TROPONIN IN EMBRYONIC CHICK SKELETAL MUSCLE CELLS IN VITRO

An Immunoelectron Microscope Study

TAKASHI OBINATA, YUTAKA SHIMADA, and RYOICHI MATSUDA

From the Departments of Biology and Anatomy, Chiba University, Chiba, Japan

ABSTRACT

The fine structural distribution of troponin on thin filaments in developing myofibrils was investigated by the use of immunoelectron microscopy. Embryonic chick skeletal muscle cells grown in vitro were treated with antibodies against each of the troponin components (troponin T, I, and C) from adult chicken muscles. Each antibody was distributed along the thin filaments with a period of 38 nm. It is concluded that these newly synthesized regulatory proteins are assembled at their characteristic position from the initial phases of myofibrillogenesis.

KEY WORDS troponin · actin filament · myofibrillogenesis · embryonic muscle cells · immunoelectron microscopy

Under appropriate salt and pH conditions in the cytoplasm of developing skeletal muscle, monomers of myosin and actin, immediately after release from the ribosomes, polymerize spontaneously and form, respectively, thick and thin filaments. These two varieties of myofilaments can be readily identified in thin sections by electron microscopy, and a number of detailed descriptions of the aggregation and organization of these contractile proteins into the adult sarcomere structure have been presented (see review by Fischman [4]). In a previous paper (14), we have demonstrated, with the use of the "decoration with heavy meromyosin" technique, that thin filaments exhibit the right polarity and spatial position in relation to thick filaments, similar to that seen in mature myofibrils, from the initial phases of myofibril assembly. This finding raises the question as to whether regulatory proteins are distributed along thin filaments in nascent myofibrils from such early stages of development. To clarify this problem, the fine structural localization of troponin on embryonic thin filaments was investigated by the use of immunoelectron microscopy. Some of the results described in this article have already appeared elsewhere (15).

MATERIALS AND METHODS

Muscle Cultures

Suspensions of embryonic skeletal muscle cells were obtained from thigh muscles of 12-d chick embryos by the standard procedure of dissociation with trypsin (16). Cell suspensions prepared by a differential cell adhesion procedure and enriched in myogenic cells (18) were used for the present cultures. For negative staining, the cells were plated at a concentration of 2×10^6 cells in 3 ml of culture medium within gelatin-coated 60-mm plastic tissue culture dishes. For thin sectioning, they were plated at a concentration of 6.6×10^5 cells in 1 ml of medium within gelatin-coated 35-mm plastic dishes. In some of these dishes, round cover slips (15 mm in Diam) were present on the bottom. These cover slips had been precoated with carbon and then with gelatin.

The culture medium consisted of Eagle's minimum essential medium with glutamine, 15% horse serum, 5% embryo extract, and penicillin-streptomycin in concen-

trations of 50 U/ml and 50 μg/ml, respectively. Cultures were kept at 37°C in an atmosphere of 5% CO_2 in air at saturation humidity. The culture medium was changed every 2–3 d. 4- and 7-d-old cultures were used for the present experiment.

Preparation of Antibodies

Troponin was extracted by the method of Ebashi et al. (3) from adult chicken breast muscles and separated into its components (troponin T [TN-T], I [TN-I], and C [TN-C]) by ion-exchange column chromatography in the presence of 6 M urea (3, 5). The purity of each of these troponin components is shown in Fig. 1. Rabbit antisera against each of these troponin components were prepared. The immunoglobulin (IgG) was fractionated from the serum by ethanol or ammonium sulfate and purified by DEAE cellulose column chromatography (2, 8). It was dialyzed against 0.15 M NaCl containing 10 mM Na-phosphate buffer, pH 7.0. The specificity of the antibodies was tested by double immunodiffusion, immunoelectrophoresis, and immunofluorescence microscopy. Each antibody formed one precipitin line against its antigenic component (Figs. 2 and 3). No cross-reaction between the different components was observed. No cross-reaction of these antibodies with any other myofibrillar proteins including α-actinin and tropomyosin was detectable in immunoelectrophoresis. Indirect immunofluorescence microscopy showed that the binding of the antibody against each of the troponin components was limited to the length of the thin filaments in adult chick myofibrils (Fig. 4).

