Studies on Cardiac Myofibrillogenesis with Antibodies to Titin, Actin, Tropomyosin, and Myosin

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Abstract. Cardiac myofibrillogenesis was examined in cultured chick cardiac cells by immunofluorescence using antibodies against titin, actin, tropomyosin, and myosin. Primitive cardiomyocytes initially contained stress fiber–like structures (SFLS) that stained positively for α actin and/or muscle tropomyosin. In some cases the staining for muscle tropomyosin and α actin was disproportionate; this suggests that the synthesis and/or assembly of these two isoforms into the SFLS may not be stoichiometric. The α actin containing SFLS in these myocytes could be classified as either central or peripheral; central SFLS showed developing sarcomeric titin while peripheral SFLS had weak titin fluorescence and a more uniform stain distribution. Sarcomeric patterns of titin and myosin were present at multiple sites on these structures. A pair of titin staining bands was clearly associated with each developing A band even at the two or three sarcomere stage, although occasional examples of a titin band being associated with a half sarcomere were noted. The appearance of sarcomeric titin patterns coincided or preceded sarcomere periodicity of either α actin or muscle tropomyosin. The early appearance of titin in myofibrillogenesis suggests it may have a role in filament alignment during sarcomere assembly.

ULTRASTRUCTURAL studies on cultured rat cardiomyocytes and embryonic hamster, rat, or sheep hearts have led to the development of a model for cardiac myofibrillogenesis in which Z band–like materials are the initiation sites of myofilament assembly (2, 13, 16). Groups of myofilaments first appear and frequently insert into Z band–like materials associated with sarcolemmal plaques or become organized in the cytoplasm ("cytoplasm condensation"). These I-Z-I–like segments gradually interconnect to each other, resulting in primitive, branching myofibrillar bundles that are characterized by loosely aggregated thick and thin filaments and expanded Z lines. During subsequent development, the Z lines narrow and the filaments become more closely packed. A and I bands appear only when myofilaments are brought into a more ordered array. H zones and M lines are not seen until late in organogenesis in the rat and after organogenesis in the hamster (16).

Another model for cardiac myofibrillogenesis is based on observations of the assembly of myofibrils in isolated chick cardiomyocytes where myofibrils apparently begin to form from structures resembling stress fibers (4, 12). These stress

fiber–like structures (SFLS)[1] were labeled with anti–nonmuscle myosin, anti–smooth muscle α actinin and phalloidin but not anti–muscle light meromyosin. SFLS were continuous with nascent myofibrils and disappeared in regions of numerous mature myofibrils. It has been proposed that preexisting SFLS serve as templates for the assembly of groups of thick and thin filaments (4). The reasons for the divergence of these two models for myofibril formation remain unclear.

Striated muscle is endowed with an elastic resistance to stretch and titin is believed to function in this capacity. Titin is an elastic filamentous protein that constitutes ∼10% of the myofibrillar proteins (17, 27). Although information on the structural organization of titin molecules in sarcomeres is limited at present, a model has been proposed in which titin is a major constituent of a set of longitudinal filaments running through the sarcomere (25, 26). These filaments would ensure structural continuity in myofibrils and may be involved in maintaining the A band in the center of the sarcomere (11). However, little is known about the mechanism and timing of titin assembly in the sarcomere in relation to the other major myofibrillar proteins.

In the present study immunofluorescence with several muscle specific antibodies (titin, actin, myosin, and tropo-

1. *Abbreviation used in this paper:* SFLS, stress fiber–like structures.

myosin) was used to investigate how myofibrils are assembled in cultured cardiac myocytes. The results indicate that sarcomeric patterns for titin are closely coordinated with the assembly of myosin into myofibrillar A bands and coincide or precede the alignment of actin and tropomyosin in the I band.

Materials and Methods

Preparation of Cardiac Cultures

The method of isolating cardiac myocytes was adapted from Sanger (20). Heart tissue was removed from 10- or 11-d chick embryos, minced, and incubated in an enzyme solution (0.17% trypsin [type II; Sigma Chemical Co., St. Louis, MO], 0.085% collagenase [type II; Sigma Chemical Co.], in calcium, magnesium-free Hank's balanced salt solution [Gibco Laboratories, Grand Island, NY], pH 7.0) for 15 min. Single cells were dissociated by trituration and collected by centrifugation. Dispersed cells were preplated for 2–3 h on uncoated dishes and then plated at 5×10^4 cells per 35-mm gelatin-coated dish. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were grown in media consisting of 84% MEM (Gibco Laboratories), 10% "selected" horse serum (Hyclone Laboratories, Logan, UT), 5% embryo extract, and 1% penicillin-streptomycin. The medium was replaced on day 2 and on alternate days thereafter.

Antibody Sources

Monoclonal Anti-Titin 9D10. 9D10 (IgM) was produced as described previously (28). This antibody did not react with rabbit skeletal muscle nebulin, myosin, M proteins, C protein, α-actinin, actin, tropomyosin or troponin in ELISA. Antibody specificity in chicken cardiac muscle was examined by immunoblot. Whole chicken cardiac muscle was dissolved in sample buffer and the extracts were applied to 10% SDS-polyacrylamide gels. Electrophoresis was conducted as described previously (8) except the stacking gel was omitted, the separating gel pH was 8.8, and the gels were run in a Mighty Small SE 250 (Hoefer Scientific Instruments, San Francisco, CA). After the proteins were separated on the gels, they were electrotransferred to a 0.22-μm nitrocellulose membrane (24) for 18 h at 0.3 A. Blots were incubated with 9D10 antibody at 5–10 μg/ml. Binding of mouse antibody on the membranes was detected by colloidal gold anti-mouse IgG with silver enhancement (Bio-Rad Laboratories, Richmond, CA).

