Immunocytochemical Studies of Cardiac Myofibrillogenesis in Early Chick Embryos. II. Generation of α-Actinin Dots within Titin Spots at the Time of the First Myofibril Formation

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Abstract. In whole mount preparations of the 9 somite stage chick embryonic hearts that were immunofluorescently double labeled for titin and α-actinin, presumptive myofibrils were recognized as rows of several periodically aligned titin spots. Within these titin spots, smaller α-actinin dots were observed. These periodical arrangements of titin spots and α-actinin dots were not found in the 7 somite stage hearts. In wide myofibrils in the 10 somite stage hearts, the α-actinin dots and titin spots simultaneously became 'lines.' To study the ultrastructural features of the titin-positive regions in the 6–9 somite stage hearts, the thoracic portions of the embryos were immunofluorescently labeled for titin and embedded in resin. Ultrathin sections were mounted on electron microscopic grids and examined in immunofluorescence optics. The titin-positive regions thus identified were then examined in the electron microscope. No readily discernable specific ultrastructural features were found in titin-positive regions of the 6 somite stage cardiac primodia. Examination of the sections of the 9 somite stage hearts, on the other hand, revealed the occasional presence of small dense bodies, Z bodies, in the titin-positive regions. These observations strongly suggest that these Z bodies are the ultrastructural counterparts of the α-actinin dots seen by immunofluorescence optics and that they are formed nearly at the time of the formation of the first myofibrils. In some of the nascent myofibrils the Z bodies were found to be considerably narrower than the myofibrils, implying that the Z bodies are required not for the assembly of myofibrils per se but for their stabilization.

Immunofluorescent labeling for titin and α-actinin revealed that the length of the shortest sarcomeres in the first myofibrils is ~1.5 μm, approximately the width of the A bands of mature myofibrils. The possibility that the A bands might define the initial length of nascent sarcomeres was indicated.

THE presence of dense amorphous material in the form of plaques in early embryonic hearts was reported in several past ultrastructural studies. Manasek (16) observed "accumulations of dense Z material surrounded by amorphous filament precursor material" in the cardiac myocytes of the chick embryo. Markwald (17) studied this subject more thoroughly in rat and hamster embryos and reported that the majority of the first myofibrils are formed in association with dense amorphous material ('precursor Z material') located as plaques along the plasma membrane of the cardiac myocytes. These authors proposed that these dense plaques serve as the centers for the assembly of myofibrils. However, the presence of such structures was observed in the chick embryo of stage 10 (16) and in the 10-d rat embryo (17), which are both in the stage of the first myocardial contractions (2, 18). Thus, these studies did not provide a clear answer to the question of whether such plaques are generated before or nearly in coincidence with the formation of myofibrils. The elucidation of the molecular nature of this ultrastructural entity is important in understanding the process of cardiac myofibrillogenesis. In skeletal myofibrillogenesis, similar structures were also studied in more detail in a larger number of reports (1, 3, 5–9, 14). Yet, the question of whether or not such plaques function as myofibril initiation sites has not been clearly answered in this system either. In part I of the present series of studies (20), we reported that by immunofluorescence microscopy, titin is present in a punctate state in the premyofibril stages. In the present study, we investigated the temporal and topological relationship of titin and α-actinin in the early stages of cardiac myofibril formation by immunofluorescence microscopy of whole mount preparations that were doubly immunolabeled for titin and α-actinin. In addition, we examined ultrathin sections of titin-labeled and resin-embedded preparations in both the fluorescence and electron microscopes to elucidate the relationship between the immunofluorescent titin spots and the ultrastructurally identifiable dense Z bodies.

