Immunocytochemical Studies of Cardiac Myofibrillogenesis in Early Chick Embryos. I. Presence of Immunofluorescent Titin Spots in Premyofibril Stages

K. T. Tokuyasu and Pamela A. Maher

Department of Biology, University of California at San Diego, La Jolla, California 92093

Abstract. Our initial attempts to immunolabel intact myocardial walls of 4–12 somite stage chick embryos were hindered by the presence of the cardiac jelly that covers the inner myocardial wall surface and prevents the access of antibodies to that surface. We overcame this difficulty by treating the specimens with hyaluronidase, which made the cardiac jelly permeable to the antibodies. An additional nonionic detergent treatment made the two or more cell layers of the myocardial wall accessible to the antibodies from both surfaces of the wall. Specimens treated in this manner were fluorescently labeled with antibodies to titin, myosin, or actin or with NBD-phallacidin for F-actin and examined as whole mount preparations or cut into semithin sections after resin embedding. These preparations and sections revealed that titin, a putative scaffolding protein of sarcomeres, is present in a punctate state and also in a diffuse form throughout the cytoplasm of cardiac myocytes in the premyofibril stages (4–7 somite stages) as well as in the early stages of myofibril formation. We interpreted the punctate and diffuse states to represent an aggregated state of several titin molecules and a dispersed state of individual titin molecules, respectively. In the 4–7 somite cardiac primodia, myosin and actin show only a uniform labeling throughout the cytoplasm of the myocytes. These observations are in contrast to a previous report that titin and myosin are tightly linked during in vitro skeletal myofibrillogenesis (Hill, C. S., S. Duran, Z. Ling, K. Weber, and H. Holtzer, 1986, *J. Cell Biol.*, 103:2185–2196). In the 8–11 somite stage hearts, the number of individual titin spots rapidly reduces, while the number of myofibrils with periodically aligned titin spots increases, which strongly suggests that the titin spots are incorporated into the newly arising myofibrils. Titin spots were seen as doublets only after titin spots were incorporated into the first myofibrils. However, the fact that the distance between the components of the narrowest doublet was close to the resolution limit of the light microscope left open the possibility that undiscernible doublets of submicroscopic separations might exist in the premyofibril stages. The myosin labeling revealed the sarcomeric periodicity in an earlier stage of myofibril development than the F-actin labeling.

In addition, we made two morphogenic observations. One was that immunolabeling of titin and ventricle-specific myosin always occurred for a short distance beyond the physical boundaries of the 8–9 somite stage heart troughs, indicating that myogenic cells occur in a wider area than that which eventually develops into the heart wall. Another observation was that in the 5–6 somite stage, the endothelial cells of the endocardium were positively labeled for ventricle-specific myosin, which implies that the endocardium is more closely related to the myocardium in origin than hitherto considered.

THE heart reaches a functional state at a very early embryonic stage. In the chick embryo, the first myocardial contractions occur at the 9–10 somite stage (Hamburger-Hamilton (H-H) stage 10; ~1.5 d of incubation) (8, 20, 24), and the blood flow through the heart begins at the 16–17 somite stage (H-H stage 12+; ~2 d of incubation) (20, 24). Because a unidirectional blood flow could only be generated by some coordinated contraction of different regions of the myocardial wall, it would follow that the functional heart of the 16–17 somite stage embryo is already highly organized both structurally and electrophysiologically. Past studies showed that several crucial events take place in the 7–10 somite stages. First, sparsely distributed myofibrils are found at the 8 somite stage (8) or H-H stage 10– (24). The Ca^{2+} + Mg^{2+} ATPase is first observed at H-H stage 9–10 (7–10 somite stage) (9). The appearance of the first rhythmic action potentials at the 8 somite stage (4) precedes the first contractions at the 9–10 somite stage. An important question is whether or not there are hitherto unidentified major myofibrillogenic events at the 7 somite or earlier stages.

Although the development of the cardiac myofibrillar net-

work (1, 2, 15, 22, 30) and the process of myofibrillar growth in vivo (21) as well as in vitro (3, 23) have been extensively investigated, the mechanism by which the first myofibrils are formed has been the subject of relatively few studies, presumably because of the intrinsic difficulty in identifying myofibrillogenic events before the actual formation of myofibrils. Several papers described the presence of sparsely distributed individual myofilaments in embryonic hearts near or at the time of the formation of the first myofibrils (1, 13). Manasek (14) and Markwald (16) observed plaques of dense Z material in the hearts of early chick (14) and rat and hamster embryos (16), but the embryos examined were already at the stage of the first myocardial contractions (1, 24). (The significance of such plaques will be analysed in part II of this series of studies.)

The short time interval from the appearance of the first myofibrils to the formation of a highly complex, functional network of myofibrils (~12 h [24]) suggested the possibility that major preparative steps leading to the formation of the first myofibrils might take place in the premyofibril stages. We reasoned that such events would not be unequivocally detected by morphological approaches but could be detected by immunolabeling of proteins participating in such events. Among known myofibrillar proteins, titin (36) was considered as a candidate for several reasons. First of all, it is found only in striated muscles (36), not in smooth muscle or nonmuscle cells, whereas other striated muscle proteins such as actin, myosin, or α-actinin are present in different isoforms in smooth muscle as well as in nonmuscle cells. Also, it is a long flexible protein and is believed to function as a scaffolding for the sarcomere (32, 33, 35). Although polyclonal (33, 35) as well as monoclonal antibodies (6, 18, 29, 34, 38) to titin typically immunolabel a region at the A-I junction, the protein is thought to extend from the H-band to the N_2-line or even to the Z line (18) and to interact with thick filaments (5, 29, 33).

Early cardiac myofibrillogenesis takes place in the curved, thin walls of the minute cardiac primodia or heart tubes, which makes it extremely difficult to assess in planar sections the phenomena that occur in three dimensions and has indeed offered a great obstacle to the investigation of myofibrillogenesis in the past. In the present study, this difficulty was overcome by observing whole mount preparations, in addition to sectioned material.

In this paper, we report that by immunofluorescent labeling, titin is found in a punctate form in the premyofibril stages in which myosin and actin still show diffuse distributions; that the titin spots are seen as singlets in the premyofibril stages and as doublets in some but not all of the first myofibrils; and that in the early stages of myofibril formation the number of titin spots is rapidly reduced, while the number of periodically aligned titin spots in the newly formed myofibrils increases.

Materials and Methods

Specimens

Fertilized eggs from White Leghorn chickens were incubated at 38 ± 0.5°C for 36 ± 4 h to yield 4–12 somite stage embryos. The embryos were removed from the eggs and immediately fixed in a 4% formaldehyde solution in 0.1 M Hepes (Sigma Chemical Co., St. Louis, MO), pH 7.4, containing 2 mM $CaCl_2$. The solution was prepared by depolymerization of paraformaldehyde (Fischer Scientific, Fair Lawn, NJ). After the completion of fixation, the embryos were washed several times in PBS containing 0.02% azide (NaN_3), for a total period of 30–60 min. They were then separated into different groups according to the somite numbers.

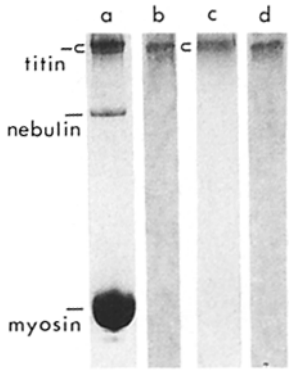


Figure 1. Analysis of SDS-PAGE by Coomassie Blue staining (lanes *a* and *c*) and immunoblotting (lanes *b* and *d*). Adult skeletal muscle myofibrils (*a* and *b*) and titin purified from the muscle (*c* and *d*) were separated by 4% SDS-PAGE, and either stained with Coomassie Blue (*a* and *c*) or transferred to nitrocellulose and immunoblotted with rabbit anti-titin, 10 μg/ml (*b* and *d*). The titin doublets in *a* and *c* are indicated with horizontal *u* markings.

Antibodies

Titin was purified by column chromatography of extracts of chick pectoralis myofibrils as described (31). Actin was partially purified from chicken gizzard as described (19) and 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. Cardiac ventricular myosin was prepared as described (11). Antibodies were produced in rabbits as described (27) and purified by affinity chromatography using the antigen immobilized on glutaraldehyde-activated Ultrogel ACA22 (LKB Instruments, Rockville, MD) (25). SDS-PAGE (12) and immunoblotting (28) were performed as described. Monospecificity of the anti-titin antibody and that of the actin and myosin antibodies are illustrated in Figs. 1 and 2, respectively. The anti-actin antibody was reactive with all isoforms of actin, as demonstrated before (refer to Figs. 2 and 4 of reference 26). Immunofluorescent labeling of semithin frozen sections of adult cardiac muscle with the anti-titin antibody produced the typical doublet pattern in myofibrils (Fig. 3).

Immunolabeling

The thoracic portions or the hearts of the fixed embryos were dissected out and were first treated with 0.1% Triton X-100 (Sigma Chemical Co.) in PBS-A (PBS with 0.02% azide, 0.01 M glycine, and 0.002% Triton X-100). Glycine was included in the buffer to quench aldehyde groups remaining in the specimens. The reason for the inclusion of 0.002% Triton X-100 will be explained later. The tissue pieces were then washed several times in the same buffer and treated with a hyaluronidase solution (2,000 U/ml; bovine testis hyaluronidase; Calbiochem-Behring Corp., La Jolla, CA) in PBS-A for 30–45 min at room temperature. The hyaluronidase treatment partially removed the cardiac jelly and made the inner surface of the myocardial wall accessible to the antibodies. This treatment was essential for the successful immunolabeling of the myocardial walls in the whole mount preparations. Collagenases tended to rapidly break down the intercellular association of cardiac myocytes, whereas hyaluronidase did so only very slowly.