Polyclonal and Monoclonal Anti-Actin. Polyclonal antibodies against the NH_2-terminal peptide of α actin were prepared in rabbits as described previously (3). Antibodies were affinity column purified and characterized by immunoblot. The antibody was specific for cardiac and skeletal muscle α actin. A monoclonal anti-α actin (designated B4) was generated against chicken gizzard actin as reported (14). This antibody reacts preferentially with the enteric smooth muscle γ actin isoform but also binds the α isoforms of cardiac, skeletal, and vascular smooth muscle. It does not react with cytoplasmic β or γ actin. The polyclonal and monoclonal actin antibodies gave identical results in all the experiments reported here.

Monoclonal Anti-Muscle Tropomyosin (CH1) and Anti-Nonmuscle Tropomyosin (CGB6). These antibodies were produced and characterized as described in Lin et al. (15). CH1 (IgG) reacted with cardiac and skeletal muscle tropomyosin but not with nonmuscle tropomyosin. CGB6 (IgM) recognized nonmuscle tropomyosin (a, b, 1, and 2) in chick embryonic fibroblasts and cross reacted with the slow component of gizzard tropomyosin, but did not cross react with cardiac or skeletal muscle tropomyosin.

Monoclonal Anti-Myosin Heavy Chain (F4/2B6). This antibody was a generous gift from Dr. Neal A. Rubinstein. This antibody (IgG) was produced using rat embryonic myosin heavy chain and it does not react with nonmuscle myosins (6).

Immunofluorescence

Chick cardiac cultures were removed 3–6 d after plating, washed in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) and fixed in selective fixatives for specific antibodies. If cells were previously fixed in formaldehyde solution, they were then treated with 0.5 mg/ml sodium borohydride in PBS for 30 min. They were further incubated with any one or two of the following antibodies at 37°C for 30–60 min: anti-titin (culture supernatants), anti-myosin heavy chain (culture supernatants), purified monoclonal anti-actin (50 μg/ml), purified polyclonal anti-actin

(50 μg/ml), anti-muscle tropomyosin (1:40 diluted ascites fluid), anti-nonmuscle tropomyosin (1:40 diluted ascites fluid). Cells were washed with PBS for 30 min and incubated with any one or two appropriate 1:20 diluted secondary antibodies (CooperBiomedical, Inc., Malvern, PA) listed as follows: fluorescein-conjugated goat anti-mouse IgG, fluorescein-conjugated goat anti-mouse IgM μ chain specific, rhodamine-conjugated goat anti-mouse IgG γ chain specific, rhodamine-conjugated goat anti-rabbit IgG. After an extensive wash, they were mounted in 2% *n*-propyl gallate (7) in 60% glycerol, 0.1 M phosphate buffer, pH 8.0. Observations were made using a Zeiss standard microscope equipped with epifluorescence illumination. Photographs were obtained using tri-X film which was developed in Microdol-X (Eastman Kodak Co., Rochester, NY).

Preparation of Myofibrils

Strips of chicken cardiac muscle were tied to plastic rods and soaked in a solution containing 75 mM KCl, 10 mM Tris (pH 6.8), 2 mM EGTA, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100 for several hours to allow the muscle to go into rigor. The tissue was then disrupted in a Polytron homogenizer (Brinkman Instruments Co., Westbury, NY) followed by a Teflon glass homogenizer and the myofibrils collected by centrifugation at 1,000 g for 5 min. The pellets were resuspended in buffer without Triton X-100 and washed several times by centrifugation and dispersion in fresh buffer. Myofibrils were stored in buffer containing 50% glycerol at −20°C before use.

Results

Characterization of D10 Anti-Titin

Previous work (28) has shown that monoclonal anti-titin 9D10 was specific for titin using ELISAs with a variety of purified myofibrillar proteins. An immunoblot using chicken cardiac tissue proteins after SDS electrophoresis on a 10% acrylamide gel is shown in Fig. 1. Positive staining was found only at the titin position.

The 9D10 antibody stained two perpendicular bands per sarcomere using chicken cardiac myofibrils (Fig. 2). The staining positions were in the I band near the A–I junctions.

Fixation and Antibody Reactivity

The ability of the various antibodies to give staining on myofibrils or tissue culture cells was dependent on the method of fixation used (Table I). Monoclonal anti-titin gave positive staining after fixation using each of the conditions
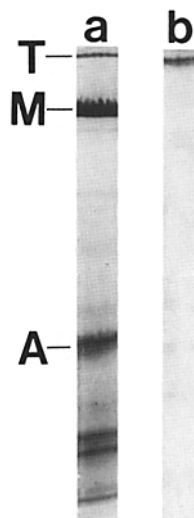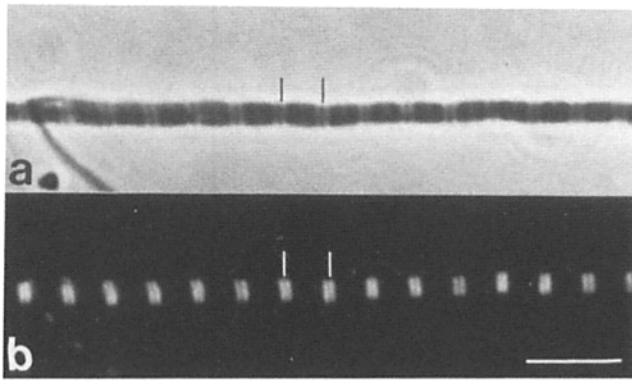

Figure 1. Western blot of an SDS gel of chicken cardiac tissue treated with anti-titin 9D10. (*a*) Coomassie Blue staining of a gel strip before transfer. *T*, titin; *M*, myosin heavy chain; *A*, actin. (*b*) Immunostaining with anti-titin of a nitrocellulose strip after transfer.