We report here that immunofluorescent α-actinin dots are formed within the titin spots, that the α-actinin dots are considerably smaller than the titin spots, that the newly formed
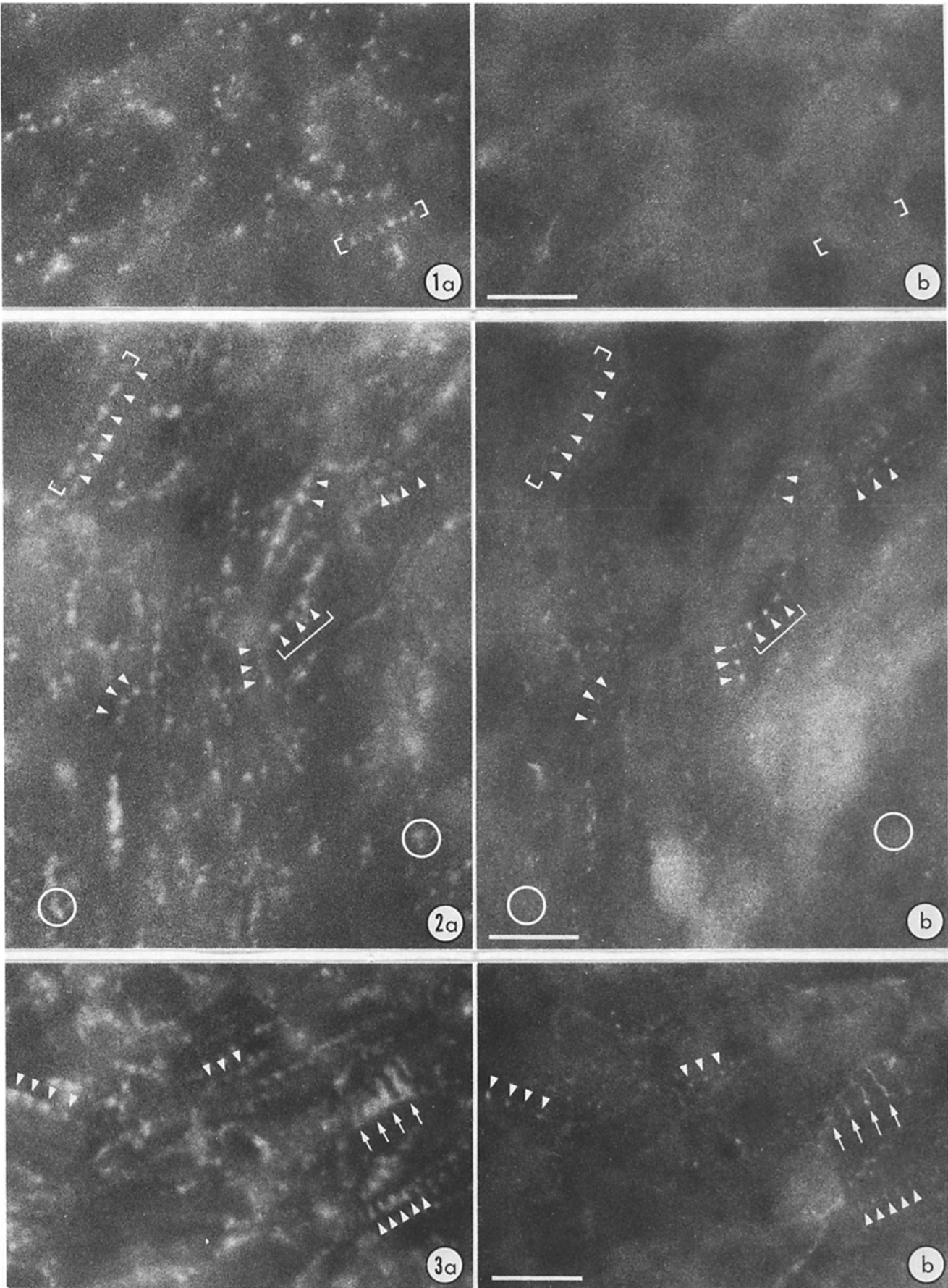
dense Z bodies are found to be significantly smaller than the titin-positive regions in the electron microscope, and that the α-actinin dots in the immunofluorescence microscope quite probably correspond to the Z bodies or dense Z material plaques seen in past studies with the electron microscope.

Materials and Methods

Specimens

Incubation of chicken eggs and fixation of the embryos were carried out as described in part I of this series.

Antibodies

α-Actinin was partially purified from chicken gizzard muscle as described (4) and then further purified by SDS-PAGE on a 7.5% gel followed by elution into 50 mM NH_4HCO_3, pH 9.0, 0.05% SDS, 1 mM EDTA. Antibodies were produced in rabbits or guinea pigs as described (22) and purified by affinity chromatography using the antigen immobilized on glutaraldehyde-activated Ultrogel ACA22 (LKB Instruments, Inc., Gaithersburg, MD) (19). SDS-PAGE (15) and immunoblotting (24) were performed as described. This antibody was used before to immunolabel α-actinin of cardiac myocytes (10). The method of titin isolation was described in part I (20).

