Immunocytochemical Studies of Cardiac Myofibrillogenesis in Early Chick Embryos. III. Generation of Fasciae Adherentes and Costameres

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Abstract. To study whether the first myofibrils are separate from or firmly bound to the myocytic cell membranes, whole mount preparations of 6–12-somite-stage chick embryonic hearts were examined by fluorescence microscopy after double labeling with antibodies to vinculin (fluorescein-conjugated) and rhodamine-phalloidin, or with antibodies to titin (rhodamine-conjugated) and nitrobenz-oxadiazole–phallacidin. When a small number of myofibrils appeared for the first time at the nine somite stage, most of them were already bound to the cell membranes through zonulae adherentes, fasciae adherentes, or costameres. In the outer of the two myocardial cell layers, in which the myocytes were closely in contact with each other along polygonal boundaries, fasciae adherentes and costameres developed at the boundaries, apparently by conversion of preexisting zonulae adherentes. On the other hand, in the inner cell layer, in which myocytes were more loosely associated with each other, both costameres and fasciae adherentes appeared to develop de novo, the former in association with the inner surface of the myocardial wall and the latter at the intercellular boundaries. The myofibrillar tracks in the inner layer followed long and smooth courses and were as a whole aligned in the circumferential direction of the tubular heart wall from the earliest stage of myofibril formation. Those in the outer layer were arranged in a pattern of two- or three-dimensional networks in the 9–10 somite stage, although many myofibrils were also circumferentially directed. The fact that the majority of the first myofibrils were already bound to the cell membranes in a directed manner suggests that myocytes at the earliest stage of myofibril formation are endowed with spatial information that directs the organization of nascent myofibrils. It is proposed that the myocyte cell membranes perform an essential role in cardiac myofibrillogenesis.

UNIDIRECTIONAL blood flow through the embryonic heart begins shortly after the initiation of myofibril formation. In the chick embryo, the time interval between the two events is only ~12 h from the 9 somite stage to the 16–17 somite stage (13, 20, 23). During such a short period of time, myofibrils must form at a rapid rate and simultaneously arrange into a highly organized network that traverses through the myocytes and is capable of the synchronized contraction which produces unidirectional blood flow. A widely accepted theory as to how nascent myofibrils are arranged into this complex, functional network is that the myofibrils are initially free from the cell membrane or bound to it only at one end, that they are then aligned in the direction of stress that is created in the myocardial wall as the embryonic heart undergoes a transformation process called looping, and that only after the completion of the alignment are myofibrils bound at both their ends to the cell membrane (15, 17). If this were the case, a simultaneous elongation of myocytes in the direction of the stress should be expected to occur, since an external force could not change the direction

of myofibrils suspended in the cytoplasm without changing the shape of the cells themselves. However, while many myofibrils were shown to be aligned in one (circumferential) direction at the 15 somite stage, which is the midpoint of the looping process (17), the cells containing them were not seen to be generally elongated in the same direction at nearly the same stage (16). Furthermore, if nascent myofibrils were not bound to the cell membranes, contractions of the heart wall would not occur, since unbound myofibrils could not transmit their contraction to the wall. However, spontaneous contractions of the wall are known to occur nearly at the time of the formation of the first myofibrils; namely, the 9 somite stage (4, 10) or H–H stage 10 (23). On the other hand, if the majority of myofibrils were bound to the cell membranes from the earliest stages of myofibril formation, the lengths of myofibrils would be directly related to those of the developing myocytes, and the myofibril network and the myocytes could not develop independently from each other. Thus, a clarification of whether or not the majority of the first myofibrils are firmly bound to the cell membranes is

crucial to understanding the basic mechanisms involved in the development of the initial myofibril network in the embryonic heart.

In mature cardiac muscle, myocytes are mutually bound through the intercalated disks. The myofibrils in the myocytes form continuous tracks via specific regions of the disks, fasciae adherentes (FAs),[1] which contain vinculin (26). The myofibrils located adjacent to the cell membrane are also laterally associated with the membrane through costameres (CMs) which are also vinculin positive (19). The continuous, beltlike intercellular junctions of epithelial cells called zonulae adherentes (ZAs) as well as the dense plaques present along the cell membrane in smooth muscle cells are also known to contain vinculin (6, 7). The feature common to all of these vinculin-positive structures is that they are closely associated with the cell membrane on one hand and with actin-containing filaments on the other. Thus, immunolabeling of vinculin can be used as an effective means to detect the points or lines of association between the cell membrane and actin-containing filaments.

Past ultrastructural studies of embryonic heart reported that "terminal bars" (11) or "junctional complexes" (13) are present in the outer of the two cell layers of the cardiac primordia and that, when the primordia are fused and myofibril formation initiated, some of the nascent myofibrils were found to be inserting into these junctions (13). On the basis of these observations, it was proposed that the intercalated disks are derived from the junctional complexes (11, 13). These ultrastructural studies, however, did not reveal whether or not ZAs were present in the junctional complexes before the initiation of myofibril formation, or whether the areas of the complexes into which myofibrils are inserted were punctate as FAs or beltlike as ZAs.

On the other hand, we recently observed the presence of F-actin continuously distributed along the intercellular boundaries in the cardiac primordia of chick embryos (24). This observation coupled with the fact that in typical epithelial cells a bundle of actin filaments is present along the cell boundaries at the level of ZA (7) suggest that ZAs might exist along the cell boundaries in the cardiac primordia and parts of these ZAs might become FAs in later developmental stages.

In the early developmental stages, the outer cell layer of the myocardium retains the epithelial organization similar to that of the mesoderm from which it is derived (13). On the other hand, the cells in the inner layer are bound to those in the outer layer but, typically, they are initially separated from each other and only later become associated with each other through punctate junctions (13, 14). Since myofibrils are formed not only in the outer layer but eventually throughout the wall, it is quite likely that FAs, CMs, or both are formed de novo in the inner cell layer.