After the enzyme treatment, the specimens were washed several times

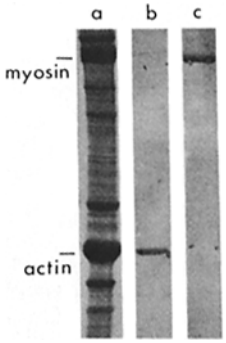


Figure 2. Analysis of SDS-PAGE by Coomassie Blue staining (lane *a*) and immunoblotting (lanes *b* and *c*). A homogenate of adult cardiac muscle was separated by 7.5% SDS-PAGE (*a*), transferred to nitrocellulose and immunoblotted with rabbit anti-actin (*b*) or rabbit anti-ventricular myosin (*c*).

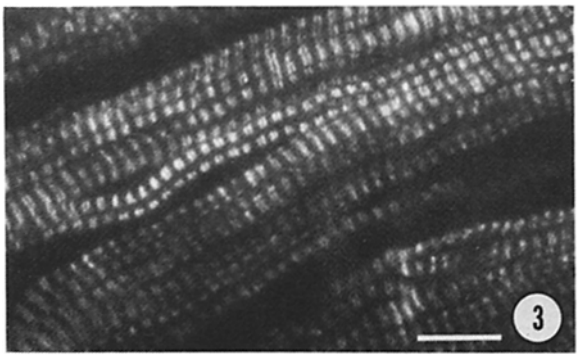


Figure 3. Semi-thin frozen section of adult chicken cardiac muscle immunofluorescently labeled for titin. Labeling in the form of doublets is found at many places. Bar, 10 μm.

in PBS-A and subjected to indirect immunofluorescent labeling. The labeling procedure was as follows.

Several specimens were immersed in a 30–60 μl droplet of a primary antibody solution (10–30 μg/ml) which was placed on the inner surface of the lid of a 35 × 10 mm Falcon culture dish (Becton Dickenson Labware, Lincoln Park, NJ). The reason for using the lid rather than the dish itself was that the inner surface of the dish is hydrophilic so that an antibody droplet placed on it tended to spread, rather than maintaining a hemispherical form. The inverted dish was then kept in a closed, moist chamber for 8–16 h in the refrigerator. Transferring of the specimens was done with a small wire loop. The inclusion of 0.002% Triton X-100 in PBS-A was important for the processing of the specimens. After the hyaluronidase treatment, the specimens became fragile and were often severely damaged by the surface tension when they were transferred through the water-air meniscus from one solution to another. They also tended to stick to the dish surface and were often destroyed when attempts were made to dislodge them from the surface. These problems were solved by the inclusion of the low concentration of detergent. The detergent also appeared to improve the penetration of the antibodies into the specimens.

After the completion of the primary immunolabeling, the specimens were washed several times in PBS-A and then kept in the same buffer for 8–16 h in a refrigerator. The secondary immunolabeling and subsequent washing steps were carried out in the same manner as just described. The secondary was rhodamine-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG (Jackson Immunoresearch, Avondale, PA). For labeling of F-actin, NBD (nitrobenz-oxadiazole)-phallacidin (Molecular Probes, Junction City, OR) was used at 10 U/ml.

All samples were examined in a Photomicroscope III (Carl Zeiss Inc., Thornwood, NY) in Nomarski optics, rhodamine fluorescence optics, and NBD fluorescence optics. The rhodamine signals were detected by using the combination of the exciter filter BP 515-560 and the barrier filter LP 590 (Zeiss item No. 487714). The NBD signals were detected by using the combination of the exciter filter BP 485/20 and the barrier filter LP 520 (Zeiss item No. 487716) with the short-wave pass filter KP 560 (Zeiss item No. 467962), which prevents the leakage of the rhodamine signal into the NBD or fluorescein signals. In addition, the different labeling patterns of F-actin (NBD-phallacidin) and titin or myosin (rhodamine) were conveniently used as controls for each other. In exceptional cases, in which a partial leakage was desirable, the examinations were carried out without the short-wave pass filter (see Fig. 11, *a–c*). The magnifications were calibrated with the standard stage micrometer.

Whole Mount Preparations

To observe the cardiac primodia or 4–7 somite stage embryos in whole mount preparations, the neural groove or canal was removed from the thoracic portion of the embryo before the initiation of the immunolabeling steps.

After the completion of the immunolabeling, the specimens were mildly compressed between the glass slide and cover slip. The mounting medium was an 80% glycerol and 20% PBS mixture with or without an antibleach reagent, *p*-phenylenediamine (Sigma Chemical Co.), at a concentration of 0.1%. To adjust the degree of compression, a piece of adhesive tape of ~0.2 mm thickness (Shamrock Scientific, Bellwood, IL) was pasted on the slide and the cover slip was placed on the tape on one end and on the slide on the other so that a slanted space was created between the slide and cover slip. By choosing the position of the specimen relative to the edge of the tape, the degree of compression could be adjusted.

Sometimes, the thoracic portion without the neural groove was cut in half along the midline or axis of symmetry. Each half was then mounted in such a way that the splanchnic mesoderm containing the cardiac primodium lay flat on the slide with the coelom-side surface directed upward.

In the 8–10 somite stages, the heart trough was formed but still attached to the foregut wall on the dorsal side. The trough was surgically separated from the foregut wall as well as from the ventral aortic roots and omphalomesenteric veins so that it was open to the outside along its dorsal side and at its cephalic and caudal ends.

After completion of the immunolabeling, the heart trough was immersed in a droplet of the mounting medium on a glass slide. When the cover slip was placed on the heart trough and moved back and forth, the trough was quite often opened up and the myocardial wall was extended so that it could be observed without folding upon itself.

Resin Embedding

It is now known that fluorescent signals can be retained in polymerized resin (10, 21*a*). We took the following steps to obtain resin-embedded specimens.

Thoracic portions of the 4–12 somite stage embryos were immunolabeled as just described. They were then dehydrated through a series of ascending concentrations of acetone, immersed in three changes of 100% acetone and embedded in Spurr's medium (Electron Microscopy Sciences, Warrington, PA). In our experience, acetone was better than ethyl alcohol and Spurr's medium was better than Epon or L. R. White acrylic resin for the retention of fluorescent signals.

The embedded preparations were cut into semithin sections of ~1 μm thickness for light microscopic examination.

Cryomicrotomy

Semithin frozen sections of adult cardiac muscle were cut, mounted on glass slides, and immunostained by the procedures described previously (26).

Results

Immunofluorescent Labeling Pattern of Titin in Sections

In 1-μm-thick sections of resin-embedded preparations, immunofluorescent titin labeling was found to be restricted to the symmetrically arranged cardiac primodial regions of splanchnic mesoderm in the 5 somite stage (Fig. 4, *a* and *b*). At high magnification, titin labeling was found to occur both diffusely throughout the cytoplasm of myocytes and in a punctate state (Fig. 4, *c–e*). The diffuse cytoplasmic labeling was clearly recognized in the myocytic cytoplasm in lightly printed micrographs (Fig. 4 *d*; compare the narrow cytoplasm around the nuclei, *n*, and a wide cytoplasmic area indicated with a black arrowhead with the endoderm, *EN*). On the other hand, the punctate labeling was more distinctly visualized in strongly printed micrographs (Fig. 4 *e*) than in lightly printed ones (compare the areas indicated with arrowheads in Fig. 4 *e* with the corresponding areas in Fig. 4 *d*).

In the 9 somite stage, titin labeling was found to be confined to the heart trough that resulted from the fusion of the primodia (Fig. 5, *a* and *b*; refer to reference 14 for cardiac morphogenesis in the early embryonic stages). At high magnification, the labeling was seen to occur both in diffuse and punctate patterns, as in the 5 somite stage (Fig. 5, *c–e*), although the overall degree of labeling appeared to be greater than in the 5 somite stage (Fig. 5, *c–e*).

Titin labeling of the cardiac primodia was seen to occur as early as the 4 somite stage (not shown). It is of interest