Immunolabeling, Whole Mount Preparations, and Resin Embedding

Immunolabeling of whole mount preparations, embedding of these preparations in resin and light microscopic examination of both the whole mount preparations and the sectioned material were carried out as described in part I (20). For the simultaneous immunolabeling of two proteins, the primary antibodies were rabbit and guinea pig antibodies and the secondary antibodies were rhodamine-conjugated F(ab')₂ fragment goat anti–rabbit IgG (Jackson Immunoresearch, Avondale, PA) in combination with fluorescein-conjugated goat anti–guinea pig IgG (10) or biotinylated goat anti–guinea pig IgG (11) followed by fluorescein-conjugated streptavidin (Amersham Corp., Arlington Heights, IL).

The same exciter filters, barrier filters, and short wave pass filters as indicated in the Materials and Methods in part I were used to discriminate fluorescein (and NBD) signals from rhodamine signals. In addition, the possibility of rhodamine signals leaking into fluorescein signals was critically checked through the evaluation of observed images. Thus, the absence of α-actinin signals in individually separated titin spots (rhodamine) was used as a built-in control for the detection of α-actinin signals (fluorescein) in periodically aligned titin spots (Figs. 1–3).

Correlated Light and Electron Microscopic Examinations of Ultrathin Sections of Resin-embedded Preparations

Ultrathin sections of the embedded preparations, 80–100 nm in thickness, were mounted on grids and then dried, as commonly done in ultramicrotomy. The observation of ultrathin sections first in the light microscope and then in the electron microscope was carried out as described before (23).

A rectangular plate of 2.5 × 4 cm size was cut out of a 0.5-mm-thick plastic slide (Laboratory Supplies Co., Hicksville, NY) and a 7-mm hole was made in it with a standard three-hole paper punch. This plate was pasted on a glass slide. A 1-μl droplet of the mounting medium was placed at the center of a cover slip and the grid was in turn placed on the droplet upside down so that the sections faced the mounting medium. The cover slip was then placed on the hole of the plastic plate on the glass slide in such a way that the grid hung on the bottom surface of the cover slip without touching the slide or the edge of the hole. After fixing the cover slip to the plastic plate with pieces of adhesive tape, the grid was examined in the light microscope.

Although the sections were hardly recognizable in Nomarski optics, they often contained sufficient fluorescent signal for examination and photography. After taking micrographs of the fields of interest and recording the reference points to identify the fields, the cover slip was removed from the slide. It was then turned over so that the grid was resting on the top surface. When a droplet of water was placed next to the grid and made contact with the mounting medium underneath the grid, the medium dissolved into the water and the grid floated on the water droplet. The grid was then washed several times with water, doubly stained with uranyl acetate and lead citrate and observed in the Philips EM-300 electron microscope. The fields that had been photographed in the light microscope were identified, examined, and photographed at various magnifications.

Results

Relationship Between the Immunofluorescent Labeling Patterns of Titin and α-Actinin

The anti–α-actinin antibody used in the present study was directed to chick gizzard smooth muscle α-actinin and reacted with the α-actinin isoforms of different muscle types (21) as well as that of nonmuscle cells (10).

In the developmental stages before the formation of myofibrils, a low but significant labeling of α-actinin was often found along the cell boundaries, particularly along the lateral cell borders (not shown), in a labeling pattern similar to that of F-actin (Figs. 10 b and 11 b of part I [20]). This occurred not only in the cardiac primodia but also in the rest of splanchnic mesoderm, which indicated that this labeling was not specific to cardiac morphogenesis but related, at least partly, to the general presence of actin filaments along the cell borders.