Since myofibrils in the earliest stages of development are not only sparsely distributed but are arranged three dimensionally in the curved, thin walls of minute heart tubes, it has been extremely difficult to study such questions as raised above by conventional examinations of planar sections of the hearts. In the present investigation, these questions were studied by examining whole-mount preparations of the hearts, prepared as described before (24), which were fluorescently labeled for F-actin and titin to visualize myofibrils and for

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1. *Abbreviations used in this paper:* CMs, costameres; FAs, fasciae adherentes; NBD, nitrobenz-oxadiazole; ZAs, zonulae adherentes.

vinculin to recognize ZAs, FAs, and CMs. Examination of the preparations in fluorescence optics revealed that (*a*) the majority of myofibrils are bound to the cell membranes through ZAs, FAs, or CMs in the earliest stages of myofibril formation; (*b*) ZAs are present along the cell boundaries in the outer cell layer of the myocardium before the initiation of myofibril formation, and they later develop into FAs and CMs; and (*c*) myofibrils newly formed in the inner cell layer of the myocardium laterally associate with the cell membranes on the inner surface of the myocardial wall through CMs, and perpendicularly or obliquely associate with the lateral intercellular boundaries through FAs.

Materials and Methods

Specimens

White Leghorn chick embryos of 6–12 somite stages were fixed in a 4% formaldehyde solution containing 0.1% $CaCl_2$ for 1 h at room temperature as described (24).

Antibodies

Vinculin was isolated from chicken gizzard and its antibody was produced in guinea pigs and affinity purified as described (5). Affinity-purified goat anti-guinea pig IgG was biotinylated as described (9). These antibodies were provided by Dr. S. J. Singer (University of California at San Diego [UCSD], San Diego, CA). Affinity-purified rabbit antibody against titin isolated from chicken pectoralis muscle was a gift of Dr. Pamela A. Maher (UCSD, San Diego, CA).

Immunolabeling

In the 6–7-somite-stage embryos, the thoracic portion from which the neural canal was removed was cut in half along the axis of symmetry. In the 8–12-somite-stage embryos, the heart trough or tube was dissected out from the embryo and cut open longitudinally along its dorsal side. These specimens were treated with nonionic detergent and hyaluronidase before immunolabeling as described previously (24). Briefly, they were treated with 0.1% Triton X-100 in a modified PBS (PBS-A) for 30 min, washed in PBS-A several times, and treated with a hyaluronidase solution in PBS-A for 30–45 min at room temperature. After washing several times in PBS-A, the specimens were indirectly immunolabeled for vinculin or titin with simultaneous labeling for F-actin.

Each step of labeling or washing in PBS-A was carried out in the refrigerator for 8–16 h. For double labeling of vinculin and F-actin, a triple-step procedure was used. Specimens were first immunolabeled with guinea pig anti-vinculin antibody, washed, treated with biotinylated goat anti-guinea pig IgG, washed again, treated with a mixture of FITC-conjugated streptavidin (Amersham Corp., Arlington Heights, IL) and rhodamine-phalloidin (Molecular Probes Inc., Junction City, OR), and then washed. For double labeling of titin and F-actin, specimens were first immunolabeled with rabbit anti-titin antibody, washed, treated with a mixture of rhodamine-conjugated F(ab')₂ fragment goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., Avondale, PA) and nitrobenz-oxadiazole (NBD)-phallicidin (Molecular Probes Inc.), and then washed.

Light Microscopy

After the completion of labeling, each specimen was mounted between the glass slide and cover slip, and observed in a mildly compressed manner as described in reference 24. The mounting medium was an 80% glycerol and 20% PBS mixture with or without an anti-bleach reagent, *p*-phenylenediamine (Sigma Chemical Co., St. Louis, MO). All samples were examined in a Microphot-FX (Nikon Inc., Garden City, NY) in rhodamine fluorescence optics, FITC fluorescence optics, and Nomarski optics. The rhodamine signals were detected by using the combination G-1B consisting of the exciter filter EX 546/10 and the barrier filter BA 590. The FITC and NBD signals were detected by using the combination B-2E consisting of the excitation filter EX 450–490 and the barrier filter BA 520–560. The focal level was adjustable in the increments of 0.5 μm or less.

When a field of a double-labeled preparation was photographed in rhodamine (such as Fig. 3 *a*) and fluorescein (such as Fig. 3 *b*) fluorescence optics

and the position of a well-defined structure (such as a corner of the polygonal intercellular boundaries in Fig. 3, *a* and *b*) in one micrograph was compared with that of the same structure in the other by using the edges of the micrograph frames as the axes of reference, the position of the structure was found to be highly reproducible in the two fluorescence optics, to the limit of the light microscopic resolution, ∼0.2 mm in the prints enlarged 1,000× (not shown). This observation gave the basis to find corresponding images of double-labeled, ill-defined structures in the micrographs taken with the two fluorescence optics by simply using the edges of the micrographs.

Results

The early stages of the embryonic heart development will be first briefly reviewed to put the present results in perspective. In the 5–6 somite stage, when the cardiac primordia are not yet fused, the myocardium consisting largely of two cell layers is highly convoluted on the inner surface but relatively smooth on the outer surface which faces the coelom (13). In the 9–10 somite stage or H–H stage 10, when the formation of the heart trough is nearly completed (10, 20), the inner surface which is now in contact with the cardiac jelly becomes smooth, whereas individual myocytes of the outer cell layer often bulge outward so that the outer surface of the myocardial wall appears as if it is composed of hemispherical domes (10, 13, 16). Circumferential or beltlike junctions are present in the outer cell layer along the intercellular boundaries at the bases of the domes, whereas spot or punctate junctions such as desmosomes are found throughout the myocardial wall (13; unpublished observations). This overall configuration of the myocardial wall is maintained until unidirectional blood flow is initiated at the 16–17 somite stage. The heart shows the first signs of looping at the 10 somite stage and it reaches the midpoint of looping, an S-shaped stage, at about the 15 somite stage (13, 17, 20). A recent electron microscopic study by Hiruma and Hirakow (10) revealed that loosely assembled myofilaments are seen at the 8 somite stage, and nascent myofibrils with definite sarcomeres are seen for the first time in a small number at the 9 somite stage and increase considerably in number at the 10 somite stage. The present study covers the period from the 6 to 12 somite stages.