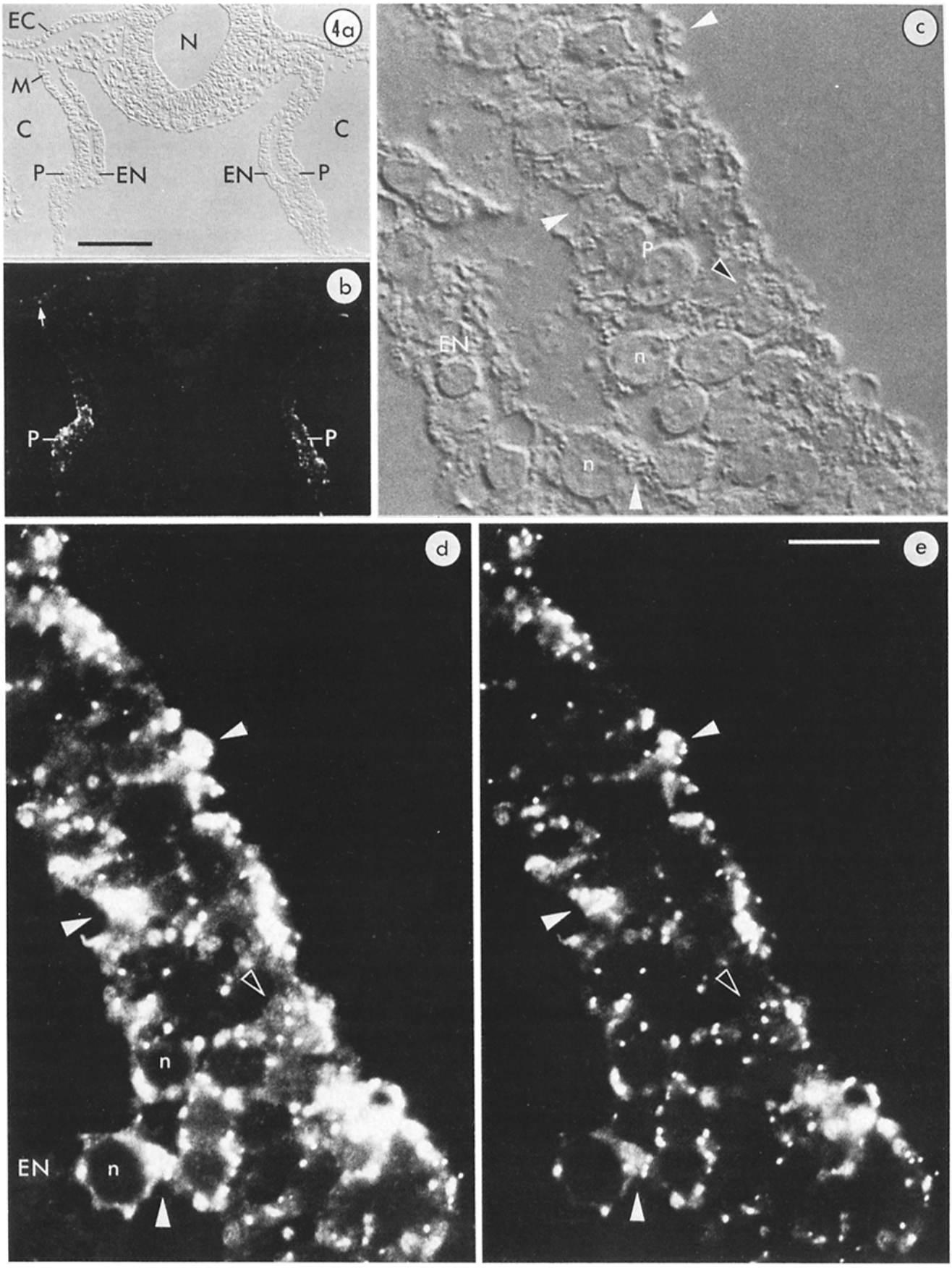


Figure 4. Cross-section of a titin-labeled and resin-embedded 5 somite stage embryo, observed in Nomarksi optics (*a*) and in fluorescence optics (*b*). Only the regions of the cardiac primodia (*P* in *a*) are seen to be positively labeled for titin in *b*. A portion of *a* is enlarged in *c*, while a part of *b* is enlarged in *d* and *e*, to the same magnification. In *d*, a lightly printed micrograph, titin labeling is found to be clearly positive in the myocytic cytoplasm above the nearly null background in the endoderm, *EN* (refer to *EN* in *a* and *c*). Both diffuse and punctate labeling forms are recognized in the narrow cytoplasmic areas around the nuclei, *n* (refer to *n* in *c*), as well as a wide cytoplasmic area indicated with a black arrowhead (refer to same area in *c*). In *e*, a strongly printed micrograph of the same negative as *d*, punctate labeling is recognizable in the wide cytoplasmic areas (*white arrowheads*) which were too bright in *d* for a clear visualization of the punctate

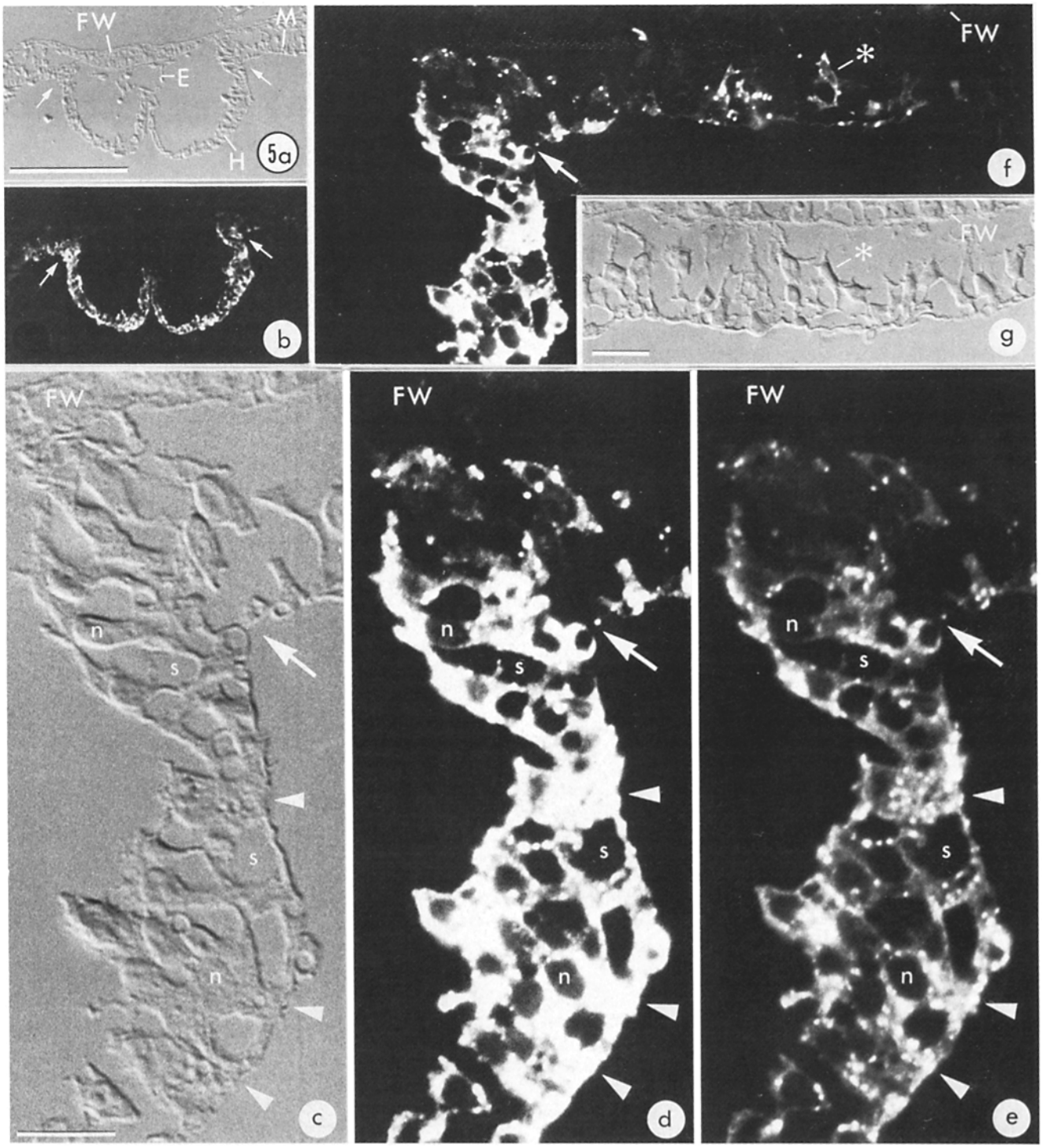
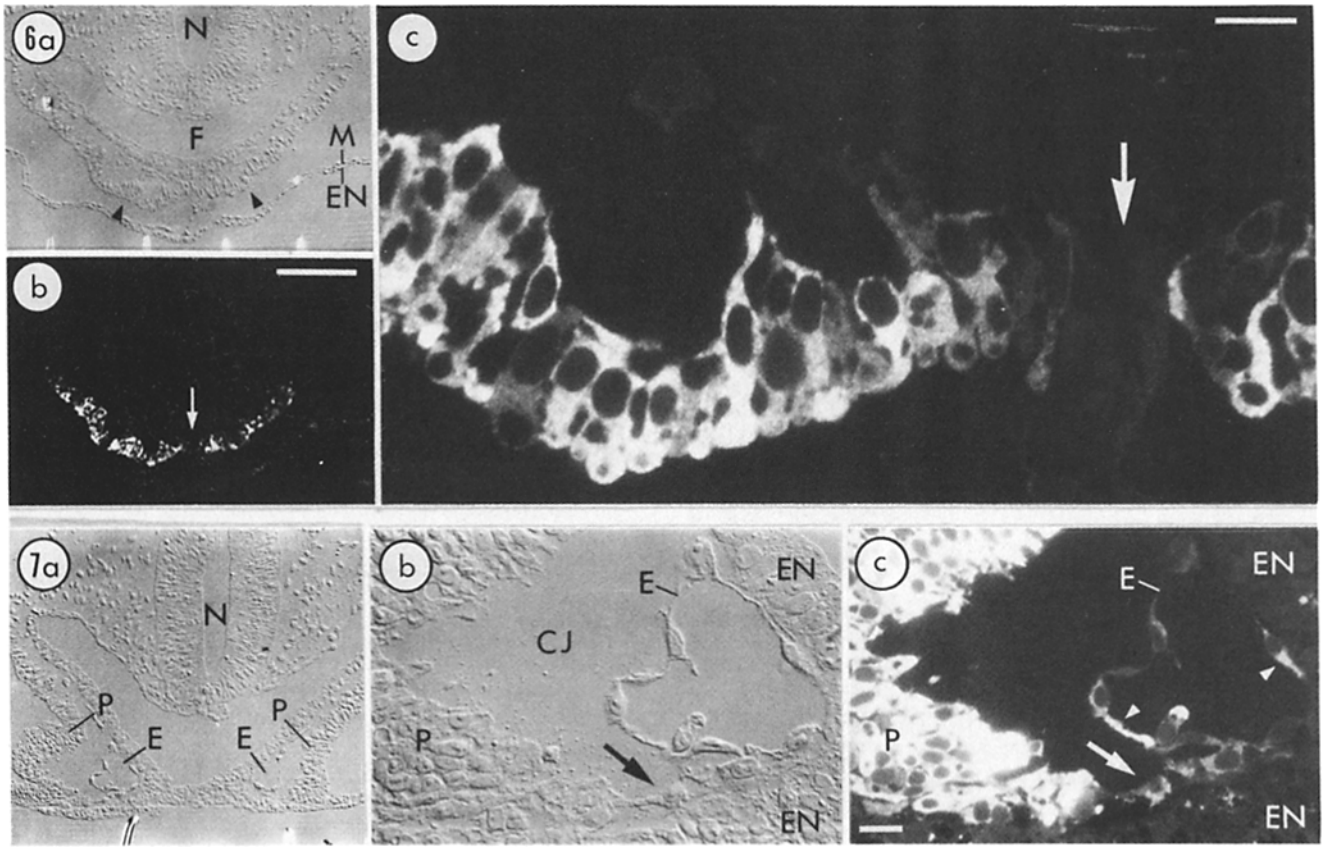