Double immunofluorescence labeling of 7 somite stage embryos for α-actinin and titin showed no recognizable labeling of α-actinin either at the individual titin spots or at the rows of nonperiodically aligned titin spots (a pair of round brackets in Fig. 1, a and b). On the other hand, in the 9 somite stage heart in which the first myofibrils were expected to be formed, minute dots of α-actinin labeling were found at the periodically arranged titin spots of the presumptive, nascent myofibrils (arrowheads in Fig. 2, a and b), but not at the sites of individual titin spots (circles in Fig. 2, a

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Figures 1–3. (Fig. 1, a and b) An area of a whole mount preparation of a 7 somite stage heart, double immunolabeled for (a) titin (rhodamine) and (b) α-actinin (fluorescein). No punctate α-actinin labeling is recognized in b, either at the sites of individual titin spots or at the spots aligned in variable intervals of 1–1.7 μm (*brackets*) that are seen in a. Bar, 10 μm. (Fig. 2, a and b) An area of a whole mount preparation of a 9 somite stage heart, double immunolabeled for (a) titin (rhodamine) and (b) α-actinin (fluorescein). At the sites of titin spots aligned in constant intervals, α-actinin dots of appreciably smaller sizes are recognized (*arrowheads*), although there is a considerable variation in the labeling intensity among α-actinin dots (compare the α-actinin dots indicated with a pair of small brackets with those marked with a large bracket). No such α-actinin dots are recognized at the sites of individual titin spots (*circles*). The distance between titin spots is constant in each row but varies among different rows from ∼1.5 to 2.4 μm. Bar, 10 μm. (Fig. 3, a and b) An area of a whole mount preparation of a 10 somite stage heart double immunolabeled for (a) titin (rhodamine) and (b) α-actinin (fluorescein). The colocalization patterns of titin and α-actinin are identical to those seen in Fig 2, a and b. Refer to the legends of Fig. 2, a and b for arrowhead-marked titin spots in a and similarly marked α-actinin dots in b. In addition, α-actinin 'lines' are found in b at the sites of titin 'lines' of a widened myofibril in a (*arrows* in a and b). Bar, 10 μm.
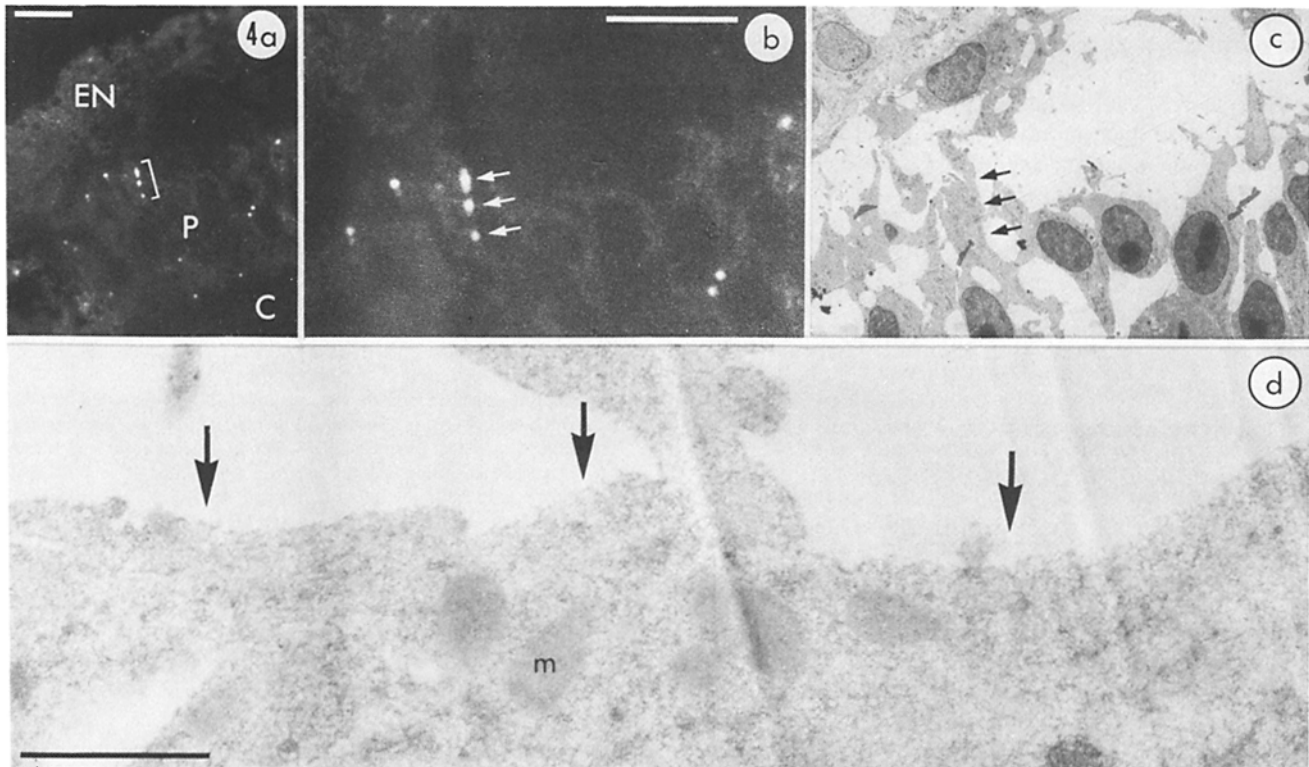
Figure 4. Ultrathin section of a titin-labeled and resin-embedded 6 somite stage heart. In *a*, a faint background labeling allows identification of the endoderm (*EN*), cardiac primordium (*P*), and coelom (C), and the observation that the punctate labeling of titin occurs only in the cardiac primordium. Three titin spots that are located on the surface of a myocyte are marked with a bracket in *a* and arrows in *b*, an enlarged portion of *a*, and the sites of these spots are indicated with arrows in *c*, an electron micrograph of the same area and magnification as *b*. The area containing these sites is further enlarged in *d*, which is turned counterclockwise by about 90° in relation to *a–c*. There are no recognizable morphological features that are common to these sites and not found elsewhere. *m*, mitochondrion. Bars in *a*, *b*, and *d*, 10, 10, and 1 µm, respectively.