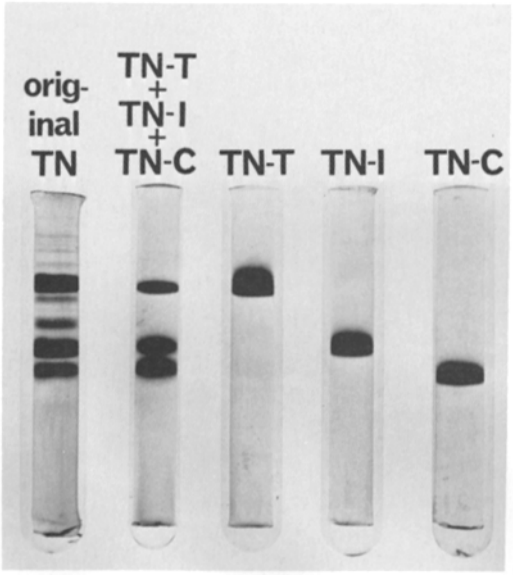

FIGURE 1 SDS polyacrylamide gel electrophoresis of the original troponin from adult chicken breast muscles, the three components separated from this troponin by ion-exchange chromatography (TN-T, TN-I, and TN-C), and the mixture of the three components (TN-T + TN-I + TN-C).

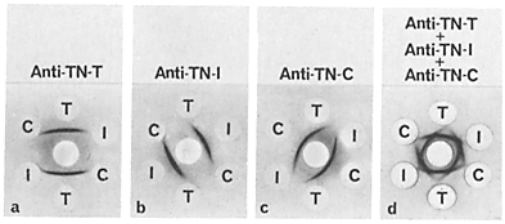

FIGURE 2 Double immunodiffusion of antisera against the troponin components. The antiserum was located in the hole at the center of the figure (Fig. 2*a*, anti-TN-T; Fib. 2*b*, anti-TN-I; Fig. 2*c*, anti-TN-C; Fig. 2*d*, mixture of anti-TN-T, anti-TN-I, and anti-TN-C). The protein components, which had a concentration of 1 mg/ml, were located in the surrounding holes (T, I, and C indicate TN-T, TN-I, and TN-C, respectively). Reactions were performed in 1% agarose gel containing 0.05% SDS, 0.9% NaCl, and 10 mM Na-phosphate buffer, pH 7.5, at 4°C.

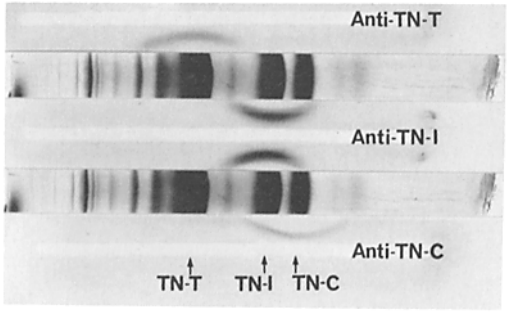

FIGURE 3 Immunoelectrophoresis of troponin from post-hatched chicken breast muscles. Electrophoresis of the original troponin on SDS acrylamide gel was followed by immunodiffusion with antiserum against TN-T, TN-I, and TN-C from adult chicken troponin. After immunoreaction, the agarose containing the immunoprecipitates was immersed in 0.9% NaCl and 10 mM phosphate buffer, pH 7.0, to remove unreacted proteins, stained with amido black, and then photographed. Photographs of the stained acrylamide gels are inserted in the exact position in the figure where unstained gels were put for immunoreaction.