Myofibrils Near the Outer Surface of the Myocardial Wall

At the 7 somite stage, before the initiation of myofibril formation, titin spots were found to be much more numerous near

the outer and inner surfaces (Figs. 1, *a* and *c*) than at the midlevel (Fig. 1 *b*) of the myocardial wall. Correspondingly, at the 9–10 somite stage, which is in the earliest stage of myofibril formation, myofibrils visualized by F-actin labeling were seen to be more abundant near the two surfaces (Fig. 2, compare *a* and *c* with *b*). These observations are concordant with the conclusion of our previous study that the titin spots formed in the premyofibril stages will be subsequently incorporated into the newly arising myofibrils (24).

We previously demonstrated the presence of F-actin along the cell borders in the stages before the fusion of cardiac primordia (Fig. 11 of reference 24). In the present study, vinculin was also localized along the cell borders in the prefusion stages (Fig. 3), which suggested the presence of ZAs along the borders as in the intestinal epithelial cells (6, 7). Optical sectioning of the primordia revealed that ZAs are located at the intercellular boundaries in the outer cell layer of the myocardium, where junctional complexes were reported to be present (11, 13). The intensity of vinculin labeling was often appreciably stronger at the corners of the polygonal cell boundaries than along the rest of the cell boundaries (Fig. 3). The presence of ZAs was not, in fact, a specific feature of the cardiac primordia but was also found in other cell types such as the mesodermal cells in the areas adjacent to the cardiac primordia and the endodermal cells forming the foregut wall (not shown).

Optical sectioning of the 9–10-somite-stage hearts revealed that myofibrils in the outer cell layer of the myocardium were present mostly at the level of the base of the cellular domes. In many somite-stage hearts, the myofibrils were still sparsely distributed but the majority of them were seen to span the entire width of the cell either along the cell borders (myofibrils indicated with arrows and arrowheads in Fig. 4, *a* and *c–e*; and those marked with broken lines in Fig. 5 *c* and shown in Fig. 5, *a* and *b*) or apart from the borders (a myofibril indicated with a double-headed arrow in Fig. 4 *c*; and those marked with white lines in Fig. 5 *c* and shown in Fig. 5, *a* and *b*). These myofibrils often terminated at the corners of the polygonal myocytic borders, and the sites of termination were frequently more strongly vinculin positive than the rest of the boundaries (points indicated with small circles and black and white v's in Fig. 4, *a–g*, and shown in Fig. 4, *b*, *e*, and *g*; and those indicated with small circles in Fig. 5 *c* and shown in Fig. 5 *d*). These facts and the observa-
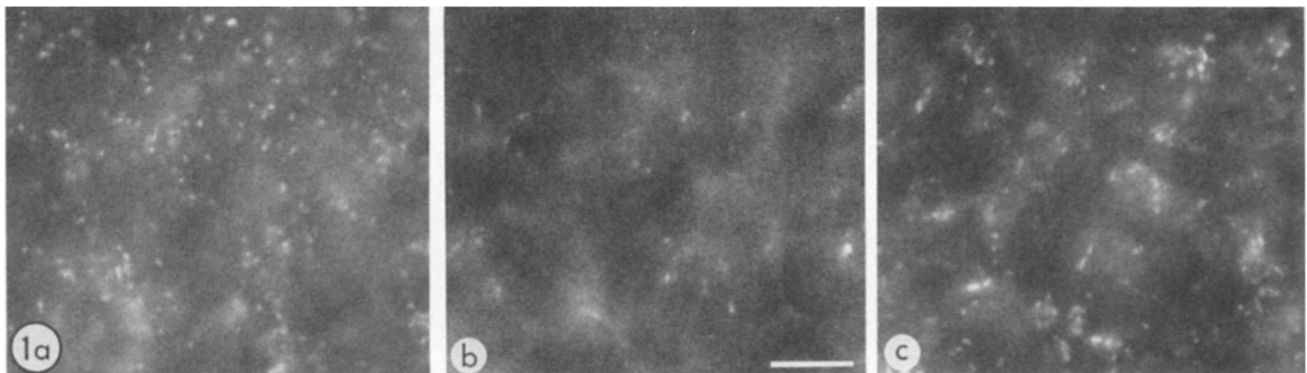

Figure 1. A 7-somite-stage cardiac primordium immunolabeled for titin and observed at three different focal levels (*a–c*). The number of fluorescent titin spots is much greater near the myocardial outer (*a*) and inner (*c*) surfaces which are separated from each other by ∼20 μm, than at a midlevel (*b*) which is separated from the outer (*a*) level by ∼14 μm. Bar, 10 μm.
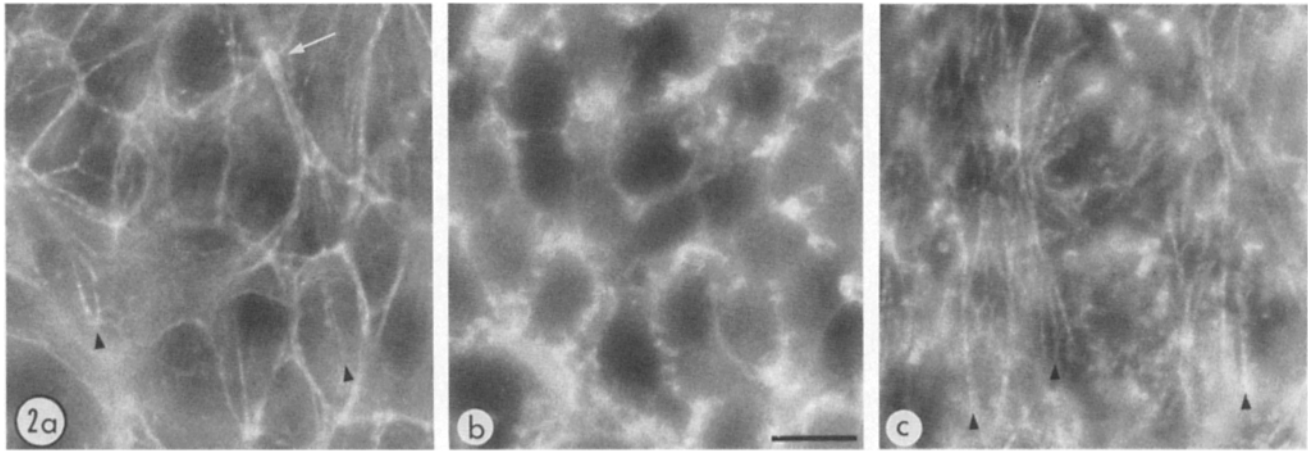
Figure 2. A 10-somite-stage heart labeled for F-actin and observed at three different focal levels (*a–c*). The myocardial wall at this stage is reduced in thickness to about half that of the 7 somite stage (13). Myofibrils (*arrowheads*) are present near the myocardial outer (*a*) and inner (*c*) surfaces which are separated from each other by ∼8 μm, but few myofibrils are seen at a midlevel (*b*) which is separated from the level of *a* by ∼4 μm. Myofibrils in *c* are aligned in the vertical direction that corresponds to the circumferential direction in the heart tube. This area represents a part of Fig. 8, observed at a slightly different focal level; the location indicated with a white arrow in *a* corresponds to that indicated with a white arrow in Fig. 8 *a*. Ruffled structures found along the cell borders in *b* are likely to represent cellular projections into the intercellular spaces which are known to be present in the myocardial wall of this stage (13). Bar, 10 μm.