and *b*). When the titin spots became lines as the myofibrils widened, the α-actinin dots increased proportionately in width and became lines (arrows in Fig. 3, *a* and *b*). As expected, these wide myofibrils were found more often in the 10 somite stage than in the 9 somite stage.

The α-actinin dots showed a noticeable variation in their labeling intensity (compare a myofibril indicated with a pair of small brackets with that indicated with large bracket in Fig. 2, *a* and *b*), probably reflecting a variation in the degree of development of the myofibrils or in their initial widths or both. Regardless of such a variation, the α-actinin dots were clearly smaller than the titin spots (compare Figs. 2, *a* and *b*, and 3, *a* and *b*).

Because the photographic processes significantly affect the apparent sizes of the fluorescent spots, the utmost care was taken to process the titin and α-actinin micrographs equally in each photographic step. Generally, in immunofluorescent microscopy, the brighter the printed spot, the larger the spot size becomes. The fact that some α-actinin dots are brighter than the corresponding titin spots but still smaller in size (compare the left two of three α-actinin dots marked by a large bracket in Fig. 2 *b* with the corresponding two titin spots in Fig. 2 *a*) indicated that the α-actinin dots are indeed smaller than the titin spots. The best geometrical evaluation of the size of the α-actinin dots gave a value of ∼0.3 µm. Assuming that the resolution of the light microscope is 0.2–0.3

µm, the actual size of the α-actinin dots is thus estimated to be close to the resolution limit of the light microscope or smaller. The results of immunolabeling experiments with an antibody that was recently raised against skeletal α-actinin were essentially identical to the results described above (not shown).

Examination of Titin Spots at the Electron Microscopic Level

The proposal made in past ultrastructural studies (16, 17) that the plaques of dense precursor Z material serve as the centers for the assembly of the first myofibrils raised the question of whether or not the titin spots represented these plaques. To study this question, the thoracic parts of 6–9 somite stage embryos were dissected out, immunofluorescently labeled for titin, dehydrated with acetone without osmium tetroxide postfixation, embedded in a resin, and cut across into ultrathin sections of 80–100 nm. The sections were mounted on electron microscope grids, and in turn the grids were mounted on glass slides as explained in Materials and Methods. The sections on the grids were then examined in the light microscope to take pictures of the parts of the myocardial wall in which immunofluorescent titin spots were abundant. Subsequently, the grids were removed from the slides and after staining with heavy metals, the sections on

them were examined in the electron microscope to identify ultrastructural features of the areas that corresponded to the titin spots recorded in the light micrographs.