Negative Staining

The muscle cultures were immersed in 50% glycerol in standard buffer (0.1 M KCl, 1 mM $MgCl_2$, and 10 mM Na-phosphate buffer, pH 7.0) and left at 0°C for 24 h. Then the cultures were stored at −20°C until used. Glycerinated cultures were rinsed with the standard buffer and gently disrupted in the same buffer with a Dounce homogenizer (Kontes Co., Vineland, N. J.). The homogenate was then centrifuged at 12,000 *g* for

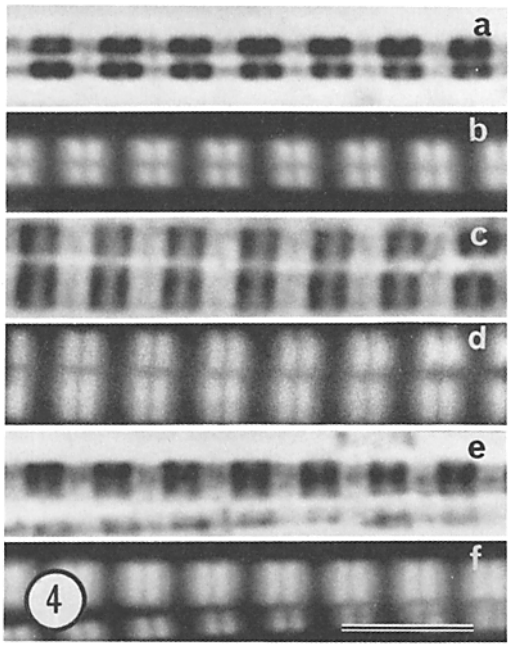

FIGURE 4 Anti-troponin staining of isolated myofibrils. Antibody for each of the troponin components was applied to myofibrils, which were then treated with fluorescein-labeled goat anti-rabbit IgG (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.). The phase-contrast micrographs, indicated *a*, *c*, and *e* correspond to the fluorescence micrographs, which are indicated *b* (anti-TN-T staining), *d* (anti-TN-I staining), and *f* (anti-TN-C staining), respectively. The I bands appear as bright doublets intersected by the Z bands. The A band shows some staining adjacent to the I band in the region of the thin and thick filament overlap. There is no stain in the center of the A band. Bar, 5 μm. × 3,500.

20 min. The resultant precipitates were re-homogenized extensively by 20–30 strokes in the tight-fitting homogenizer in a relaxing medium containing 0.1 M KCl, 10 mM Na-phosphate buffer (pH 7.0), 0.5 mM ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) and 2–4 mM adenosine triphosphate (ATP). This myofibrillar suspension was then mixed with antibody (1–2 mg/ml), incubated for 1 h at room temperature, and centrifuged at 6,000 *g* for 20 min. The precipitates formed were washed with the relaxing buffer and resuspended in the same solution. The materials were placed on carbon-coated, collodion films on 400-mesh copper grids, washed twice in 0.1 M KCl containing 0.3 mM $NaHCO_3$, and stained with 2% uranyl acetate.

Thin Sectioning

The cultures were treated with 0.1% saponin in standard buffer for 20 min at room temperature (12, 14). They were then incubated overnight at 4°C with antibody. Subsequently, myosin was extracted for 15 min in Guba-Straub solution (0.3 M KCl, 0.15 M K-phosphate buffer [pH 6.5], and 3 mM ATP with 0.5 mM EGTA). The cultures were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 25°C, postfixed in 1% OsO_4 in the same buffer at 4°C, and en bloc stained with 0.5% uranyl acetate in 0.05 M maleate buffer, pH 5.2, for 1 h at 25°C. After dehydration in an ascending ethanol series, the fixed material on plastic dishes was treated in hydroxypropyl methacrylate (1), and that on coverslips was processed in propylene oxide (16). Both materials were embedded in Epon 812. Thin sections were collected on carbon-coated, collodion-covered 400-mesh grids, and stained with uranyl acetate and lead citrate. All specimens were examined with a Hitachi H-700 electron microscope operated at 200 kV.