Figure 2. Immunofluorescence staining of a chicken cardiac myofibril with monoclonal anti–titin 9D10. (a) Phase contrast. (b) Anti-titin. The antibody stained two transverse zones per sarcomere in the I band near the A–I junction. Vertical lines indicate position of Z lines. Bar, 5 μm.

listed. The results presented in the table were all obtained after borohydride reduction with fixatives using formaldehyde. The reduction step has subsequently been found to be unnecessary with formaldehyde fixation. Positive staining can be obtained with both anti–α actin antibodies using the sequential formaldehyde and methanol fixatives without borohydride reduction. In this report the myosin plus titin staining (Fig. 6) was performed after 1% formaldehyde plus methanol fixation while all other staining combinations were conducted after 3.7% formaldehyde plus methanol fixation.

Appearance of Actin and Tropomyosin Filament Bundles in Immature Cardiomyocytes

Cardiomyocytes at varying stages of differentiation were always encountered in the cultures and they could be distinguished from fibroblasts by doubly labeling with anti–nonmuscle tropomyosin plus any one of the other muscle-specific antibodies (anti–titin, polyclonal or monoclonal anti–α actin, anti–muscle tropomyosin, anti–myosin). None of the five muscle-specific antibodies used in this study labeled any cells that stained strongly with anti–nonmuscle tropomyosin.

Immature cardiomyocytes were defined as cells that did not exhibit nonmuscle tropomyosin staining but contained no or only a few myofibrils. Figs. 3, a and b revealed two immature cardiac myocytes that were stained with anti–muscle tropomyosin but not with anti–nonmuscle tropomyosin. Several presumptive fibroblasts showed strong staining with anti–nonmuscle tropomyosin but no staining with the muscle antibody. Filament bundles (SFLS) of varying lengths consistently appeared in the cardiomyocytes. These bundles contained detectable muscle tropomyosin and usually muscle actin but not muscle myosin. They were stained with anti-titin with variable intensities.

Some cells were labeled with anti–muscle tropomyosin but not anti–α actin. This pattern did not depend on the sequence of primary antibody incubation. In Fig. 3, c and d, cells were incubated with anti–α actin and then anti–α–muscle tropomyosin followed by secondary antibody incubation. In Fig. 3, e and f, the sequence of primary antibody incubation was reversed, i.e., anti–muscle tropomyosin first and then anti–α actin. Thus, although α actin staining might become visible using higher titer antibodies, there appear to be variations in the ratios of muscle tropomyosin to α actin in different filament bundles from different cells.

Distribution of Filament Bundles in More Mature Cardiomyocytes and Their Relation to Myofibril Formation

As the actin and tropomyosin containing SFLS became abundant they could be classified into two categories, central and peripheral, according to their location and their titin antibody reactivity.

Peripheral SFLS were distributed in outer regions of the cytoplasm (as viewed from above). They were positive for α actin (Fig. 4 b) and muscle tropomyosin but stained very weakly for titin (Fig. 4 a) and muscle myosin (data not shown). In some cases the staining with anti–α actin or anti–muscle tropomyosin showed striations (Fig. 4 b, arrow). SFLS were intimately associated with membranous ruffles (also positive for α actin and muscle tropomyosin) and often formed three-dimensional polygonal networks be-

Table 1. Effects of Fixation Method on Antibody Reactivity in Cultured Chick Cardiac Cells*

Primary antibody	Fixative				
	1% formaldehyde, then methanol	1% formaldehyde	3.7% formaldehyde, then methanol	Methanol	3.7% formaldehyde
Monoclonal anti-titin	+	+	+	+	+
Monoclonal anti-MHC	+	+	−	−	−
Monoclonal anti-mTM	−	−	+	−	−
Monoclonal anti-nmTM	ND	ND	+	−	+
Monoclonal anti-α actin	−	−	−	+	−
Polyclonal anti-α actin	−	−	+	+	−