In sections of the 6 somite stage hearts, the regions corresponding to the titin spots (compare Fig. 4, *a* and *b*, with Fig. 4, *c* and *d*) did not show any readily discernable ultrastructural features that were common among them and clearly distinguished them from the surrounding areas (compare regions indicated with arrows with surrounding areas in Fig. 4 *d*). In the 9 somite stage hearts, there were titin spots corresponding to the Z areas of nascent myofibrils lying within the plane of the section (two titin spots indicated with arrows in Fig. 5 *a*, corresponding to two arrow-marked regions in Fig. 5 *b* and further to two broken line circles in Fig. 5 *c*). Such areas often contained a small dense structure of ∼0.1 μm width which is referred to as a 'Z body' in this paper (Fig. 5 *c*). In two adjacent sections separated by ∼0.2 μm but containing parts of the same myofibrils (large arrows in Figs. 5 *c* and 6 *b*) and showing identical patterns of titin labeling (compare arrow-marked titin spots in Fig. 5 *a* with those in Fig. 6 *a*), the Z bodies were seen in one section (Fig. 5 *c*) but not in the other (Fig. 6 *b*). Such observations indicated that the newly formed Z bodies were smaller than the titin-positive areas, that is, the titin spots in immunofluorescent microscopy. In fact, in some nascent myofibrils that appeared to be slightly more developed than those described above, the width of the Z bodies was seen to be considerably smaller than the width of the myofibrils (arrowheads *A–C* in Fig. 7). These observations corresponded to the previously described immunofluorescent observation that the α-actinin dots were smaller than the titin spots in nascent myofibrils and strongly suggested that the newly formed Z bodies were the ultrastructural counterparts of the α-actinin dots. A small number of the Z bodies showed a width nearly equal to that of the myofibrils (arrowhead *D* in Fig. 7). These were believed to represent a more advanced stage than the small Z bodies.

The filaments that were visualized at a relatively low magnification in the nascent sarcomeric space between Z bodies (large arrow in Fig. 5 *c* and small arrowheads in Fig. 7) showed widths of 15–20 nm and thus were very likely myofibrillar thick filaments. This conclusion is concordant with the ultrastructural observation that the earliest stage in which thick filaments are found is nearly coincident with the stage when the first myofibrils appear, that is, the 8–9 somite stage (13). The unusual wavy feature of these filaments (Figs. 5 *c* and 7) was identified as an artifact induced during the specimen preparation and observation processes, because no such feature was seen in the sections of conventionally embedded preparations of glutaraldehyde-fixed and osmium-treated 9–10 somite stage hearts (13, 16).

In the 9 somite stage heart, a large number of titin spots appeared to be still individually separated. No readily discernable specific ultrastructural features were found in such spots (dotted line circles in Figs. 5 *c* and 6 *b* which correspond to fluorescent titin spots indicated with forks in Figs. 5 *a* and 6 *a*, respectively). Sometimes, several titin spots appeared to be laterally packed and form groups (brackets in Figs. 5 *a* and 6 *a*). Electron microscopic examinations of the areas corresponding to these spots revealed that they were not laterally connected to each other by longitudinally lying thick filaments and that they were similar to individually separated titin spots in that no specific ultrastructural features were detected in them (not shown). The significance of the apparent lateral packing of these spots is not clear at the present time.

Discussion

A major question that we addressed in this paper was whether the dense Z material plaques reported in past ultrastructural studies function as myofibril organizing centers. We have just demonstrated that α-actinin dots are formed within the titin spots that were periodically arranged at the time of, but not before, the formation of the first myofibrils and that the α-actinin dots are clearly smaller than the titin spots. Electron microscopic examination of the regions of the periodically arranged immunofluorescent titin spots revealed the presence of small dense structures, Z bodies, in some but not all of these regions. Such Z bodies were considerably smaller than the titin-containing regions. These facts together with the fact that no such structures were observed in the earlier stages strongly suggest that these structures are the ultrastructural counterparts of the immunofluorescent α-actinin dots and that they are formed not earlier than but nearly in synchrony with the formation of myofibrils. Furthermore, the fact that the Z body width was much smaller than the myofibrillar width in some of the nascent myofibrils (Fig. 7) suggests that α-actinin is required not for the initial assembly of myofibrils per se but for their stabilization. Thus, these observations do not support the proposal from earlier ultrastructural studies (16, 17) that dense Z material plaques, that is, the present Z bodies, are formed first and function as the myofibril organizing centers. Rather, our observations support the view of the investigators of skeletal myofibrillogenesis that the actin and myosin filaments are already organized before the appearance of the dense Z material (8, 9).