RESULTS

Myotubes grown in vitro for 4 d possess large numbers of myofibrils at the initial phases of their formation (14). Namely, some myofibrils are aggregates of thick and thin filaments arranged in parallel or nearly in parallel, but sarcomere structure is not well formed. Well-defined Z and M bands are absent, but, occasionally, darkly staining dense bodies are seen which appear to be the precursors of the Z bands. Other myofibrils are slightly larger in diameter, and the Z bands appear to zig-zag across the width of the fibril. At 7 d in vitro, myofibrils have increased in number and diameter, but they have not yet filled the sarcoplasmic space within the developing myotube. Z and M bands, which are clearly discernible at this stage, run straight across the width of the myofibril. In the present study, the distribution of troponin at these two representative stages in culture was investigated.

By treating the homogenate of glycerinated 4-d myogenic cell cultures with the antibody against each troponin component, bundles of thin filaments are precipitated. Along the whole bundle, cross-striations with a periodicity of 38 nm are invariably formed as the result of the antibody binding (Fig. 5*a*, anti-TN-T staining; Fig. 5*b*, anti-TN-I staining; Fig. 5*c*, anti-TN-C staining). The width of the bands formed by the three antibody varieties ranges from 9 to 14 nm. Bundles of thin filaments released from 4-d cultures usually measure ~1 μm in length. The Z band structures are not clearly discernible at this developmental stage; this probably reflects the observation that in thin sections they are absent or exhibit an immature appearance (6, 14).