tion that the intensity of vinculin labeling was greater at the corners of the myocytic polygons in the premyofibril stage (Fig. 3) suggest that the formation of the first FAs at the corners is an event programmed before the initiation of myofibril formation.

The polygonal intercellular boundaries at the 9 somite stage were generally vinculin positive as in the premyofibril stage (compare Fig. 4 with Fig. 3). On the other hand, at the 9 somite stage, the myofibrils which ran along the boundaries were located so close to the boundaries that they appeared as if they were superimposed with the boundaries (Fig. 4). These facts strongly suggest that such nascent myofibrils are bound to the cell boundaries through ZAs. At some portions of the boundaries, vinculin labeling showed a periodic pattern (*arrowheads*; Fig. 4, *f* and *g*), which may indicate the formation of the nascent CMs.

At the 9–10 somite stage, some myocytes contained a large number of thin filaments which did not clearly show regular cross-striations when labeled for F-actin (Fig. 6 *a*). When examined by optical sectioning or sometimes in a single focal plane, these filaments were also quite often seen to traverse the lengths of myocytes and terminate on vinculin-positive cell boundaries (Fig. 6, *a* and *b*). Such filaments were titin positive (*arrowheads*; Fig. 7, *a* and *b*), indicating that they were very early myofibrils, not nonmuscle-type microfilaments. In fact, in the present study, we never observed F-actin–positive filaments that were negative for titin.

By the 10 somite stage in many of the hearts, myofibrils had already formed complex networks (Fig. 8 *a*) but the myofibrillar tracks were still narrow in width and the strongly vinculin-positive areas of the cell boundaries into which the myofibrils terminated in an end-on fashion oc-
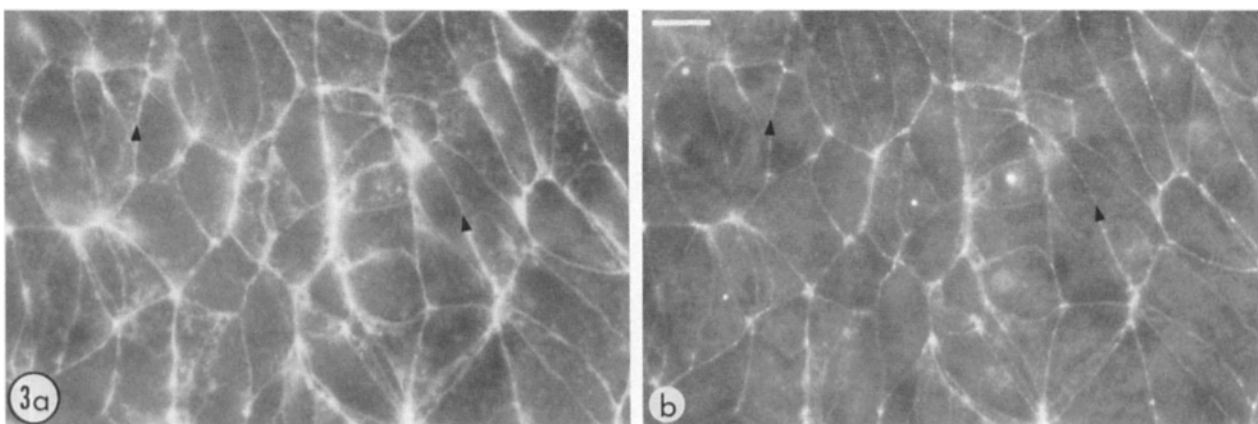