We observed by immunofluorescent labeling for titin and α-actinin that the shortest sarcomere length seen in the nascent myofibrils was ∼1.5 μm, approximately the length of the A band of mature striated muscle (Figs. 2 and 3). Whether this is a mere coincidence or whether the A band width is the factor that defines the initial sarcomeric length of the first myofibrils is not clear at this time. Recently, Hill and co-workers (12) observed that in taxol-induced pseudo-striated myofibrils there were no recognizable thin filaments and Z bands, but the thick filaments comprising the A band of such myofibrils showed a normal length of 1.6 μm. They interpreted these observations to suggest "that the assembly of 1.6-μm-long thick filaments in vivo and in vitro might be relatively autonomous." This possibility needs to be further investigated.

It was reported in part I that several titin spots were occasionally aligned in variable intervals of ∼1–1.7 μm in the 6 somite stage (Fig. 10 *a* of part I) when no myofibrils were expected to be present, and indeed no actin-containing filaments (Fig. 10 *b* of part I) were observed at the sites of these rows. Here, it was demonstrated that similar rows of titin spots, aligned in variable intervals in the 7 somite stage, were not associated with α-actinin dots (Fig. 1, *a* and *b*). Whether such rows of titin spots are fortuitously formed or have a myofibrillogenic significance remains to be investigated.
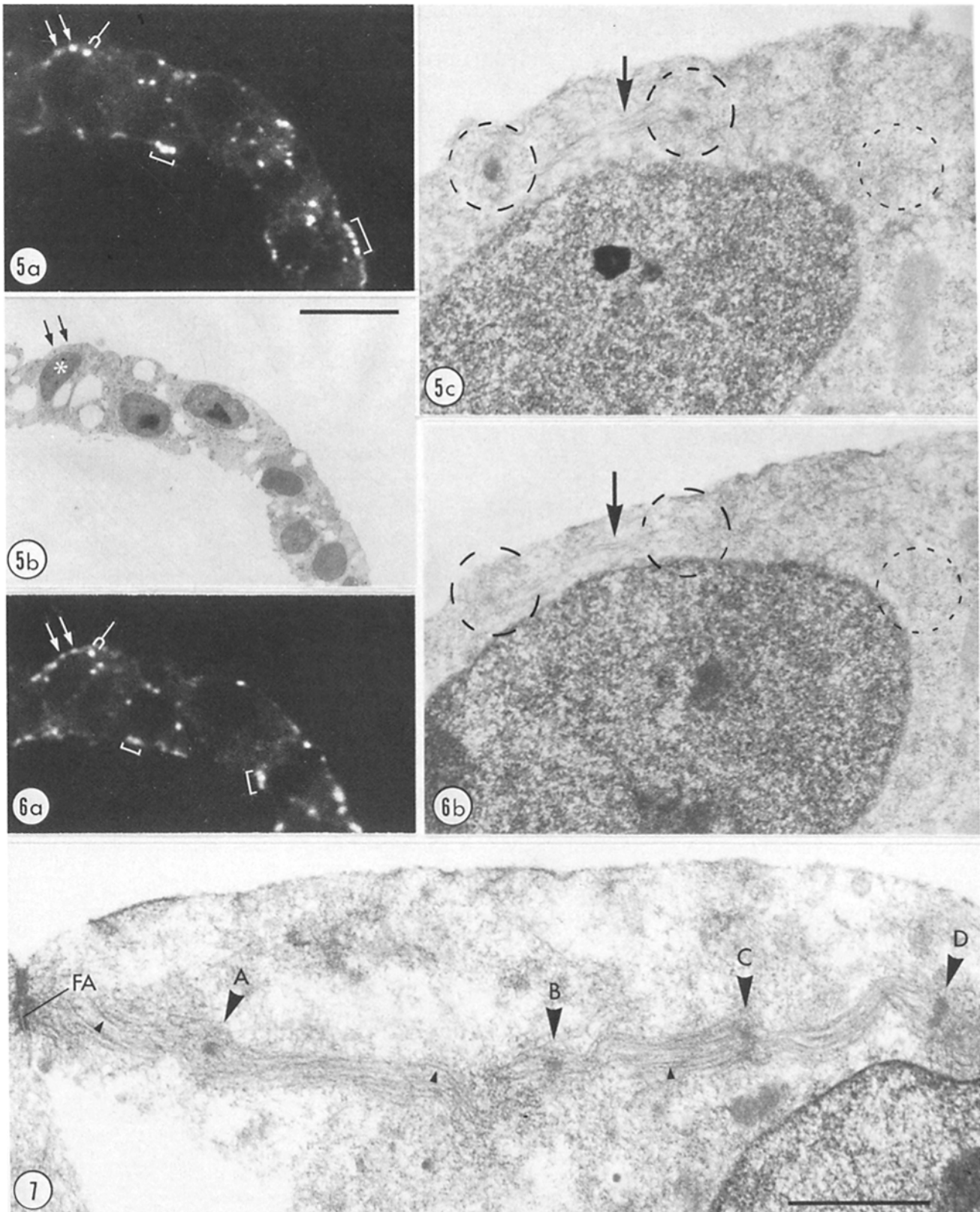
Figures 5-7. (Fig. 5, *a–c*) Ultrathin section of a titin-labeled and resin-embedded 9 somite stage heart. A faint background labeling in *a*, an immunofluorescence micrograph, makes it possible to match the nuclei and the features of the heart wall surfaces in it with the corresponding structures in *b*, an electron micrograph of the same area and magnification (see marked nucleus (*) in *b*). This in turn makes it possible to relate the locations of chosen titin spots in *a* (*arrows*) to specific sites in *b* (*arrows*). In *c*, an enlarged portion of *b*, these sites are found to correspond to the regions encircled with broken lines. These regions, separated by a distance of ~1.5 μm, and the thick filaments spanning between them (*large arrow*) form a nascent sarcomere. A dense Z body is recognized in each of the regions. In the region of *c* that corresponds to the titin spot indicated with a fork in *a* (*dashed circle* in *c*), no specific ultrastructural features are readily