Figure 5. Heart region of a titin-labeled and resin-embedded 9 somite stage embryo in cross-section, observed in Nomarski optics (*a*, *c*, *g*) and in fluorescence optics (*b*, *d–f*). A portion of *a* is enlarged in *c*, and a corresponding portion of *b* is enlarged in *d* and *e*. A part of *b* is also enlarged in *f*, to a lower magnification than *c–e*, and a segment of *f* is shown in Nomarksi optics in *g*. The heart trough (*H*) is strongly positive for titin labeling, whereas the wall of the foregut (*FW*) is negative (compare *b* with *a*). In *d*, a lightly printed micrograph, titin labeling is seen to be clearly positive in the myocyte cytoplasm in the heart trough, particularly in wide areas indicated with arrowheads (refer to the same areas in *c*), whereas it is found to be negative in the myocyte nuclei (*n* in *c–e*), the extracellular spaces (*s* in *c–e*) as well as the foregut (*FW* in *a*, *c–e*). In *e*, a strongly printed micrograph of the same negative as *d*, both punctate and diffuse titin labeling patterns are recognized in the wide cytoplasmic areas indicated with arrowheads, which were too bright in *d* for a clear visualization of the punctate labeling. Positive labeling is found in the adjacent areas of mesoderm (*M* in *a*), for a considerable distance beyond the boundaries of the heart trough (*arrows* in *a–f*). This is seen particularly well in *f* and *g* (a titin-positive cell indicated with asterisks). The endocardium (*E* in *a*) is now found at the center of the heart trough. Bars in *a*, *c*, and *g*, 100, 10, and 10 μm, respectively.

pattern. Fluorescent spots in the ectoderm (*arrow* in *b*) represent autofluorescent, unidentified structures that occur throughout the early embryos. *N*, neural groove; *EC*, ectoderm; *M*, splanchnic mesoderm; *C*, coelom. Bars in *a* and *c*, 100 and 10 μm, respectively.



Figures 6 and 7. (Fig. 6, *a–c*) Cross-section of a myosin-labeled and resin-embedded 6 somite stage heart at a level near the cephalic end of the heart, observed in Nomarksi optics (*a*) and in fluorescence optics (*b* and *c*). Only the cardiac primodia (*arrowheads* in *a*) are seen to be positively labeled for myosin (adult ventricular myosin) in *b*. In *c*, an enlarged portion of *b*, the myosin labeling shows a certain variation among the different myocytes but it is uniform throughout the cytoplasm of each cell. The cardiac primodia are now closely apposed to each other but still separated by a thin area of the endoderm (*arrows* in *b* and *c*; see the whole mount preparation of a 6 somite stage heart in Fig. 9 *b*). *N*, neural groove; *F*, foregut; *M*, splanchnic mesoderm; *EN*, endoderm. Bars in *b* and *c*, 100 and 10 μm, respectively. (Fig. 7) Another cross-section of the 6 somite stage heart of Fig. 6 *a*, at a level more caudal than that of the section in Fig. 6 *a*. In this section, the endocardial primodia (*E* in *a*) are recognized (see that the primodia are fused into a single endocardium in the nine somite stage heart in Fig. 5 *a*). In *b*, an enlarged portion of *a*, the endothelial cells of the endocardial primodium (*E*) form a lumen and are separated from the cardiac primodium (*P*) by a wide space containing the cardiac jelly (*CJ*; refer to [14] for the cardiac jelly of this stage). In *c*, some of the endothelial cells (*arrowheads*) show a strong myosin labeling, and the cardiac primodium (*P*) and the endothelial cells appear to be connected by a bridge of cells which are positive for myosin labeling (*arrow*). *N*, neural groove; *EN*, endoderm. Bar in *c*, 10 μm.

to note that in the 9 somite stage, titin labeling was found not to be tightly restricted to the heart trough (two arrows in Fig. 5, *a* and *b* indicating the boundaries of the heart trough, and an arrow in Fig. 5, *c–f* indicating the right boundary). Indeed, titin labeling occurred for a considerable distance beyond the trough boundaries (see a mesodermal cell with a low but positive titin labeling, indicated with an asterisk in Fig. 5, *f* and *g*, a Nomarksi optics micrograph of a portion of Fig. 5 *f*). Nevertheless, the overall degree of labeling was seen to be abruptly reduced beyond the boundaries of the heart trough (Fig. 5, *b–d*, and *f*).

Immunofluorescent Labeling Patterns of Myosin and Actin in Sections

In an early stage such as the 5–6 somite stage, the cardiac primodia were already strongly labeled by the anti-adult ventricular myosin antibody, against the negative background of the rest of the embryo (Fig. 6, *a* and *b*). This was much earlier than had been previously studied (39). At high magnification, the myosin labeling was seen to occur throughout the cytoplasm of each myocardial cell in a uniform manner (Fig. 6 *c*), although a certain degree of variation in the labeling intensity was observed among the cells of this early stage. It was rather surprising to find that the endothelial cells of the endocardium in the 5–6 somite stage showed a low but significant labeling for cardiac muscle myosin (Figs. 7, *a–c*) as well. In the stages later than the 9 somite stage, such labeling was not observed (not shown).

When the heart trough was formed at the 8 somite stage, the cells became uniform in the intensity of myosin labeling and specific myosin labeling was seen to occur, for a short distance, beyond the physical boundaries of the heart trough (not shown), as in the case of the titin labeling (see Fig. 5, *g* and *f*).

When an antibody that was reactive with all isoforms of actin was used for labeling, the cells comprising the cardiac

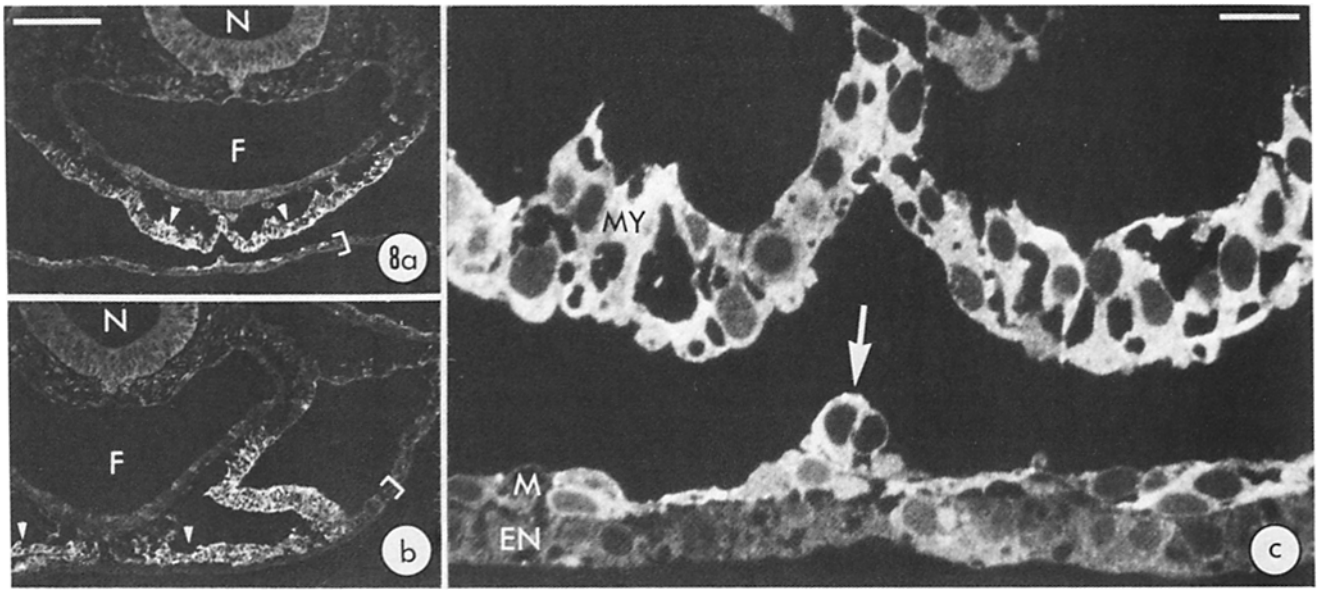


Figure 8. Cross-sections of an actin-labeled and resin-embedded 7 somite stage embryo seen in immunofluorescence optics, at a level near the cephalic end of the heart in *a* and at a more caudal level in *b*. The cardiac primodia (*arrowheads*) are fused at the level of *a* but not yet at the level of *b*. Actin labeling occurs throughout the embryo but more strongly in the cardiac primordia at the levels of both *a* and *b*. In *c*, an enlarged portion of *a*, actin labeling is seen to occur uniformly throughout the cytoplasm of the myocardial cells (*MY*). A part of the splanchnic mesoderm (*arrow*) which closely faces the myocardial wall is labeled for actin as strongly as the myocardial wall. *N*, neural tube; *F*, foregut; *M*, splanchnic mesoderm; *EN*, endoderm. Brackets in *a* and *b*, superimposed layers of mesoderm and endoderm. Bars in *a* and *c*, 100 and 10 μm, respectively.