Figure 3. A 6-somite-stage cardiac primordium double labeled for F-actin (*a*) and vinculin (*b*), and observed near the myocardial outer surface. Cell boundaries which are identified by the presence of F-actin (see reference 24 for F-actin labeling of cell boundaries) in *a* (*arrowheads*) are seen to be vinculin positive in *b* (*arrowheads*). The intensity of vinculin labeling is often stronger at the corners of polygonal-shaped myocytes than along the sides. Bar, 10 μm.
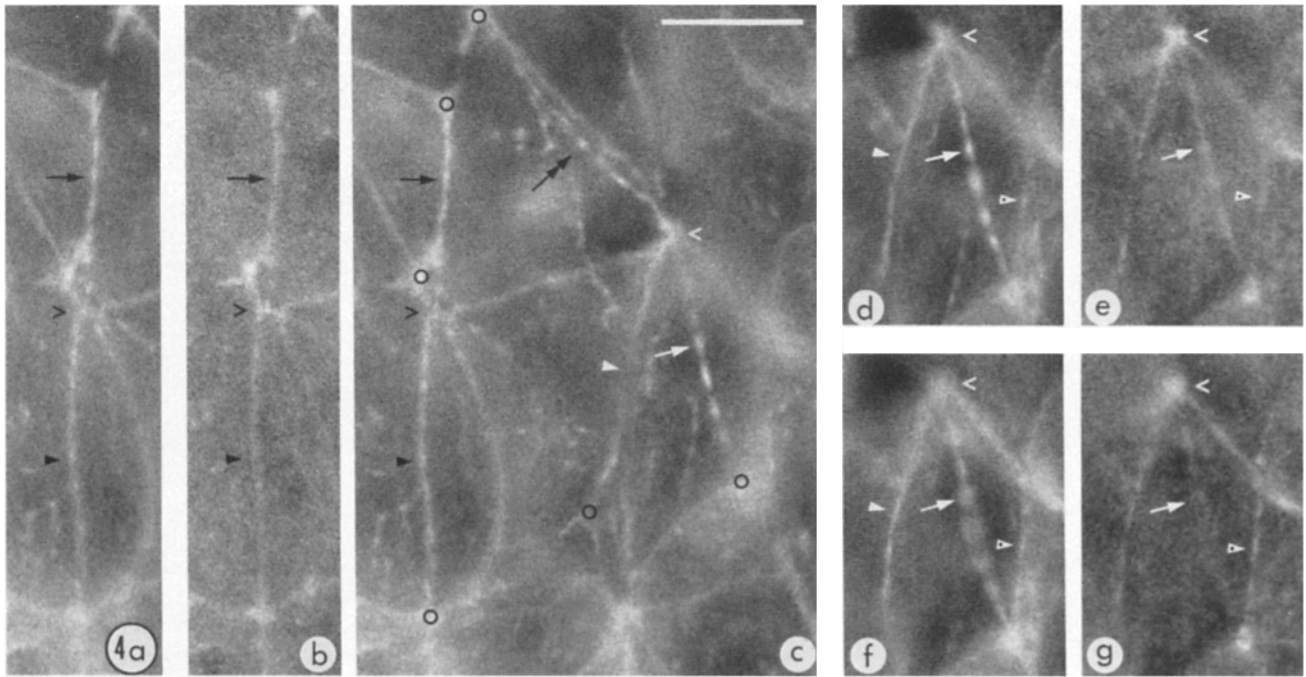
Figure 4. A 9-somite-stage heart double labeled for F-actin (*a, c, d,* and *f*) and vinculin (*b, e,* and *g*). The area shown in *c* was observed at four close focal levels near the myocardial outer surface. The left one third of the area was shown in *a* and *b,* while a central portion of the right one half was shown in *d–g. b* and *c, d* and *e,* and *f* and *g* are at the same focal levels. The focal differences between *a* and *b, d,* and *f* are 0.5, 0.8 and 1.0 μm, respectively. Several myofibrils (black and white arrows and arrowheads in *a, c, d,* and *f*) are present along vinculin-positive, polygonal boundaries of the myocytes (arrows and arrowheads in *b, e,* and *g*) and terminate at the corners of the boundaries (small circles, black v, and white v in *c;* and v's in *a, b,* and *d–g*). These corners are generally stronger in the intensity of vinculin labeling than the rest of the cell boundaries (compare *b* with *c, e* with *d,* and *g* with *f*). A myofibril indicated with a double-headed arrow in *c* spans from a corner to an adjacent corner but appears to be separated from the cell border. Many myofibrils are oriented nearly in the vertical direction that corresponds to the circumferential direction of the tubular heart. This area and that of Fig. 11 belong to the same heart. Bar, 10 μm.
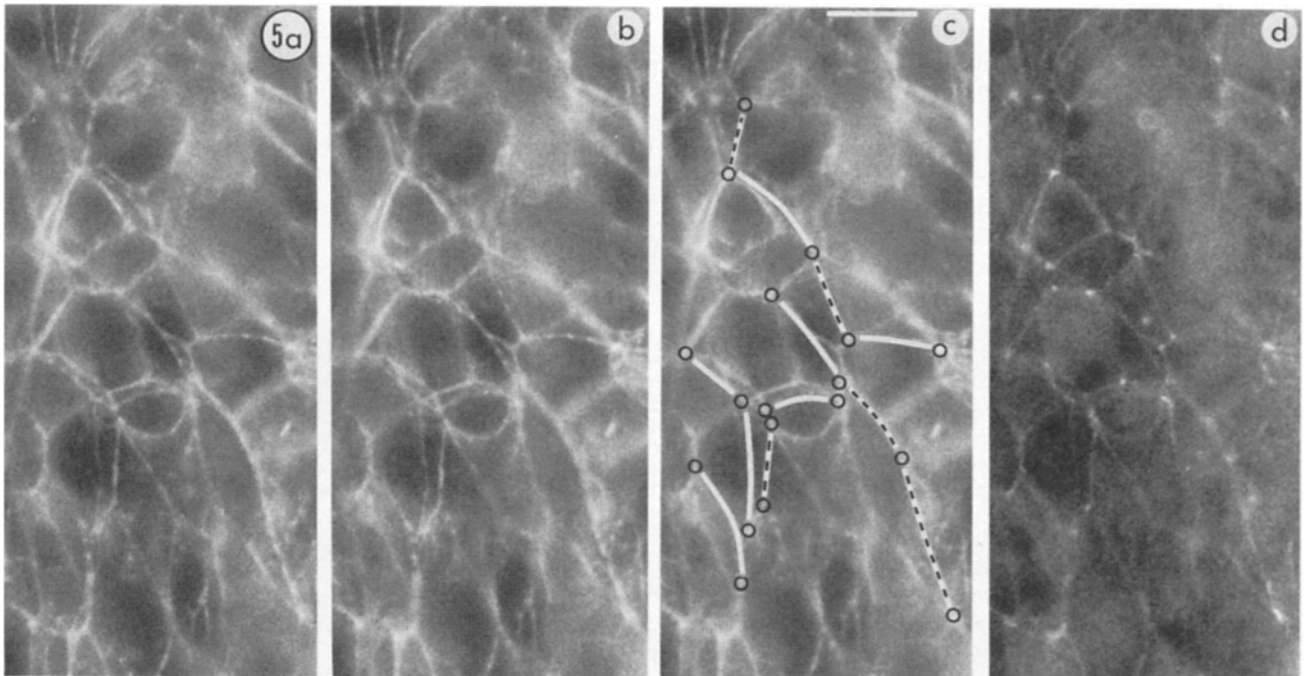