MHC, myosin heavy chain; mTM, muscle tropomyosin; nmTM, nonmuscle tropomyosin.
* The results presented refer to samples that were treated with sodium borohydride after fixation. Both anti–actin antibodies gave positive staining with the sequential formaldehyde and methanol fixatives if the borohydride treatment was omitted.
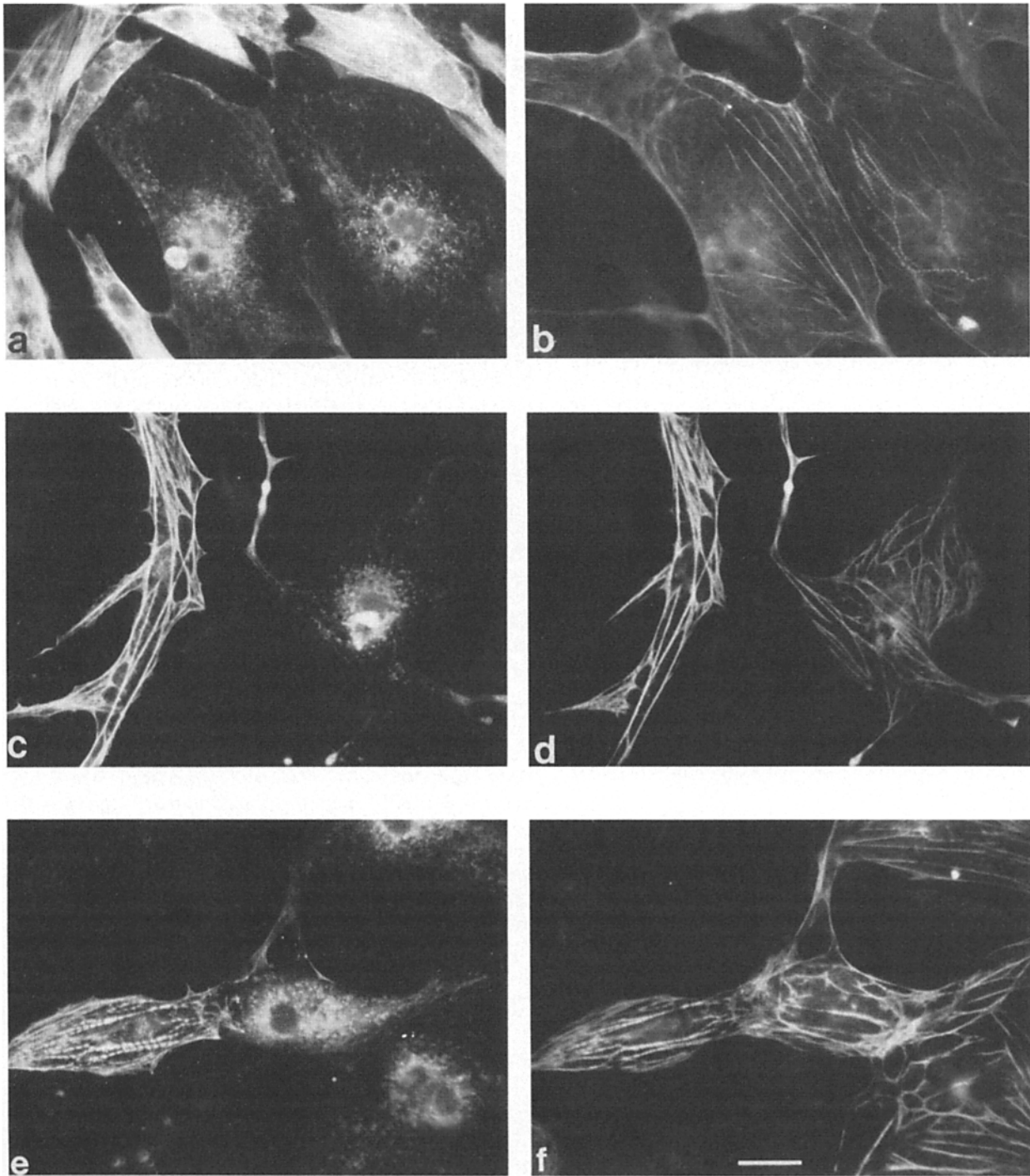
Figure 3. Immunofluorescent staining of cultured cardiac cells with antibodies against tropomyosin and actin. (*a*) Staining with anti–nonmuscle tropomyosin. Strong staining of the cells along the edges of the photograph (presumptive fibroblasts) was evident while much weaker staining occurred in the two large centrally located cells (presumably cardiomyocytes). (*b*) Staining with anti–muscle tropomyosin (same as *a*). Positive staining occurred only in the cardiomyocytes. Striated myofibrils and SFLS are visible. The cell membrane regions appeared to be more strongly stained. (*c*) Staining with polyclonal anti–α actin. The cell on the left showed intense staining on the myofibrils, stress fibers, and cell borders while the one on the right was poorly stained. (*d*) Staining with anti–muscle tropomyosin (same as *c*). Myofibril and stress fiber staining was extensive in both cells. For *c* and *d*, the cells were treated with polyclonal anti–α actin followed by anti–muscle tropomyosin. (*e*) Staining with polyclonal anti–α actin. The region at the left shows a strong striational staining pattern while that on the right shows no distinct SFLS. (*f*) Staining with anti–muscle tropomyosin (same as *e*). The antibody gave intense staining in cells and/or regions of cells which did not stain with anti–α actin. Striation patterns are visible on SFLS which have negligible staining for α actin. For *e* and *f*, the cells were treated with anti–muscle tropomyosin followed by polyclonal anti–α actin. Bar, 20 μm.
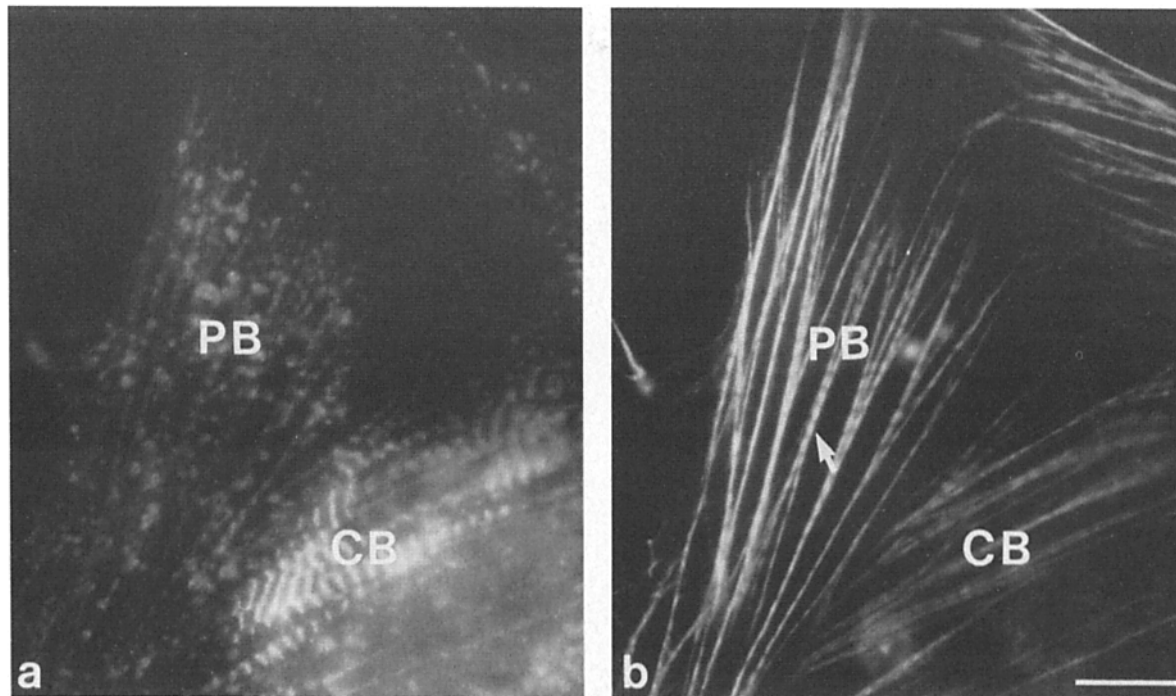
Figure 4. Immunofluorescent staining of cardiomyocytes with anti-titin and anti-α actin. (*a*) Anti-titin. Staining occurred on longitudinal bundles. The central bundles (*CB*) showed sarcomeric striations and were usually found near the middle of the cells (as viewed from above). Peripheral bundles (*PB*) were found closer to the cell border and were more weakly stained. (*b*) Polyclonal anti-α actin (same as *a*). Both central and peripheral bundles were strongly stained. Bar, 10 μm.

