie most likely outside the thick filaments, although the direct mode of arrangement may be a matter of debate. Evidence that the titin filaments could extend to the M line are based on immunoelectron micrographs. Maruyama et al. (24) found, with polyclonal antibodies, the stripe closest to the M line at a distance of 0.15 μm from the center of the A band, and we observed the closest stripe at 0.2 μm using the mAb T23.

Wang et al. (41) have reported in abstract form on the location of four titin epitopes defined by mAbs. They also found that some epitopes along the I band exhibit an elastic stretch dependence, while epitopes within the A band remain fixed upon change in sarcomere length. Thus the elastic titin filaments are stretchable only over a certain part of their I domain. This seems to correspond to the area between the A–I junction and the N1 line. We suggest, like Wang et al., that there is a yet undefined anchoring mechanism for the titin filaments along the A band structure.

The combined data allow for two reasonable models. In both cases the linkage of titin to the Z line and its presence close to the M line are responsible for the expected integration of the I–Z–I units and the A bands. In model *A*, titin filaments have half-sarcomere length and are polar with their tails attached to the Z lines. At the M line there could be a head-to-head interaction to provide additional stability of the elastic system. In model *B*, titin filaments could have sarcomere length. They would be bipolar with central symmetry at the M line. Hence both physical ends would attach to the Z line. The alternative possibility of sarcomere length titin molecules connecting neighboring M lines seems unlikely as antibodies specific to titin TI map at the Z line and seem to involve a physical end of the molecule. Distinction between models *A* and *B* requires an accurate length estimate of the native titin molecule, which is currently not easy to obtain. Metal-shadowed TII molecules have been analyzed in several reports and all agree on the existence of technical difficulties. Length values of up to 1.2 μm were reported by Wang (40), while the histograms given by Trinick et al. (31) cover 0.2–1 μm with a peak ~0.6 μm. In Maruyama's histograms, the peak is just below 1 μm (21). Using high resolution gel permeation chromatography, we have separated TII into two species. One fraction, characterized by a very homogeneous ultrastructural appearance, measures 1 μm, while the less homogeneous fraction seems to have length values ~1.2–1.3 μm (Nave, R., unpublished). As the parent, TI molecule could have a length appreciably longer than this, we favor model *A*. However, model *B* can only be eliminated if it is excluded that titin strings are not shortened due to shearing of the molecules. One obvious future avenue is indicated by the linear epitope map; i.e., the molecular characterization of proteolytically excised regions purified by differential antibody binding. In addition, further biophysical characterization of titin is of obvious importance.

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