Filament bundles with cross-striations are simi-

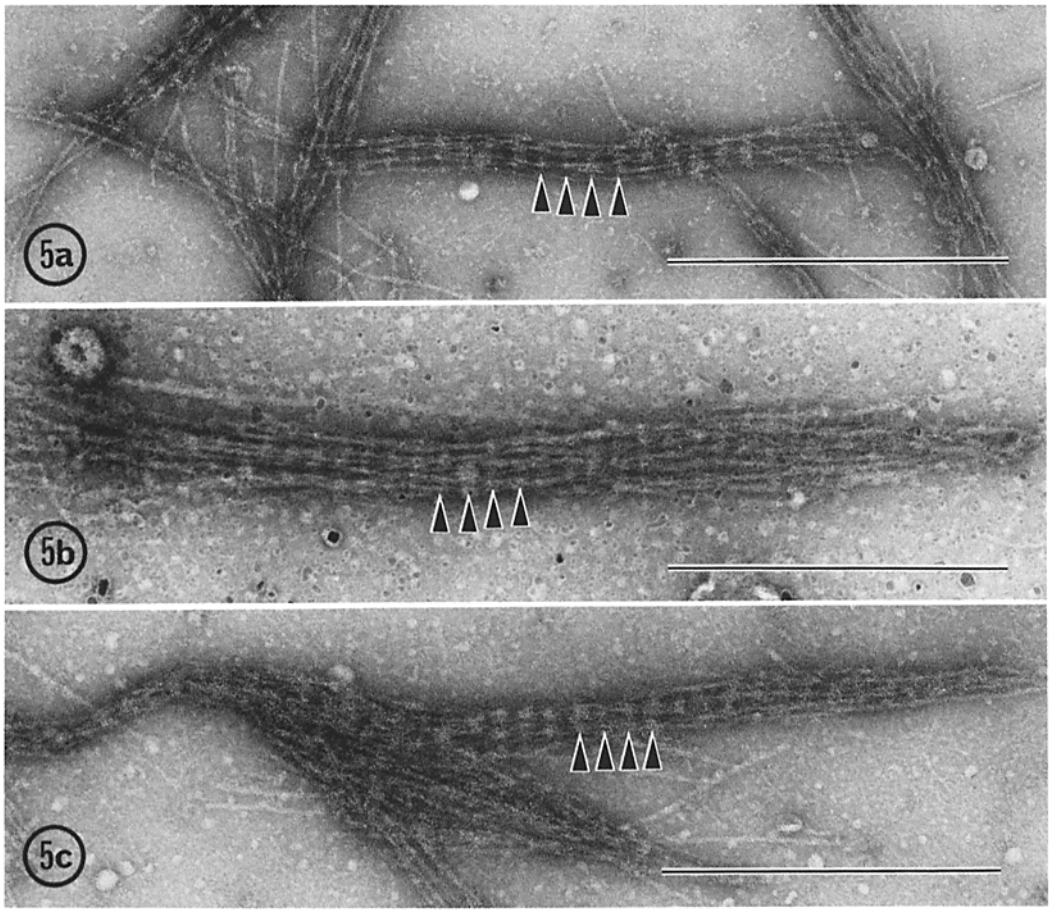

FIGURE 5 Bundles of thin filaments precipitated with antibodies against (*a*) TN-T, (*b*) TN-I, and (*c*) TN-C from the homogenate of glycerinated 4-d myogenic cell cultures. Cross-striations with 38-nm intervals are formed by the antibody treatment (arrows). Negative staining with 2% uranyl acetate. Bar, 0.5 μm. × 90,000.

larly obtained by treatment of 7-d cultures with the antibody against each component. At this stage of culture, thin filaments extend from the Z band material, and cross-striations are seen along the thin filament bundle on either side of the Z band structure (Fig. 6*a*, anti-TN-T staining; Fig. 6*b*, anti-TN-I staining; Fig. 6*c*, anti-TN-C staining). Separated thin filaments remain in the form of bundles near the Z band, while at the other end they become split. At these areas where free filament ends do not overlap, striations are hardly visible. Thus, it is difficult to count the number of striations along the entire length of the filaments; at least 20 can be counted along one thin filament region extending from the Z band.

Longitudinal sections of myotubes at 4 d in vitro treated with saponin and then stained with anti-TN-C antibody are shown in Fig. 7. As stated earlier, myofibrils with a small number of filaments (Fig. 7*a*) as well as those with a somewhat larger diameter (Fig. 7*b*) are present in the myotubes at this stage. Clear periodic striations are formed in these myofibrils. This staining pattern is essentially the same as that seen with the separated thin filaments. With the present embryonic material, if the thick variety of filaments are present in myofibrils, the cross-striations formed by antibodies are extremely difficult to observe. Thus, thick myofilaments have been extracted (see Materials and Methods). Consequently, thin filaments from each end of the sarcomere overlap and we were not able to count the entire number