tween myofibrils. They were also present in large numbers in pseudopodia.

Central SFLS were located in regions where the developing sarcomere patterns were visible (Fig. 4, *a* and *b*). Sarcomeric periodicity was frequently observed at multiple sites on the central SFLS, resulting in striated (*S*) and nonstriated (*NS*) regions (Fig. 5 *a*). The nonstriated regions usually did not bind anti-myosin but were labeled intensely with anti-α actin and anti-muscle tropomyosin and with varying intensities using anti-titin. Fig. 5 *b* demonstrates anti-muscle tropomyosin staining in the nonstriated regions. More intense anti-myosin staining could be located in regions where the new sarcomeres were apparently being formed (Fig. 6, *b* and *d*). The striated regions consisted of small groups of sarcomeres numbering from 2 to 20 (Fig. 6, *a–d*). In the striated regions at the two or three sarcomere stages, the segregation of titin on the SFLS into doublets appeared to be closely related to the alignment of myosin into A bands (Fig. 6, *a–d*) and actin and tropomyosin into I bands (Fig. 7, *a* and *b*). In Fig. 6, *e* and *f*, two areas of apparent sarcomere assembly were visible. Titin and myosin staining are almost continuous in the gaps (Fig. 6, *e* and *f*, sarcomeres 2 and 3) between well-formed sarcomeres (Fig. 6, *e* and *f*, *1* and *4*). Surprisingly, the titin staining corresponds to a single A-I junction in developing sarcomeres two and three. This pattern is in contrast to the pairs of titin bands visible in Fig. 6 *a* and indicates that the pair of titin bands in a sarcomere may be assembled either separately or at approximately the same time. The level of focus was not responsible for obscuring the staining patterns; i.e., sarcomeres two and three in Fig. 6, *e* and *f* remained blurred at all focal planes.

Fig. 7, *c* and *d* show anti-actin staining in developing cardiac myocytes. In very short sarcomeres (myofibrils 1 and 2), anti-actin stained the A band regions. In the right half of myofibril 3 where the sarcomere lengths were between 1.6–1.8 μm, full-length A band staining was unexpectedly found. In the middle region of the myofibril 3 where the sarcomeres had approximately the same lengths, typical I band staining was seen.