Figure 5. A 9-somite-stage heart double labeled for F-actin (*a–c*) and vinculin (*d*) and observed at two close focal levels near the outer surface of the myocardial wall. *b–d* are at the same focal level; *c* is a reproduction of *b. a* and *b–d* are different in focal level by 0.5 μm. In *c,* the locations of myofibrils seen in *a* and *b* and the positions of vinculin spots seen in *d* are indicated with white or broken line segments and small circles, respectively. Most of the vinculin spots are found at corners of polygonal cells and are more intensely labeled than the sides of the cells. Myofibrils which appear to be on cell boundaries are indicated with broken line segments. Myofibrils are variable in direction but many of them are oriented nearly in the vertical direction which corresponds to the circumferential direction of the tubular heart. Bars, 10 μm.
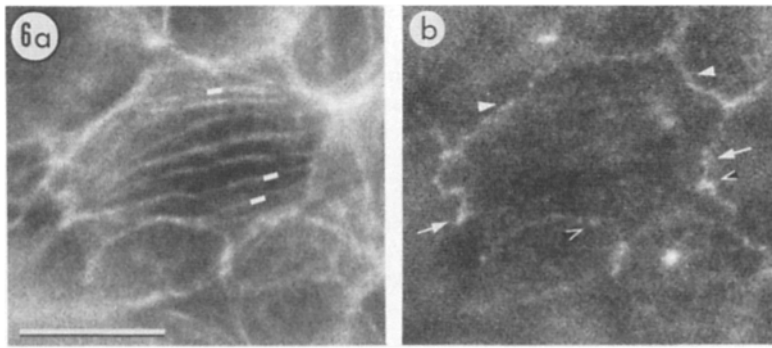
cupied only a small portion of the cell boundary (Fig. 8 b). Nevertheless, at this stage, such areas (i.e., FAs) were not confined to the corners of the myocytic polygons but found also on the sides of the polygons (Fig. 8 b). Occasionally, myofibrils appeared to be laterally associated with the cell membrane through CMs which were vinculin positive and periodically arranged along the cell membrane (*arrows*; Fig. 8, a, b, and *insets*).

In some 10-somite-stage hearts the myofibril networks were increased in width and the portions of the cell boundaries that were perpendicular or oblique to the direction of the myofibrillar tracks were found to be extensively labeled for vinculin (pairs of black or white arrowheads in Fig. 9, a and b). Sometimes these tracks appeared to converge toward a myocyte (*asterisks*; Fig. 9, a and b). However, whether this is a common feature of all early embryonic hearts or whether there are more than one such centers of convergence in early embryonic hearts is not clear at this time. The portions of the cell boundaries which were perpendicular or oblique to the direction of the myofibrillar tracks often appeared as if consisting of broken lines (Fig. 9, a and b). At higher magnification, in these (not shown) and older hearts (Fig. 10), the areas of cell boundaries which were devoid of vinculin were found to correspond to the areas at which myofibrils did not terminate. The cellular lengths, as measured from FA to FA in each cell, were often 10–20 μm but occasionally were as short as 5 μm (* in Fig. 10, a and b).

Myofibrillar tracks in the outer cell layer often followed zig-zag courses from cell to cell but, as a whole, appeared to be oriented circumferentially, that is, in the direction perpendicular to the longitudinal axis of the tubular myocardial wall (Figs. 4 c, 5 c, 8 a, and 9 a).

Myofibrils Near the Inner Surface of the Myocardial Wall

Optical sectioning of whole mount preparations of 9–10 somite stage hearts labeled for F-actin revealed that myofibrils were also present along the inner surface of the myocardial wall, as described above (Fig. 2). When the labeled preparations were systematically examined in fluorescent optics and in Nomarski optics in the focal intervals of 0.5–1 μm, it became clear that the majority of the myofibrils were present very close to the inner surface of the myocardial wall, within the 0.5-1-μm-thick subplasmalemmal space, at this early stage (not shown). Because of this proximity and the slight undulations of the inner surface, only small portions of the

myofibril networks on this surface could be visualized in a single optical plane. For instance, in the 9-somite-stage heart shown in Fig. 11, a and b, which was double labeled for F-actin and vinculin, myofibrils or vinculin spots were recognized only in two narrow areas (A and B). Myofibrils and corresponding vinculin spots were, in fact, found on the whole inner surface of this heart, when the surface was systematically examined by successively changing the focus (not shown). In A and B areas shown in Fig. 11 a, the myofibrils became largely unrecognizable when the focus was changed by 1 μm away from or into the myocardial wall.

The myofibrillar tracks present along the inner surface were more unidirectionally aligned (compare Fig. 2 c with Fig. 2 a and Fig. 11 a with Fig. 8 a) and stretched smoothly for longer distances than those found near the outer surface (40- and 60-μm-long myofibrils indicated with pairs of black and white arrowheads, respectively, in Fig. 11 a). Systematic examination revealed that these tracks branch and form large loops (not shown) but as a whole, ran circumferentially even at the 9 somite stage before the initiation of heart looping.