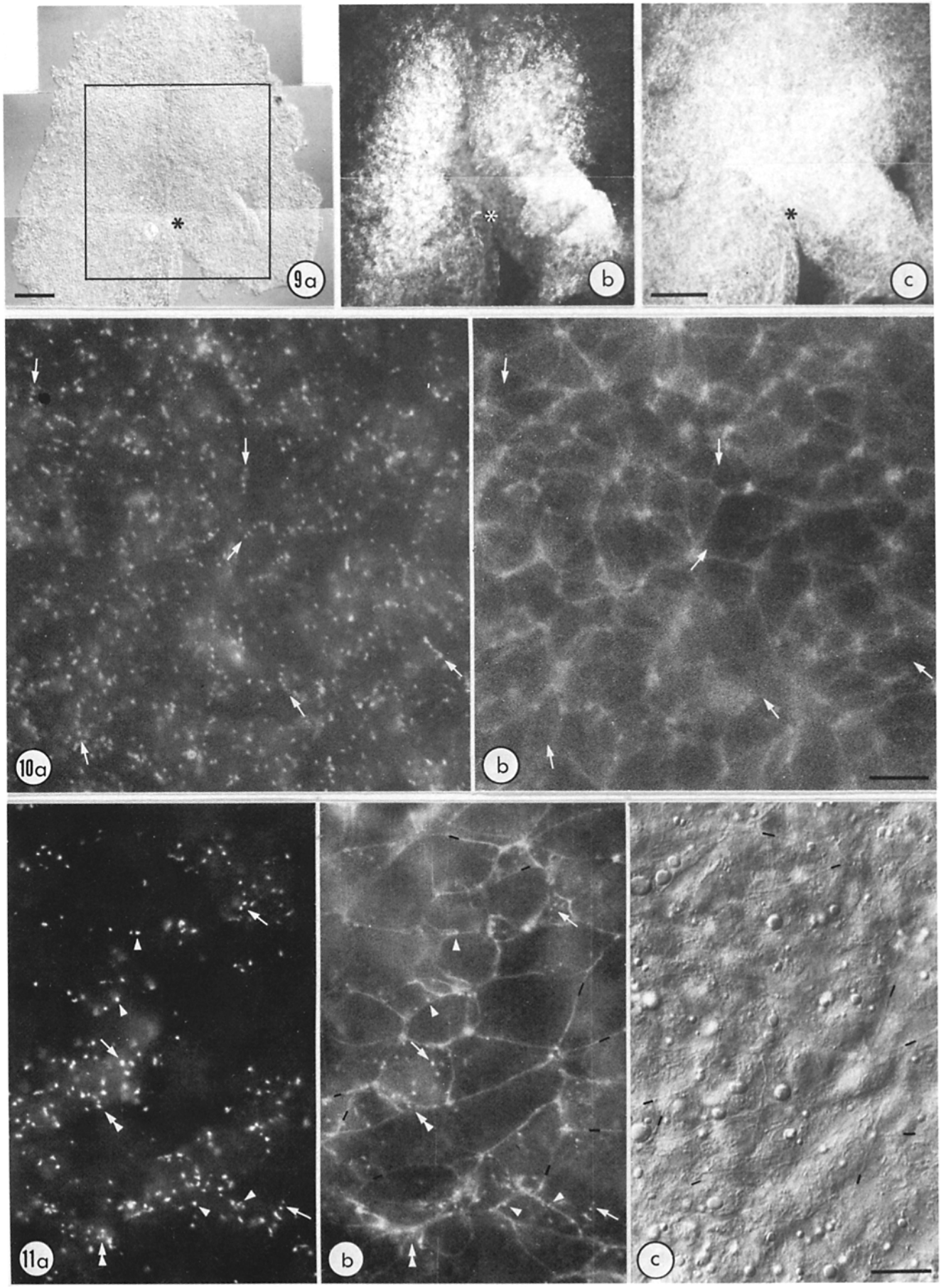
primodia were not clearly distinguishable from the rest of the embryo in the 5 somite stage but became more strongly labeled than the rest of the embryo in the 7 somite stage (Fig. 8, *a* and *b*) and much more strongly labeled in the 9 somite stage (not shown). At high magnification, the labeling was seen to occur uniformly throughout the cytoplasm (Fig. 8 *c*) in a manner similar to that seen for the myosin labeling (Fig. 6 *c*). In the 7 somite stage embryo shown in Fig. 8, *a–c*, the part of the splanchnic mesoderm which was located close to the newly fused portion of the myocardium was also more strongly labeled than the rest of embryo (arrow in Fig. 8 *c*). Quite probably, this portion had been a part of the cardiac primodia before the occurrence of the fusion.

All of the control labeling experiments using normal rabbit IgG in place of the antibodies gave rise to negative results (not shown). The clearly different labeling patterns of titin and myosin or actin also served as controls for each other.

Labeling Patterns of Titin and F-Actin in Whole Mount Preparations

In the whole mount preparations of the thoracic portions of 6 somite stage embryos (refer to Materials and Methods for the preparative method), the cardiac primodia were recognized as two titin-labeled regions that were still separated from each other along the embryonic midline (Fig. 9, *a* and *b*; refer also to Fig. 6 for the structure of the thoracic portion of the 6 somite stage heart). F-Actin labeling with NBD-phallacidin, on the other hand, occurred throughout the embryo (compare Fig. 9, *b* and *c*). At high magnification, titin spots were recognized as singlets (Fig. 10 *a*), not as doublets as seen in relaxed myofibrils of adult striated muscles (Fig. 3). Most of the titin spots were individually separated but there were occasional rows of several spots that were aligned in variable intervals of 1–1.7 μm (arrows in Fig. 10 *a*). NBD-Phallacidin labeling revealed the presence of F-actin meshworks which appeared to occur along the lateral cell borders but no F-actin containing filamentous structures were observed at the sites of the aligned titin spots (arrows in Fig. 10 *b*, indicating the sites corresponding to the locations of rows of titin spots in Fig. 10 *a*).

In the 4–5 somite stages, the thick central area of the cardiac primodium consists of two or more layers of cells (14), while the thin peripheral areas consist of one or two cell layers. To study the relationship of F-actin labeling with the cell borders and with the sites of titin spots more thoroughly than just described, we examined the peripheral areas of the 5 somite stage primordia more closely. It then became quite clear that the F-actin meshworks were indeed present along the lateral cell borders (compare Fig. 11, *b* and *c*). F-Actin was quite likely present beneath the entire cell surface but it was clearly recognizable only along the lateral cell borders that lie perpendicular or oblique (see areas indicated with double arrowheads in Fig. 11 *b*) to the glass slide surface. When the filter system of the light microscope was adjusted so that the rhodamine signals of titin labeling partially leaked into and were superimposed with the NBD signals of F-actin labeling, some of the titin spots were recognized along the lateral cell borders visualized by the NBD signals (arrowheads along the perpendicular borders and double arrowheads along the oblique borders in Fig. 11, *a* and *b*). Other titin spots that were located away from the lateral cell borders gave an opportunity to determine whether or not F-actin–containing filamentous structures similar to stress fibers of non-muscle cells were present in association with the titin


9a
b
c
10a
b
11a
b
c

spots. Such examinations provided a clearly negative answer (arrows in Fig. 11, *a* and *b*). It is quite possible that some or all of such titin spots that were seen as being away from the lateral cell borders were in fact closely associated with the top and bottom surfaces of the cells, whose positions could not be clearly ascertained in the light microscope. This possibility was not further investigated in the present study.

Incorporation of Titin Spots into Nascent Myofibrils

In the whole mount preparations of the 8 somite stage hearts, most of the titin labeling was seen as individual spots (Fig. 12 *a*), and F-actin labeling, as visualized by NBD-phallacidin labeling, was found to occur almost totally along the cell borders (Fig. 12 *b*).

In the 10 somite stage, a considerable number of rows of periodically arranged titin spots were seen to coexist with individually separated titin spots (pairs of brackets in Fig. 12 *c*). Actin filaments were observed at the sites of these rows (brackets in Fig. 12 *d*), but not at the individual titin spots. Because the first myofibrils are known to be formed at this stage (8), such rows of titin spots and actin filaments quite likely represent parts of nascent myofibrils. F-Actin labeling did not show the sarcomeric periodicity as clearly as titin labeling, in agreement with a past finding (7). The number of nascent myofibrils showed a local variation which agreed with past morphological observations (8, 14).

In the 11 somite stage, that is, only ~2 h after the 10 somite stage, the great majority of titin spots were found as parts of myofibrils (Fig. 12, *e* and *f*), although in some areas individually separated titin spots were still recognizable (not shown). These observations strongly suggested that individual titin spots generated in the premyofibril stages incorporate into newly arising myofibrils. At this time, F-actin labeling now reveals a sarcomeric periodicity in most myofibrils (Fig. 12 *f*).

It is of interest to note that in the 11 somite stage, some myofibrils were already branched (arrowheads in Figs. 12, *e* and *f*).

Presence of Myosin in the First Myofibrils

We reported above the coincidental occurrence of the periodic alignment of titin spots and the formation of F-actin filaments. In the present investigation, we were not able to study by double immunolabeling the question of whether or not the periodic alignment of titin spots in the first myofibrils and the incorporation of myosin into the first myofibrils occur coincidentally, because both of our anti-titin and anti-myosin antibodies were raised in rabbits. However, taking advantage of the fact that F-actin labeling with NBD-phallacidin does not show a sarcomeric periodicity in the earliest stage of myofibril development, we carried out a double-labeling study to compare the labeling patterns of F-actin and myosin in the nascent myofibrils.

In the 10 somite stage, the concentration of monomeric myosin in the myocytic cytoplasm was still high and myosin labeling of nascent myofibrils was less distinct than F-actin labeling (compare Fig. 13, *a* and *b*). Nevertheless, it was possible to observe three different combinations of F-actin and myosin labeling patterns. The first one was the apparent absence of a periodic pattern in both F-actin and myosin labeling (*x* in Fig. 13, *a* and *b*). The second was the apparent absence of a periodic pattern in the F-actin labeling but the presence of a periodic pattern in the myosin labeling (*y* in Fig. 13, *a* and *b*). The third was the presence of a periodic pattern in both the F-actin and myosin labeling (*z* in Fig. 13, *a* and *b*). These observations indicated that myosin is indeed incorporated into myofibrils in their earliest stage of development and also that the sarcomeric periodicity in nascent myofibrils is visualized by the myosin labeling in an earlier stage of myofibrillar development than by the F-actin labeling. The sarcomeric periodicity became recognizable by the myosin labeling when it reached a length of 1.6–1.7 μm (dots in Fig. 13 *b*).