Discussion

In the present study we have used muscle-specific antibodies against titin, myosin, actin, and tropomyosin to examine the alignment of these proteins into sarcomeres in cultured chick cardiomyocytes. SFLS containing muscle tropomyosin and α actin first appeared in immature cardiomyocytes before titin and muscle myosin could be detected in these cells. As the cells matured, these actin- and tropomyosin-containing SFLS could be classified as peripheral or central. Peripheral SFLS contained primarily muscle actin and muscle tropomyosin and had minimal amounts of titin and muscle myosin. They were also found in the pseudopods, attached to membranous ruffles, and were interconnected to form fine polygonal networks between developing myofibrils, thus appearing to be involved in cytoskeletal functions. In contrast, the central bundles appeared to be the site of sarcomere assembly. The central location of sarcomerogenesis is only defined by observation of cells from above; it may occur immediately adjacent to the cell membrane at the top or bottom of the cell. Because of the multiple sites of sarcomere formation along these bundles, they frequently possessed striated and non-
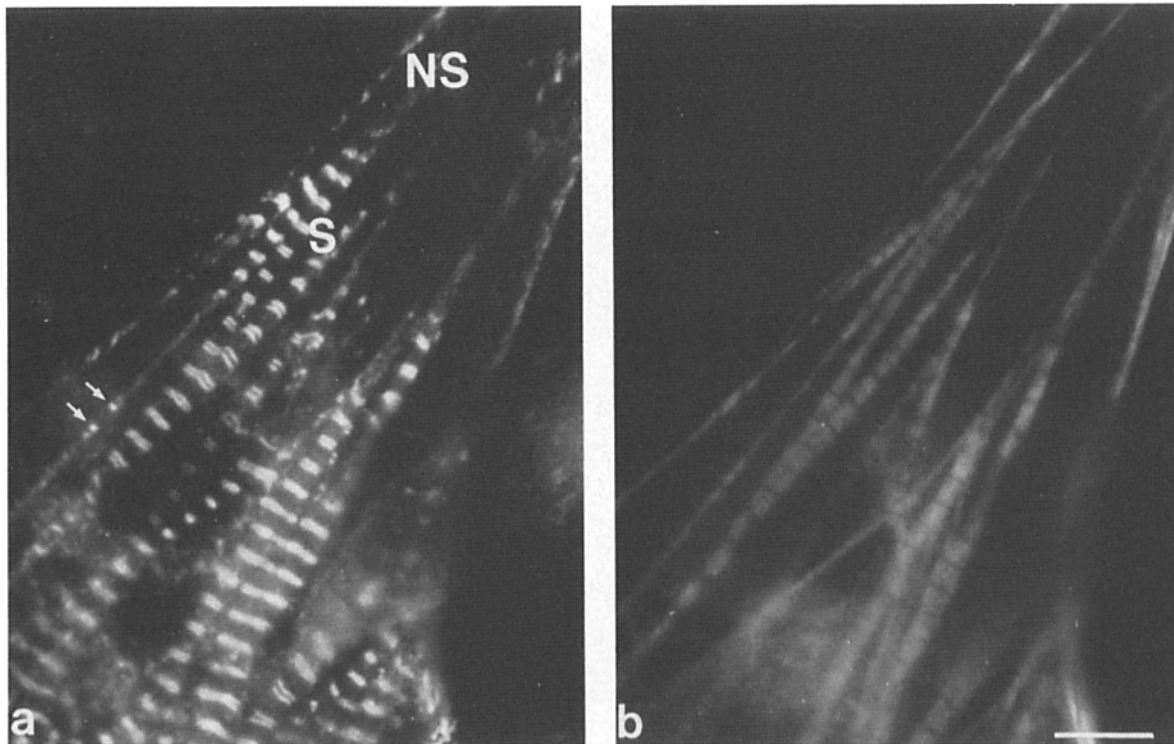
Figure 5. Immunofluorescent staining of cardiomyocytes with anti-titin and anti-muscle tropomyosin. (*a*) Anti-titin. SFLS with the staining pattern of mature striated myofibrils (*S*) were continuous with bundles which were nonstriated (*NS*). Note the strong staining of a pair of titin spots which presumably occurs at the position of the first sarcomere in that region (*arrows*). (*b*) Anti-muscle tropomyosin (same as *a*). Staining was visible in both the striated and nonstriated regions. Bar, 10 μm.

striated regions. Nonstriated regions were labeled intensely with anti-muscle actin and anti-muscle tropomyosin and at variable intensities with anti-titin. Anti-muscle myosin only stained the regions where primitive sarcomeres were organizing. These nonstriated regions were presumably transformed into sarcomeres. It is also possible that the peripheral bundles might become sites for sarcomere formation as the cardiomyocyte matures.

The most interesting observation from this report was the striking coordination of myosin and titin alignment into apparently newly formed sarcomeres. Even at the two or three sarcomere stage the alignment of titin and of myosin into A bands appeared tightly linked. In rare cases a stained titin band was found at only one end of a developing sarcomere, suggesting separate titin assembly in each half sarcomere. It has been shown previously that muscle myosin replaces cytoplasmic myosin during myofibrillogenesis (1, 5). Also the coordination of myosin and titin assembly in cultured skeletal muscle cells has been reported (10) by workers who found that during myofibril formation myosin and titin were colocalized but myosin and desmin were not. Their study, however, was at the whole cell level and provided no evidence for coordinated alignment at the two or three sarcomere stage.

The proposed role of titin in filament alignment during sarcomere formation is consistent with recently reported studies on intact chick hearts (22, 23). These investigations showed that small titin spots were visible before any myosin or actin periodicity and before α actinin staining was visible. They were not, however, able to double label with antibodies

against titin and myosin since both of their antibodies were produced in rabbits.

The present study suggests that new myofibrils are derived from preexisting filament bundles or SFLS as proposed previously (4). They showed that the nonstriated regions of SFLS were labeled with anti-nonmuscle myosin, anti-smooth muscle α actinin, and phalloidin (which binds both muscle and nonmuscle actins), but not with anti-muscle light meromyosin. With these probes, they were unable to detect any difference in the protein compositions of peripheral SFLS and nonstriated regions of SFLS. We have classified SFLS into two categories, peripheral and central, based on their different antibody reactivity. We did not observe significant anti-titin and anti-muscle myosin staining on the peripheral SFLS. Moreover, anti-titin generally labeled the nonstriated regions of central SFLS. Thus our results strongly suggest that the protein compositions of peripheral bundles and nonstriated regions of central bundles differ.