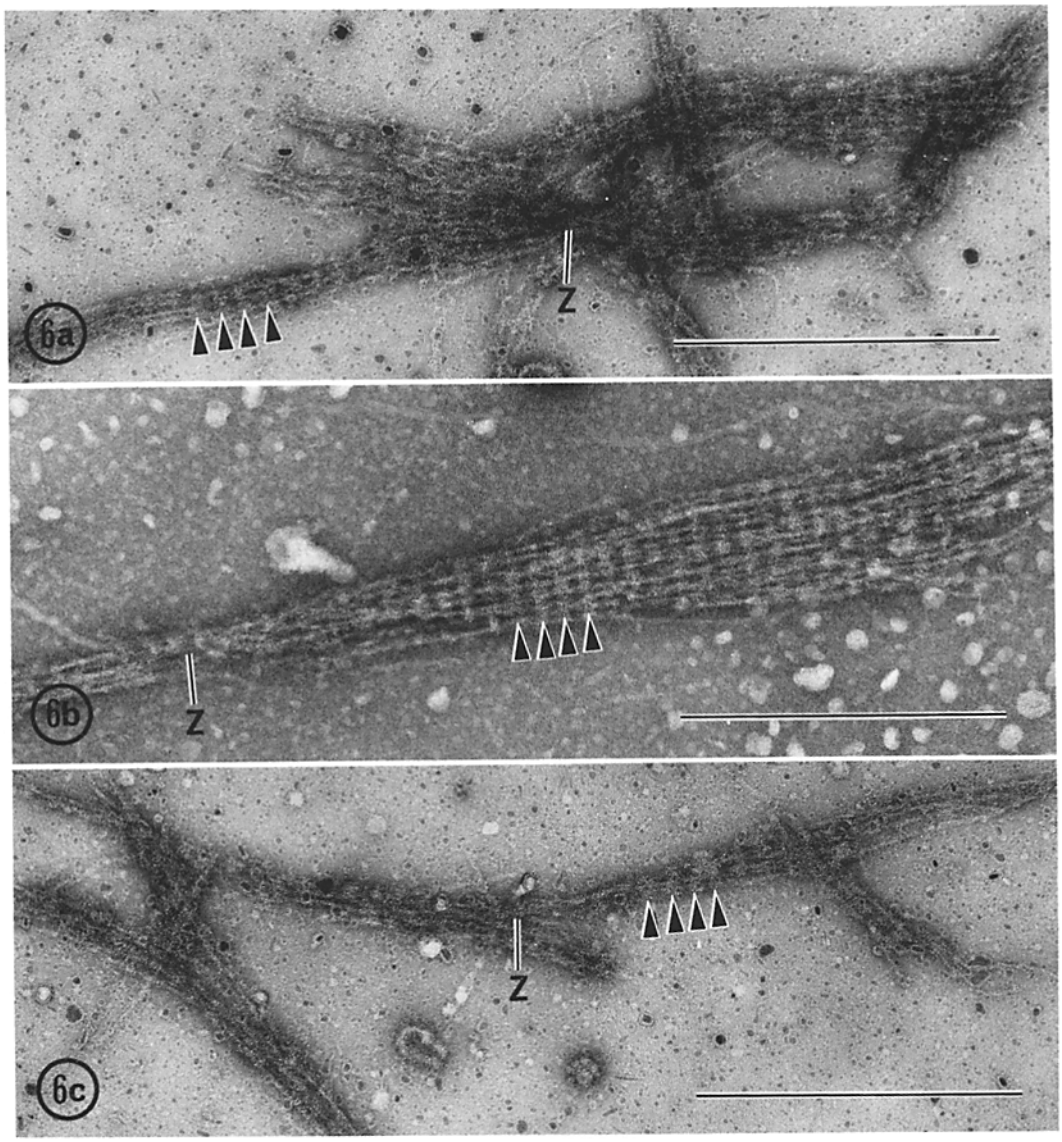

FIGURE 6 Bundles of thin filaments from 7-d muscle cultures precipitated by treating with antibodies against (*a*) TN-T, (*b*) TN-I, and (*c*) TN-C. Striations with the same intervals as those seen in Fig. 5 are observed (arrows). Negative staining with 2% uranyl acetate. *Z*, Z band. Bar, 0.5 μm. × 86,000.

of striations along one thin filament region on one side of the Z band structure.

Without the antibody treatment, isolated thin filament bundles are disordered in the relaxing medium and are not precipitated. Cross-striations are not seen along the filament bundles in thin sections without treatment with antibodies (Fig. 8*a*) or addition of γ-globulin from non-immune serum (Fig. 8*b*).