Close inspection of the preparations double labeled for F-actin and vinculin revealed that vinculin spots or their tracks were located exactly on the tracks of myofibrils (compare A and B in Fig. 11 a with corresponding areas in Fig. 11 b). Since, as indicated in the Introduction, vinculin is a
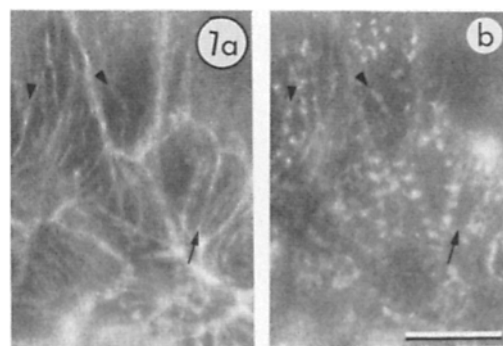

Figure 7. A 10-somite-stage heart double labeled for F-actin (a) and titin (b) and observed at a level near the myocardial outer surface. The majority of myofibrils in this area do not show definite striations (arrowheads in a) but they are titin positive (arrowheads in b). F-actin–positive and titin-negative structures such as those shown with an arrow in a and b are believed to represent cell boundaries. Bar, 10 μm.
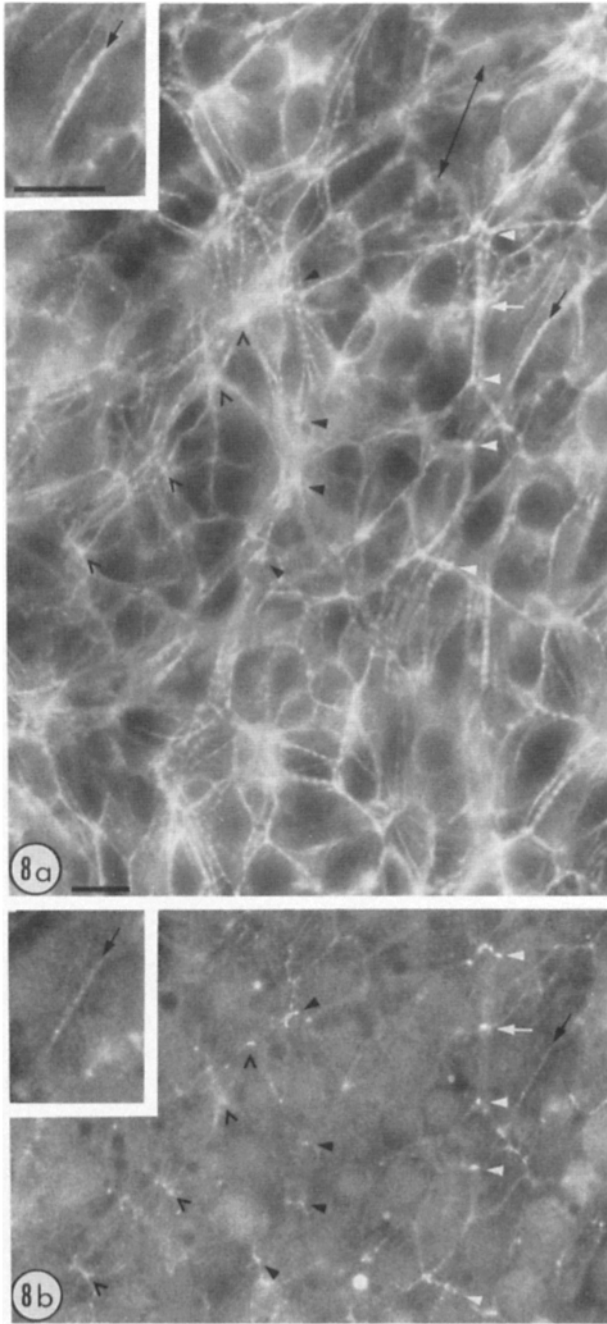
Figure 8. A 10-somite-stage heart double labeled for F-actin (*a*) and vinculin (*b*, a partial area of *a*) and observed at a level near the myocardial outer surface. Myofibrillar tracks that follow zig-zag courses and form a network (*a*) are bound to the cell membranes at vinculin spots (*b*; compare black and white arrowheads, black v's, and white arrow in *a* with those in *b*). An area of *a* is shown in Fig. 2 *a* at a slightly different focal level. This figure is rotated clockwise by ∼20° in relation to Fig. 2 and the circumferential direction of the tubular heart, that is the direction of myofibrils in Fig. 2 *c*, is indicated with a bidirectional arrow in *a*. Many myofibrils or myofibrillar tracks in *a* are directed approximately in the direction of the arrow. A myofibril and a vinculin-positive portion of the cell membrane along which the myofibril appears to be located are indicated with black arrows in *a* and *b*, as well as in the insets, respectively. Bars, 10 μm.

protein found specifically at the points of contact between actin filaments and the cell membrane (5–7, 19, 26), these vinculin spots are believed to represent CMs, through which myofibrils are bound tangentially to the inner wall surface, or FAs, through which myofibrils are bound perpendicularly or obliquely to the lateral intercellular boundaries at a level near the inner surface.

F-actin labeling did not clearly reveal the cell shapes at the innermost surface of the myocardial inner layer (*A* and *B*, Fig. 11 *a*), probably due to the obliqueness of the cell boundaries to the inner surface. Nevertheless, immediately below the surface the cell boundaries were recognizable by F-actin labeling (see the area to the left of *A* in Fig. 11 *a*), and the lengths of the myocytes located on the inner surface could be estimated to be similar to those in the outer cell layer, ∼10–20 μm. This estimate agreed well with the cell lengths measured by electron microscopic examinations of ultrathin sections of the walls (not shown). Since myofibrils found on the inner surface in this early stage were already often 40 μm or longer, as shown above, some of the vinculin spots are believed to represent FAs present at intercellular boundaries.

In the 12-somite-stage hearts, in which myofibrillar tracks were considerably more numerous than in the 9–10-somite-stage hearts, rows of vinculin spots were often recognized exactly at the locations of the myofibrils, as in the earlier hearts (Fig. 12, compare *a* or *c* with *b* or *d*, respectively). There were a large number of small spots (*line segments*, Fig. 12) and a much smaller number of larger spots (Fig. 12, *arrowheads*). Both types of spots were superimposable on myofibrils (Fig. 12, compare *a* or *c* with *b* or *d*, respectively). The most likely interpretation is that the small and large spots represent CMs and FAs, respectively. The fact that vinculin spots were not always present along the entire length of the myofibrils (Fig. 12), on the other hand, suggests that myofibrils were not associated with the cell membranes along their entire lengths.