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discernible. Groups of laterally packed titin spots are indicated with brackets in *a*. Magnification of *c* is the same as that of Fig. 7. Bar in *b*, 10 μm. (Fig. 6, *a* and *b*) Ultrathin section ~0.2 μm apart from the section shown in Figs. 5, *a–c*. In *a*, an immunofluorescent micrograph, two titin spots marked with two arrows are found in the same locations as the two arrow-marked titin spots in Fig. 5 *a*, along the surface of the same nucleus (see * in Fig. 5 *b*). In *b*, an electron micrograph of an area in *a*, thick filaments of ~15 nm in width, indicated with a large arrow, are also found in the same area as the arrow-indicated thick filaments in Fig. 5 *c*. These facts indicate that this section includes the same sarcomere as observed in the section of Fig. 5, *a–c*. In *b*, however, no Z body is observed in the regions (*line circles*) corresponding to the arrow-marked titin spots in *a*. In the region of *b* that corresponds to the titin spot indicated with a fork in *a* (*dashed circle* in *b*), no easily detectable, specific ultrastructural features are found. Groups of laterally packed titin spots are indicated with brackets. Magnifications of *a* and *b* are same as those of Fig. 5 *b* and Fig. 7, respectively. (Fig. 7) An ultrathin section of the same 9 somite stage heart as shown in Figs. 5 and 6. Three Z bodies on the right-hand side, marked with arrowheads *B–D*, are found in the Z areas of a myofibril. The myofibril with a Z body on the left, arrowhead *A*, does not appear to be continuous with the above myofibril. The Z bodies marked with arrowheads *A–C* are much narrower than the myofibrils, whereas the one marked with arrowhead *D* appears to be composed of two Z bodies and nearly spans the whole myofibril width. Thick filaments of 15–20 nm widths are indicated with small arrowheads. *FA*, fascia adherens. Bar, 1 μm.