It is not clear why muscle actin and muscle tropomyosin are apparently associated with nonmuscle myosin (see reference 4) in peripheral SFLS and nonstriated regions of central SFLS. Muscle myosin is gradually incorporated into the nonstriated regions of central SFLS but anti-muscle myosin staining was weak or absent in peripheral bundles. Thus it is unclear whether a replacement of nonmuscle myosin with muscle myosin occurs in these structures or they are totally disassembled as cardiomyocytes mature. The recent studies showing myosin exchange between thick filaments in vitro (19) and in vivo (30) suggest a possible mechanism for such transformations. The exchange of myosin light chains with
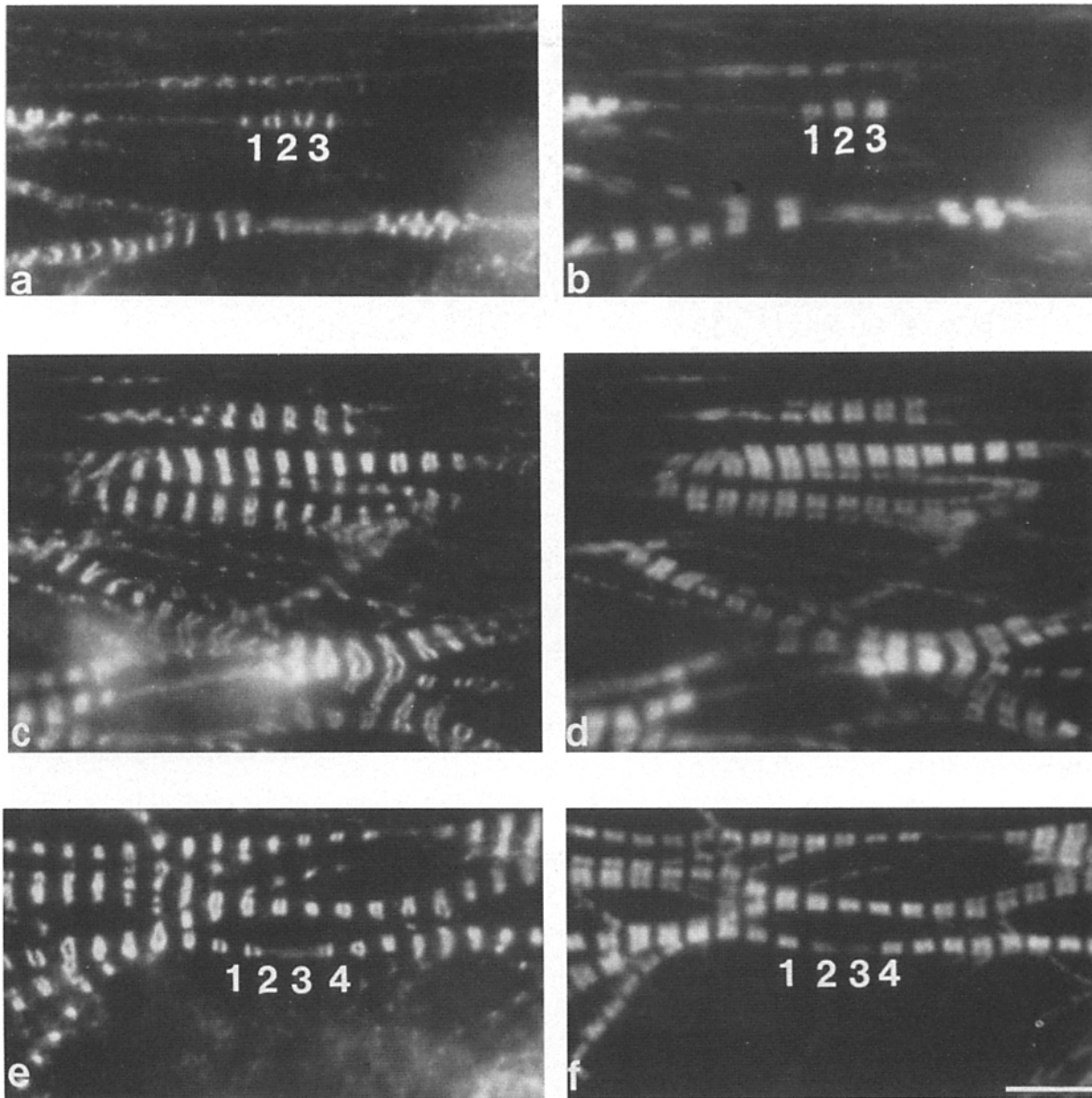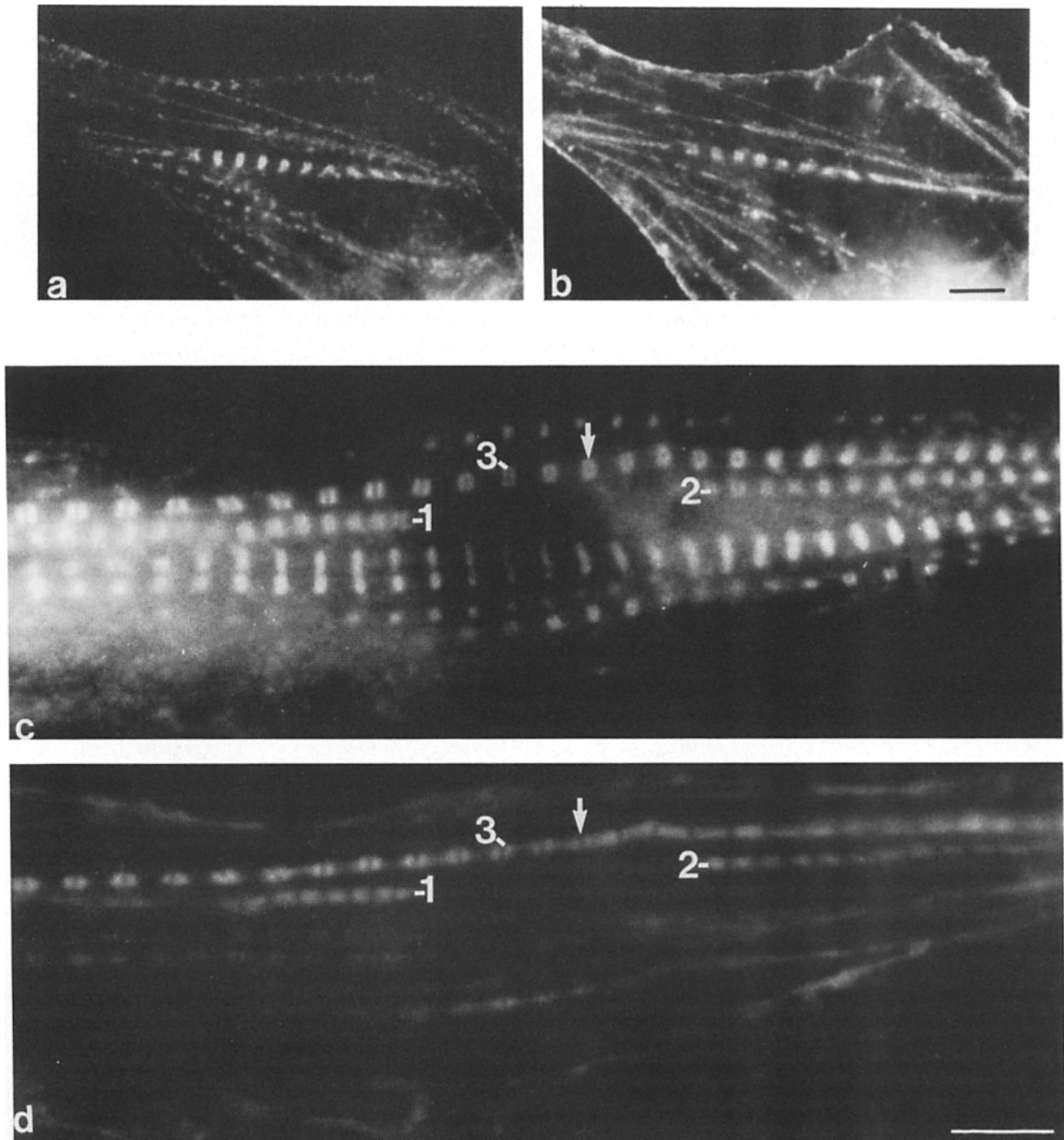
Figure 6. Immunofluorescence staining of developing sarcomeres with anti-titin and anti-myosin. (*a*) Anti-titin. Sarcomeric titin patterns were visible at multiple sites along the same SFLS. Between these sites the titin occurred as either irregular aggregates or diffuse patches. (*b*) Anti-myosin (same as *a*). Well-formed A bands were present at multiple sites on the bundles and were coincident with the sarcomeric titin pair. Groups of sarcomeres as small as two or three were visible. The three numbered A bands had six titin stripes (see *a*). (*c*) Anti-titin. (*d*) Anti-myosin (same as *c*). The precise correlation between the presence of A bands and the titin pair is evident. (*e*) Anti-titin. The final two sarcomeres (*2* and *3*) appear to be in the process of alignment. Note that in this case the titin band at the left of sarcomere 2 is clearly visible while its right partner is not, thus suggesting that the titin in each half sarcomere can be assembled independently. (*f*) Anti-myosin (same as *e*). The numbered A bands correspond to the numbered positions in *e*. Bar, 10 μm.