Appearance of Titin Doublets at the Time of the First Myofibril Formation

In the stages earlier than the 7 somite stage, all the titin spots were seen as singlets. In the 8–10 somite stage, that is, in the stage of the formation of the first myofibrils, the majority of titin spots were still seen as singlets (Fig. 12 *c*) but some of them were now seen as doublets for the first time (arrowheads and an arrow in Fig. 14; see also Fig. 12 *e* of the 11 somite stage). The center-to-center distance of the components of the narrowest doublets was measured as ~0.3–0.4

Figures 9–11. (Fig. 9, *a–c*) Whole mount preparation of the thoracic portion of a titin- and F-actin (phallacidin) labeled 6 somite stage embryo, mildly compressed between the glass slide and cover slip after the removal of the neural groove and the foregut wall on the dorsal side. It is observed in Nomarski optics (*a*), in rhodamine fluorescence optics for titin labeling (*b*; rectangle-marked area of *a*), and in NBD fluorescence optics for F-actin (*c*; the same area as *b*). Titin labeling is restricted to two areas in *b*, whereas F-actin labeling occurs throughout the embryo in *c*. The variation in the intensity of F-actin labeling appears to be related to local variations in specimen thickness. The position of the foregut portal, which is wrinkled in this whole mount preparation, is indicated with asterisks. Bars, 100 μm. (Fig. 10, *a* and *b*) A portion of Fig. 9 *b* is shown in *a* and the corresponding portion of Fig. 9 *c* in *b*, at high magnification. The punctate feature of titin labeling is obvious in *a*, whereas F-actin labeling forms a network along the lateral cell borders in *b* (see Fig. 11, *b* and *c*, and legend). In *a*, rows of several titin spots aligned in variable intervals of 1–1.7 μm are recognized (*arrows*). In *b*, no F-actin containing structures are found at the locations corresponding to these rows (*arrows*). Bar, 10 μm. (Fig. 11, *a–c*) A peripheral area of a 5 somite stage cardiac primordium double labeled for titin and F-actin. It is observed in rhodamine fluorescence optics for titin labeling (*a*), in NBD fluorescence optics for F-actin labeling (*b*), and in Nomarksi optics (*c*). The network of F-actin lines in *b* is found to be coincident with the network of the lateral cell boundaries in *c*, as indicated by short line segments at the corresponding locations in *b* and *c*. The filter system of the fluorescence microscope was adjusted so that the punctate titin signals seen in *a* are partially leaked into and superimposed with the F-actin signals seen in *b*. The titin spots that are located along the lateral and oblique cell borders are indicated with arrowheads and double-arrowheads, respectively, in *a* and *b*. At the locations of the titin spots that are away from the lateral and oblique cell borders (*arrows* in *a* and *b*), no F-actin-containing filamentous structures are recognized (*arrows* in *b*). Bar, 10 μm.

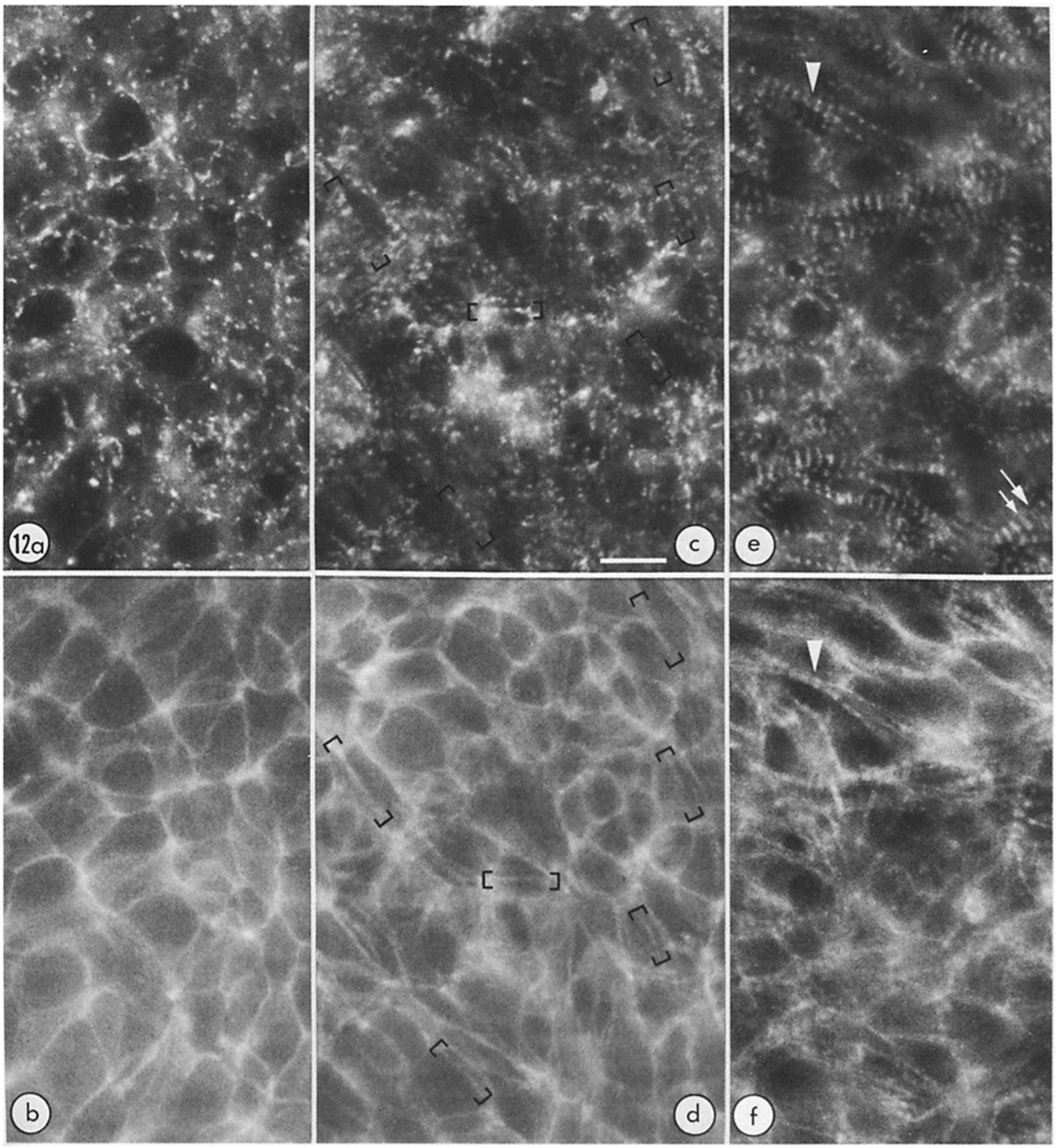


Figure 12. Parts of whole mount preparations of 8 (*a* and *b*), 10 (*c* and *d*), and 11 (*e* and *f*) somite stage hearts, double labeled for titin (rhodamine, *a*, *c*, *e*) and for F-actin (NBD-phallacidin, *b*, *d*, *f*). Titin labeling is seen almost entirely as individual spots in the 8 somite stage (*a*), partly as individual spots and partly as rows of periodically aligned spots of presumptive nascent myofibrils (pairs of brackets) (*c*), and almost entirely as rows of periodically aligned spots or lines in the 11 somite stage (*e*). F-Actin labeling of the same fields, on the other hand, is seen mostly as subplasmalemmal labeling in the 8 somite stage (*b*; compare with Figs. 4b and 5b of the previous stages), both as subplasmalemmal labeling and as actin filaments of presumptive myofibrils in the 10 somite stage (d; compare pairs of brackets in *c* and *d*), and mostly as actin filaments of myofibrils in the 11 somite stage (*f*). A myofibril in the 11 somite stage heart appears to be branched (*arrowheads* in *e* and *f*). In the 10 somite stage, F-actin labeling does not show the sarcomeric periodicity of nascent myofibrils as clearly as titin labeling (compare *c* and *d*). At the right lower corner of *e*, the components of a wide doublet (*large arrow*) and those of a narrow one (*small arrow*) are seen to be separated by ~0.8 and 0.45 μm, respectively. Bar, 10 μm.