DISCUSSION

When isolated myofibrils from embryonic muscle cells in vitro are treated with antibodies against each of the three troponin components, regular striations with a periodicity of 38 nm are formed along the thin filaments. The same result was obtained with longitudinal thin sections of myofibrils treated with the antibody. This staining pat-

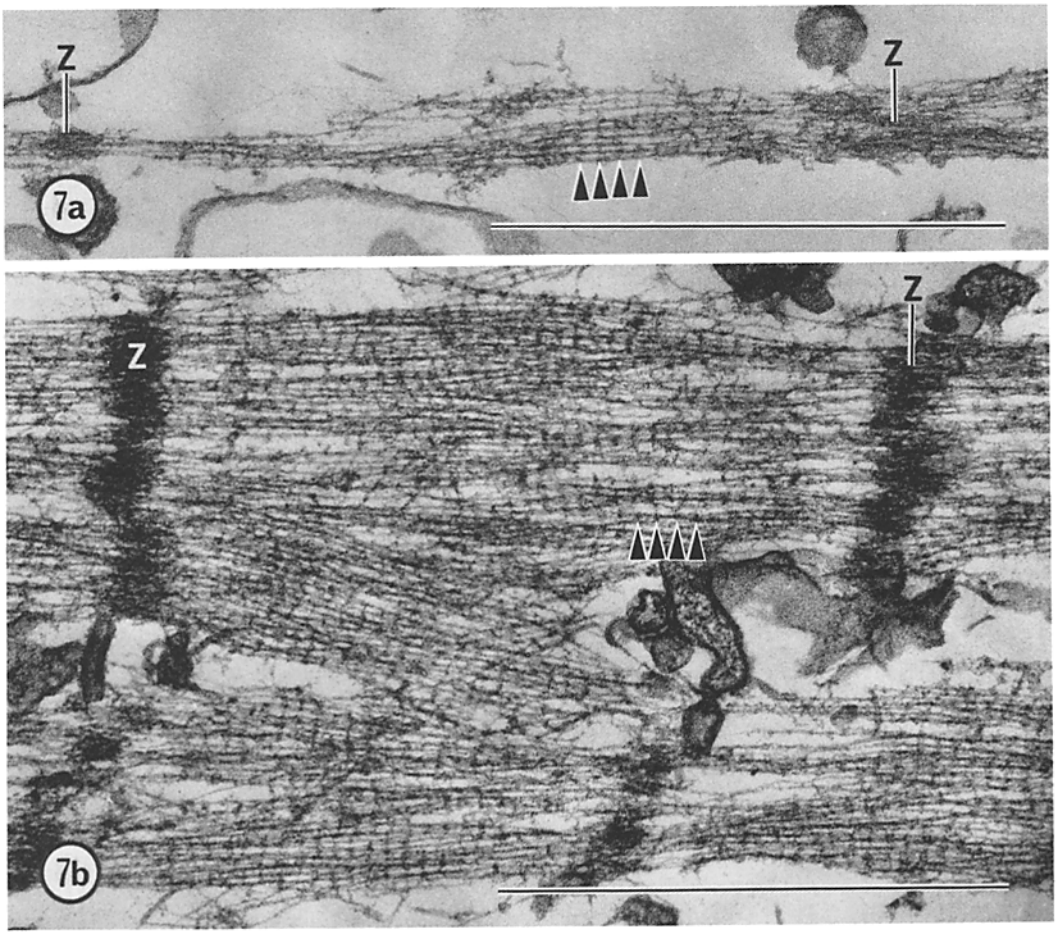

FIGURE 7 Thin sections of myofibrils at the initial phases of their formation from 4-d myogenic cell cultures treated with anti-TN-C antibody. Regular transverse striations along the entire thin filament region are seen (arrows). The length of each period is ~38 nm. Myosin has been extracted in Guba-Straub solution. Myofibrils with a small diameter (*a*) and a somewhat larger diameter (*b*) are shown. *Z*, Z band. Bar, 1.0 μm. × 68,000.

tern is identical to those observed in adult myofibrils in vivo (9–11). The present results indicate that every component of embryonic troponin reacts with the antibody against the respective troponin component from adult muscles and that these regulatory proteins are distributed along the thin filament at a periodicity identical to that in mature myofibrils.