muscle and nonmuscle myosin in living cells has also been demonstrated (18). It will be important to determine whether both muscle and nonmuscle myosins can coexist in the same thick filament. One might postulate that the reason for alignment of thick filaments during sarcomerogenesis is the specific association of titin with muscle myosin filaments and not with those containing the nonmuscle isoform.

It was interesting to note that a few cardiomyocytes showed lack of coordination of muscle actin and muscle tropomyosin staining (Fig. 3, *c–f*). The lack of α actin staining in cells

that showed sarcomeric tropomyosin staining was surprising. Our initial hypothesis that the order of antibody treatment might somehow be responsible for this proved to be incorrect. Also, since the tropomyosin antibody stained more structures than the actin antibodies, it cannot be postulated that the tropomyosin was inadequately fixed and thus was washed off the thin filaments (if actin was preferentially released, there would be nothing to anchor the tropomyosin). The recent demonstration of sarcomeric periodicity in cardiomyocytes stained with antibody against γ actin and which

Figure 7. Immunofluorescence staining of developing sarcomeres with anti-titin and anti-actin. (*a*) Anti-titin. (*b*) Polyclonal anti–α actin. The segregation of staining on the SFLS into sarcomeric patterns appeared to be in step with the titin alignment seen in *a*. (*c*) Anti-titin. (*d*) Monoclonal anti–α actin (same as *c*). Actin staining occurred in several different patterns. Typical I band staining is present in the left portion of myofibril 3. A different pattern not seen in isolated mature myofibrils is present in the right part of myofibril 3 (transition point marked by the *arrow*) where the sarcomere lengths are ∼1.6 μm but the actin staining extends between the Z lines (the difference in sarcomere lengths can be contrasted with myofibril 2). Bars: (*a* and *b*) 10 μm; (*c* and *d*) 5 μm.

did not stain with α actin (9) appears to explain the results reported here. In rare instances there was positive staining for α actin and no staining with muscle tropomyosin antibody. Because of the precise 7:1 molar stoichiometry of actin to tropomyosin in the thin filament, it would be expected that the ratio of the staining intensities would remain constant in cells and regions of cells stained at the same time. The apparent uncoupling of the quantity of muscle specific proteins synthesized or assembled was unexpected. It remains to be determined whether such uncoupling occurs in vivo or is some artifact due to cell culture.

Recent studies with microinjected fluorescent α actinin

have suggested that minisarcomeres ($\sim$1.0 μm) are first formed during myofibrillogenesis and that they subsequently elongate to the mature pattern (21). Although our present observations do not rule out such a mechanism, short sarcomeres were fairly rare. Sarcomeric periodicities in the range of 2.0 μm (determined from titin staining spacing) were present even when two or three developing sarcomeres occurred. Even an apparent single sarcomere is sometimes observed (Fig. 5 a). The earliest distinguishable A bands were always 1.6 μm long. The transition in actin patterns seen in Fig. 7 d (myofibril 3) was accompanied by a slight length change ($\sim$0.2–0.3 μm) between the right and left halves. Occasionally very short sarcomere patterns were observed (Fig. 7, c and d, myofibrils 1 and 2), but these may have resulted from breakage during fixation. Whether sarcomeres are formed via minisarcomeres or full-length or both remains to be determined.

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