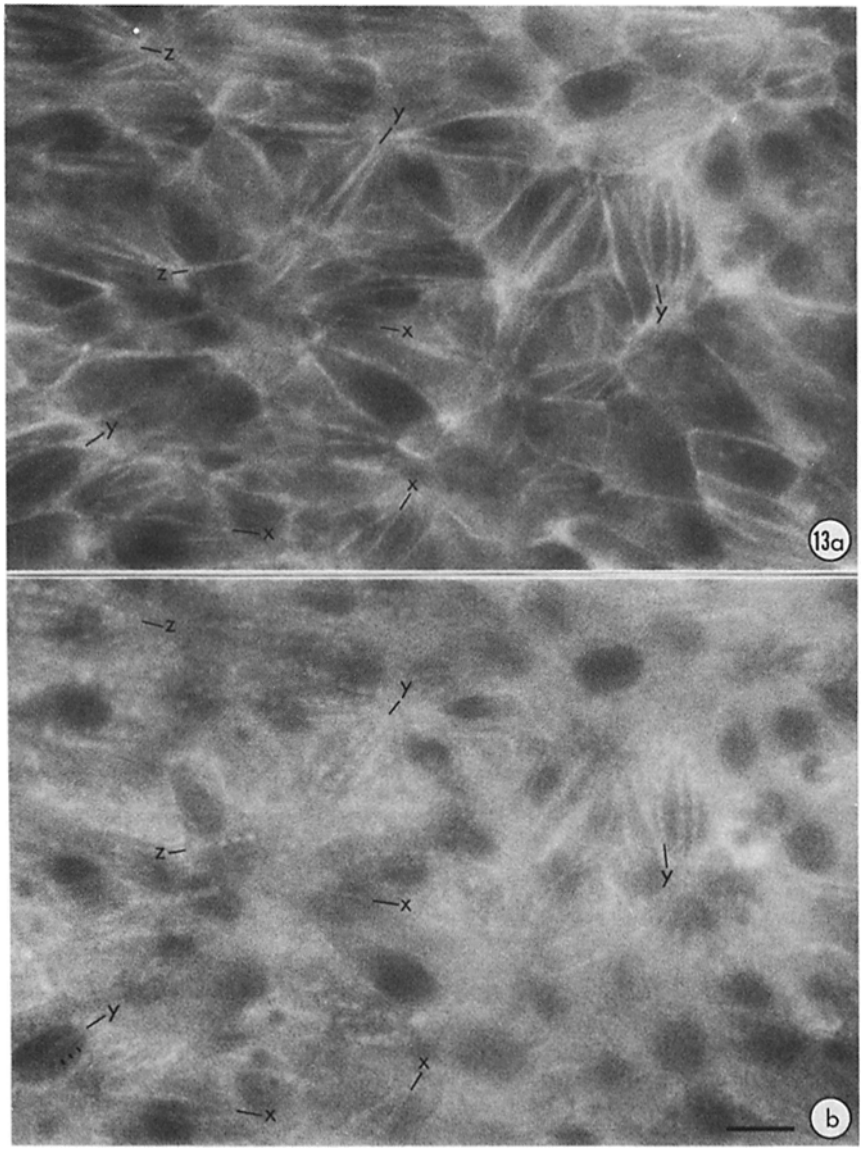


Figure 13. Part of a whole mount preparation of a 10 somite stage heart, double labeled for F-actin (NBD-phallacidin; *a*) and myosin (rhodamine; *b*). Myofibrils are seen less clearly in *b* than in *a*, due to the presence of a high concentration of monomeric myosin in the myocytes of this stage, but the majority of myofibrils that are recognized by F-actin labeling are observed by myosin labeling. In *x*-marked myofibrils, the sarcomeric periodicity is revealed neither by F-actin labeling nor by myosin labeling. In *y*-marked myofibrils, the periodicity is recognized by myosin labeling (*b*) but not by F-actin labeling (*a*). In *z*-marked myofibrils, the periodicity is recognized by both F-actin labeling and myosin labeling. Dots mark the positions of the A bands in myofibrils that show the discernible, shortest periodicity, 1.6–1.7 μm. Bar, 8 μm.

μm (arrow in Fig. 14), nearly the resolution limit of the light microscope. In fact, the images of such doublets easily changed from doublets to singlets by a slight shift of focus and were rather difficult to record on the photographic film. By the 11 somite stage, some of the titin doublets already showed a separation of ~0.8 μm (large arrow in Fig. 12 *e*), similar to that which was often found for the titin doublets of relaxed, mature myofibrils (Fig. 3).

Discussion

We reported above that titin is present in a punctate state and also in a diffuse form throughout the cytoplasm of myocytes, before and at the time of the formation of the first myofibrils. The punctate and diffuse states quite probably represent an aggregated state of several titin molecules and a dispersed state of individual titin molecules, respectively. It is very unlikely that individual titin molecules, which measure only ~4 nm in width (17, 29, 37), have a sufficient number of epitopes to be detected as distinct immunofluorescent spots by such antibodies as ours which react only with the A-I junction portion of titin in mature myofibrils. Thus, we interpret the present observations to reveal that titin spots, that is, aggregates of several titin molecules, are present in a large number in cardiac myocytes before the formation of the first myofibrils.

Recently, Hill and co-workers (7) reported a tight temporal and topological linkage between titin and myosin throughout skeletal myofibrillogenesis in vitro. Obviously, in this aspect, cardiac myofibrillogenesis is quite different from the skeletal process. This difference might be directly related to the necessity for the assembly of the first myofibrils in the embryonic heart to occur at a rapid rate to meet the requirement that the embryonic heart should become functional within a very short period of time. In the chick embryo, only 12 h separate the onset of myofibril assembly from the initiation of unidirectional blood flow through the heart (20, 24). The presence of a large number of titin spots at the time of the onset of myofibril assembly might be essential for the fast assembly of myofibrils. We showed that in the initial stages of

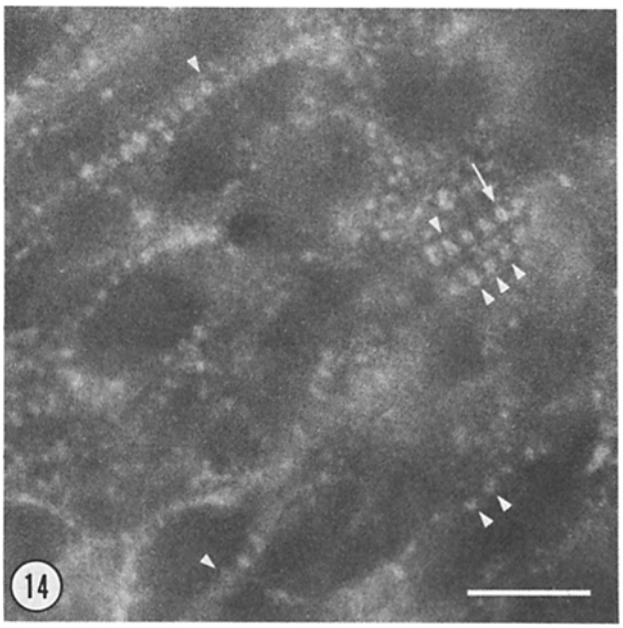


Figure 14. Part of a whole mount preparation of a titin-labeled, 10 somite stage heart. Titin doublets are indicated by arrowheads and a particularly clear one by an arrow. The components of the latter are separated by a distance of ~0.35 μm. Bar, 10 μm.

myofibril formation, that is, in the 9–11 somite stages, the number of individually separated titin spots is rapidly reduced, as the number of periodically aligned titin spots in newly formed myofibrils increases. This fact strongly suggests that individual titin spots generated in the premyofibril stages are incorporated into newly arising myofibrils.

In skeletal myofibrillogenesis, myofibrils are aligned in one direction, that is, in parallel with the asymmetry axis of the cells from the time they are first formed in postmitotic myoblasts (7). On the other hand, myofibrils in cardiac myocytes are arranged in a much more complex manner. They branch and are interconnected through intercellular junctions, fasciae adherentes, to form a complex, three-dimensional organization. This is true in the earliest functional embryonic heart (2; Tokuyasu, K. T., and P. A. Maher, our unpublished observation) as well as in the mature heart. The numerous titin spots formed in the premyofibril stages could contribute to the generation of the three-dimensionality of the cardiac myofibril organization. It was observed in the present study that rows of several titin spots aligned in variable intervals are occasionally present even in the premyofibril stages (Fig. 10 *a*) but the linear alignment of titin spots into a periodic pattern occurs only at the time of formation of the first myofibrils (Fig. 12 *c*). In other words, titin spots appear to be brought into a periodic alignment only in the presence of other components of the myofibrils. Thus, a cooperative association of newly forming myofilaments and titin spots is likely to occur at the time of myofibril formation. In fact, ultrastructural studies revealed that the formation of thick filaments slightly precedes the onset of the formation of the first myofibrils (8). These facts suggest that titin spots may provide a source of initiation sites for myofibril assembly.

In mature myofibrils, each half of an immunolabeled titin doublet is localized at the A-I junction and aligned in parallel to the Z line present at the center of the doublet (32, 33, 36). The titin molecules are believed to run parallel to the direction of the myofibrils (32, 33). In other words, once the titin doublets are formed, not only their location but also their direction is fixed in relation to the myofibrils. Whether titin doublets are formed before or only at the time of the first myofibril assembly is an intriguing question. We found that when titin doublets are first seen at the time of the first myofibril assembly, the center-to-center distance of the components of the narrowest doublet is close to the resolution limit of the light microscope (Fig. 14). We also found that titin doublets are seen not in separation from but, always as parts of, newly forming myofibrils in the 8–10 somite stages. These facts suggest that the structural or organizational changes of titin singlets into doublets might be linked to the formation of myofibrils. However, it is still possible that undiscernible doublets of submicroscopic separations exist in the premyofibril stages and become discernible only when they are incorporated into myofibrils. The question of whether or not the titin spots have a directionality before being incorporated into myofibrils is related to the question of whether or not the distribution of titin spots in the premyofibril stages directly contribute to the generation of the three-dimensional networks of myofibrils.

We found that sparsely distributed rows of titin spots aligned in variable intervals of about 1–1.7 μm are present in as early a stage as the 6 somite stage (Fig. 10 *a*) and that they are not associated with actin-containing filaments (Fig. 10 *b*). These observations raise the questions of how titin spots are aligned into such rows and whether or not such rows have any morphogenic significance. A related question is whether, in the premyofibril stages, the entire titin molecule is located within the immunolabeled titin spots or whether the major portion of the molecule is in the adjacent, unlabeled regions. To study these questions, it would be important to produce antibodies that label multiple segments of the titin molecules. At the present time, the distribution of titin molecules as well as the internal organization of titin spots can be studied only through immunocytochemical approaches, because no ultrastructural entity of titin in situ has so far been established.

Hill and co-workers noted in their study of skeletal myofibrillogenesis in vitro (7) that "though clearly distributed in a sarcomeric periodicity . . . , the titin-positive bands in nascent myofibrils rarely form a doublet bordering the region where the Z band should be assembling. The significance of this observation regarding either the assembly of the myofibrils or possible contraction is not clear." A possible explanation for this observation is, as they pointed out, that even in mature myotubes, "in moderately contracted myofibrils the distance between adjacent A bands becomes so narrow that the two fluorescent lines appear as a single band in the fluorescence microscope." Another possible explanation is that the titin spots in skeletal muscle are initially generated as singlets in a manner similar to that seen in cardiac myocytes and become doublets only after their incorporation into myofibrils and as myofibrils mature. In the skeletal postmitotic myoblasts, however, the generation of the titin spots and the formation of myofibrils apparently occur nearly simultaneously. Then, it would not be easy to observe the titin spots unaccompanied by other myofibrillar components.