According to Ohtsuki (11), the anti-TN-T antibody forms a broad band in adult muscles and sometimes splits into a pair of lines, whereas the striations formed by the antibodies against the other troponin components are found to be narrow lines. In the present study with embryonic muscles, the anti-TN-T treatment did not form such wide bands, although it formed wide striations with adult chicken breast muscles.[1] Ohtsuki (11) has described that in adult muscles TN-T appears to be composed of two antigenic sites. Further, Perry and Cole (13) and Wilkinson (17) have shown the difference in the molecular weights of the TN-T components from chicken breast and leg muscles. Thus, because the stained filaments are from embryonic leg muscles and the immunogen is from adult breast muscles, it is possible that with these materials only some of the TN-T antigenic sites at each 38-nm interval react

[1] Shimada, Y., T. Obinata, and R. Matsuda. Unpublished observations.

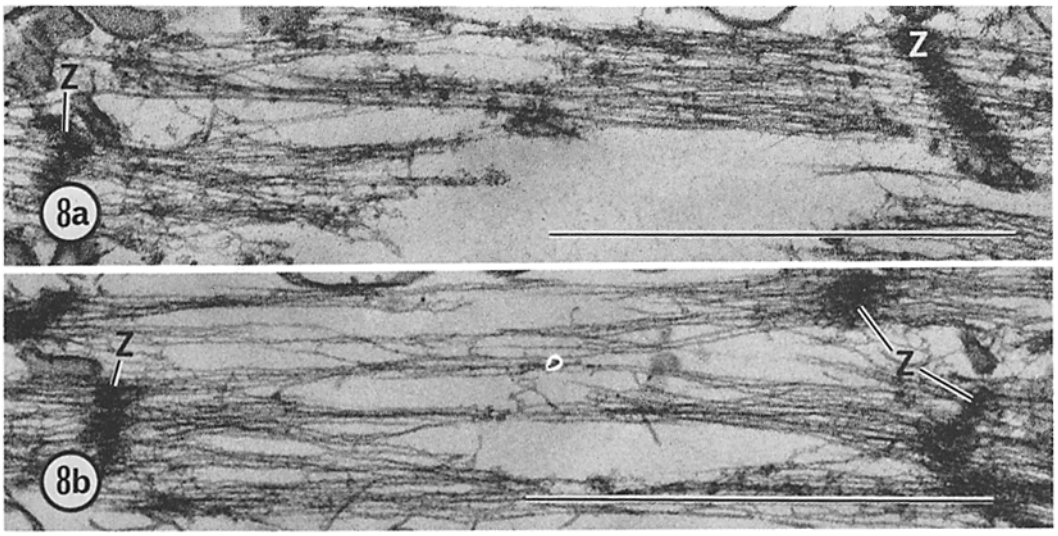

FIGURE 8 Thin sections of myofibrils from 4-d cultures, (*a*) without the treatment of the antibody, and (*b*) with the addition of γ-globulin from nonimmune serum. No cross-reactions are seen along the thin filaments. Myosin has been extracted. *Z*, Z band. Bar, 1.0 μm. × 62,000.

with the antibody. This means that the width of individual striations formed by anti-TN-T is narrower than that in mature myofibrils. However, other additional or alternative factors cannot be ruled out. Further studies are required to clarify this problem.

We have shown previously that, from the initial phases of myofibrillar assembly, thin filaments are arranged at the right polarity and spatial position in relation to thick filaments in developing muscle cells (14). In this study, we further demonstrated the presence of the troponin components, from such early developmental stages, at regular 38-nm intervals on thin filaments. Masaki and Yoshizaki (7) have shown, by using the fluorescent antibody technique, that the synthesis of various myofibrillar proteins of skeletal muscle is initiated simultaneously in the somite of the neck regions of chick embryos. From our earlier (14) and present observations, it can be concluded that these newly synthesized muscle proteins are assembled at their characteristic position from the initial phases of myofibrillogenesis, so far as the thick-thin filament association and the troponin distribution are concerned.

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