We observed that the sarcomeric periodicity in the earliest stage of myofibril development is clearly visualized by titin

labeling but not by F-actin labeling with NBD-phallacidin (Figs. 12, *c* and *d*). Similar findings were reported in vitro skeletal myofibrillogenesis by Hill and co-workers (7). If the thin filaments of the first myofibrils had the same length as those of mature myofibrils, an overlapping of thin filaments over a 0.3-μm span would be expected to occur at the midlevel of the nascent 1.5-μm-long sarcomeres (refer to Figs. 2 and 3 of Part II of this series for the shortest sarcomeres) and would be detected as a positive line at the midlevel of the nascent sarcomere when labeled for F-actin. The presence of such lines, however, has neither been reported previously nor observed by us. Whether this is due to the failure of observing such a narrow line or whether the thin filaments in the nascent sarcomere may not have yet attained the length of those of mature myofibrils is not clear at the present time.

The labeling of titin and myosin was not tightly restricted to the heart trough of the 8–9 somite stage but occurred for a short distance beyond the physical boundaries of the trough (Fig. 5 *b* and our unpublished data). In fact, actin labeling was also seen to occur similarly beyond the boundaries of the 9 somite stage trough (not shown). These facts suggested that in the development of the heart, a margin of error is built into cardiac morphogenesis, a process which involves the fusion of the two initially widely separated regions of cardiac primodia.

In the present study, the endothelial cells of the endocardium in the 5-6 somite stage embryos were positively labeled by the cardiac myosin-specific antibody. This observation suggested that the endothelial cells of the heart are not only derived from the splanchnic mesoderm, as past morphological studies reported (14, 30), but in fact closely related to the myocardium in origin. This might be developmentally important in assuring that the endocardium is formed at a location close to the cardiac primodia.

We wish to thank Mrs. Margie Adams and Mrs. Rebecca Windle for their excellent technical assistance, and to Dr. Xing Xing Luo on visit from Wuhan University for her special assistance. We also thank Dr. S. J. Singer and Dr. Sidney S. Sobin for their invaluable advice and critical reading of the manuscript. This paper was considerably improved by the advice of the reviewers and the monitoring editor, Dr. Donald A. Fischman.

This study was supported by grants HL-30282 to Dr. Tokuyasu, CA-38006 to Dr. Maher, and GM-15971 to Dr. S. J. Singer from the National Institutes of Health.

Received for publication 11 May 1987, and in revised form 28 August 1987.

References

1. Chaco, K. J. 1974. Observations on the ultrastructure of developing myocardium of rat embryos. *J. Morphol.* 150:681–710.
2. Challice, C. E., and S. Viragh. 1973. The embryonic development of the mammalian heart. *In* Ultrastructure of the Mammalian Heart. C. E. Challice and S. Viragh, editors. Academic Press, New York. 91–126.
3. Dlugosz, A. A., P. B. Antin, V. P. Nachmias, and H. Holtzer. 1984. The relationship between stress fiber-like structures and nascent myofibrils in cultured cardiac myocytes. *J. Cell Biol.* 99:2268–2278.
4. Fujii, S., A. Hirata, and K. Kamino. 1981. Optical indications of pacemaker potential and rhythm generation in early embryonic chick heart. *J. Physiol. (Lond.).* 312:253–263.
5. Gruen, L. C., N. L. King, L. Kurth, and I. J. McKenzie. 1982. Studies on the structure of connectin in muscle. *Int. J. Pept. Protein Res.* 20:401–407.
6. Hill, C. S., and K. Weber. 1986. Monoclonal antibodies distinguish titins from heart and skeletal muscle. *J. Cell Biol.* 102:1099–1108.
7. Hill, C. S., S. Duran, Z. Ling, K. Weber, and H. Holtzer. 1986. Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. *J. Cell Biol.* 103:2185–2196.
8. Hiruma, T., and R. Hirakow. 1985. An ultrastructural topographic study on myofibrillogenesis in the heart of the chick embryo during pulsation onset period. *Anat. Embryol.* 172:325–329.
9. Jorgensen, A. O., and R. Bashin. 1984. Temporal appearance and disappearance of the Ca^{2+} + Mg^{2+} ATPase of the sarcoplasmic reticulum in developing chick myocardium as determined in immunofluorescence labeling. *Dev. Biol.* 106:156–165.
10. Karr, T. L., and B. M. Alberts. 1986. Organization of the cytoskeleton in early Drosophila embryos. *J. Cell Biol.* 102:1494–1509.
11. Kielley, W. W., and W. F. Harrington, 1959. A model for the myosin molecule. *Biochem. Biophys. Acta.* 41:401–421.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
13. Lim, S.-S., M. N. Woodroofe, and L. F. Lemanski. 1983. An analysis of contractile proteins in developing chick heart by SDS polyacrylamide gel electrophoresis and electron microscopy. *J. Embryol. Exp. Morphol.* 77:1–14.
14. Manasek, F. J. 1968. Embryonic development of the heart. I. A light and electron microscopic study of myocardial development in the early chick embryo. *J. Morphol.* 125:329–366.
15. Manasek, F. J. 1970. Histogenesis of the embryonic myocardium. *Am. J. Cardiol.* 25:149–168.
16. Markwald, R. R. 1973. Distribution and relationship of precursor Z material to organizing myofibrillar bundles in embryonic rat and hamster ventricular myocytes. *J. Mol. Cell. Cardiol.* 5:341–350.
17. Maruyama, K., S. Kimura, H. Yoshidomi, H. Sawada, and M. Kikuchi. 1984. Molecular size and shape of β-connectin, an elastic protein of striated muscle. *J. Biochem. (Tokyo).* 95:1423–1433.
18. Maruyama, K., T. Yoshioka, H. Higuchi, K. Ohashi, S. Kimura, and R. Natori. 1985. Connectin filaments link thick filaments and Z lines in frog skeletal muscle as revealed by immunoelectron microscopy. *J. Cell Biol.* 101:2167–2172.
19. Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *Methods Cell Biol.* 24:271–289.
20. Patten, B. M. 1927. The Early Embryology of the Chick. Blakiston Co., Philadelphia. 1–228.
21. Peng, H. B., J. J. Wolosewick, and P.-C. Cheng. 1981. The development of myofibrils in cultured muscle cells: a whole mount and thin section electron microscopic study. *Dev. Biol.* 88:121–126.

21a. Rieder, C. L., and S. S. Bowser. 1985. Correlative immunofluorescence and electron microscopy on the same section of Epon-embedded material. *J. Histochem. Cytochem.* 33:165–171.

22. Rumyantsev, P. P. 1978. Interrelations of the proliferation and differentiation process during cardiac myogenesis and regeneration. *Int. Rev. Cytol.* 51:186–273.
23. Sanger, J. M., B. Mittel, M. Pochapin, and J. W. Sanger. 1986. Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. *J. Cell Biol.* 102:2053–2066.
24. Sissman, N. J. 1970. Developmental landmarks in cardiac morphogenesis: comparative chronology. *Am. J. Cardiol.* 25:141–148.
25. Ternynck, T., and S. Avrameas. 1976. Polymerization and immobilization of proteins using ethylchloroformate and glutaraldehyde. *Scand. J. Immunol.* 3(Suppl.):29–35.
26. Tokuyasu, K. T. 1986. Application of cryoultramicrotomy to immunocytochemistry. *J. Microsc. (Oxf.).* 143:139–149.
27. Tokuyasu, K. T., P. A. Maher, and S. J. Singer. 1984. Distributions of vimentin and desmin in developing chick myotubes *in vivo*. *J. Cell Biol.* 98:1961–1972.
28. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
29. Trinick, J., P. Knight, and A. Whiting. 1984. Purification and properties of native titin. *J. Mol. Biol.* 180:331–356.
30. Viragh, S., and C. E. Challice. 1973. Origin and differentiation of cardiac muscle cells in the mouse. *J. Ultrastruct. Res.* 42:1–24.
31. Wang, K. 1982. Purification of titin and nebulin. *Methods Enzymol.* 85: 264–274.
32. Wang, K. 1982. Myofilamentous and myofibrillar connections: role of titin, nebulin, and intermediate filaments. *In* Muscle Development: Molecular and Cellular Control. M. L. Pearson and H. F. Epstein, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 439–452.
33. Wang, K. 1985. Sarcomere-associated cytoskeletal lattices in striated muscle. *In* Cell and Muscle Motility. J. W. Shay, editor. Plenum Publishing Corp., New York. 6:315–369.
34. Wang, S.-M., and M. L. Greaser. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. *J. Muscle Res. Cell Motil.* 6:293–312.
35. Wang, K., and R. Ramirez-Mitchell. 1979. Titin: possible candidate as components of putative longitudinal filaments in striated muscle. *J. Cell Biol.* 83:389a. (Abstr.)
36. Wang, K., J. McClure, and A. Tu. 1979. Titin: major myofibrillar components of striated muscle. *Proc. Natl. Acad. Sci. USA.* 76:3698–3702.
37. Wang, K., R. Ramirez-Mitchell, and D. Palter. 1984. Titin is an extraordinarily long, flexible and slender myofibrillar protein. *Proc. Natl. Acad. Sci. USA.* 81:3685–3689.
38. Wang, S.-M., E. Schultz, and M. L. Greaser. 1984. Immunofluorescence studies with a monoclonal anti-titin antibody on skeletal muscle myofibrillogenesis. *J. Cell Biol.* 99:436a. (Abstr.)
39. Zhang, Y., S. A. Shafiq, and D. Bader. 1986. Detection of a ventricular-specific myosin heavy chain in adult and developing chicken heart. *J. Cell Biol.* 102:1480–1484.