Optical sectioning of the myocardial wall revealed that the subplasmalemmal zone in which myofibrils were present thickened to ∼2 μm at the 12 somite stage from 1 μm or thinner at the somite stage (not shown).

Discussion

The present study revealed that as early as the 9–10 somite stage, myofibrils already form networks that traverse through myocytes and are, in most parts, oriented in the circumferential direction of the tubular heart. These observations suggest that individual myocytes in the early stage of myofibril formation may be endowed with spatial information by which the nascent myofibrils are laid down. In the inner cell layer of the myocardial wall, the nascent myofibrils are laterally bound to the cell membrane through CMs on the inner surface of the myocardial wall as described above. Thus, in the inner layer, the cell membrane may serve as a stable structure upon which the myofibrils are fixed. Nevertheless, it is not clear how they are aligned in the specific, circumferential direction. In the outer cell layer, many nascent myofibrils are also laterally associated with the cell membrane through ZAs but the association occurs at the sides of the myocytic polygons which have definite directions. Thus, in this case, the cell membrane not only supports the nascent myofibrils but also defines their directions. Some nascent myofibrils in this
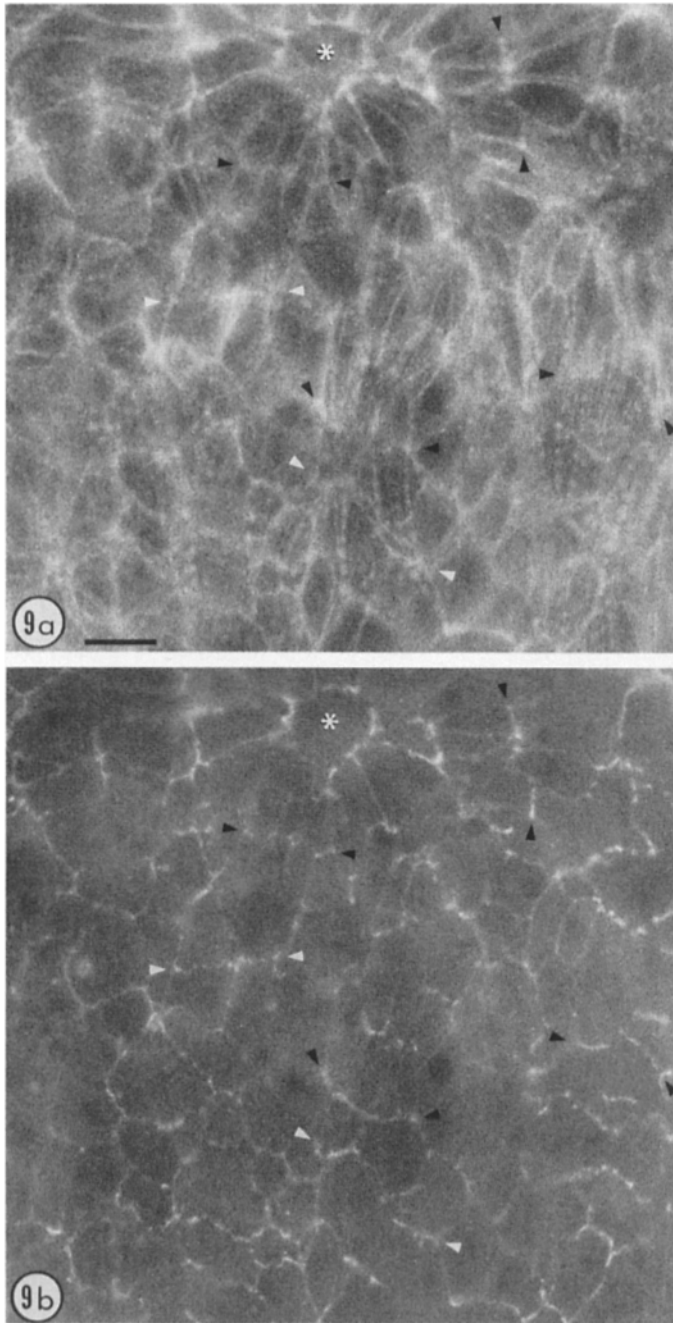
Figure 9. A 10-somite-stage heart double labeled for F-actin (*a*) and vinculin (*b*) and observed at a level near the myocardial outer surface. In the bottom two thirds of *a*, wide bands of myofibrillar tracks are as a whole aligned in the vertical direction that corresponds to the circumferential direction of the tubular heart. In the top one third of *a*, the myofibrillar tracks appear to converge toward a myocyte marked with an asterisk. In *b*, the portions of cell boundaries which are perpendicular or oblique to the direction of the myofibrillar tracks appear as if composed of broken lines. This is believed to indicate the absence of vinculin at the areas of cell boundaries at which myofibrils did not terminate (see Fig. 10). Bars, 10 μm.

layer, on the other hand, span through the cytoplasm and terminate into the corners of the myocytic polygons. Here too, the myocytic cell membrane is directly involved in defining the directions of the nascent myofibrils. However, it is not clear how such myofibrils present in the cytoplasm come to terminate into the specific points of the cell membrane. Nevertheless, it is conceivable that such myofibrils are initially associated with the cell membrane but subsequently become separated from the membrane by an unknown process while remaining attached to the membrane at both their ends. Since ZAs are not present in the mature heart, the portions of ZAs with which the myofibrils are laterally as-

sociated may either be converted to CMs or subsequently become nonjunctional so that the myofibrils are separated from the cell membrane. In summary, the interaction of the myofibrils with the cell membrane appears to be an essential part of cardiac myofibrillogenesis from the very beginning. It should be noted that the association of nascent myofibrils with the cell membrane had been reported in the study of skeletal myofibrillogenesis in vivo as well (3). In fact, cardiac and skeletal muscles appear to share many common features. CMs were observed in both types of muscles (18, 19) and vinculin was localized at the myotendinous junction in skeletal muscle (22) as well as at FA in cardiac muscle (7,
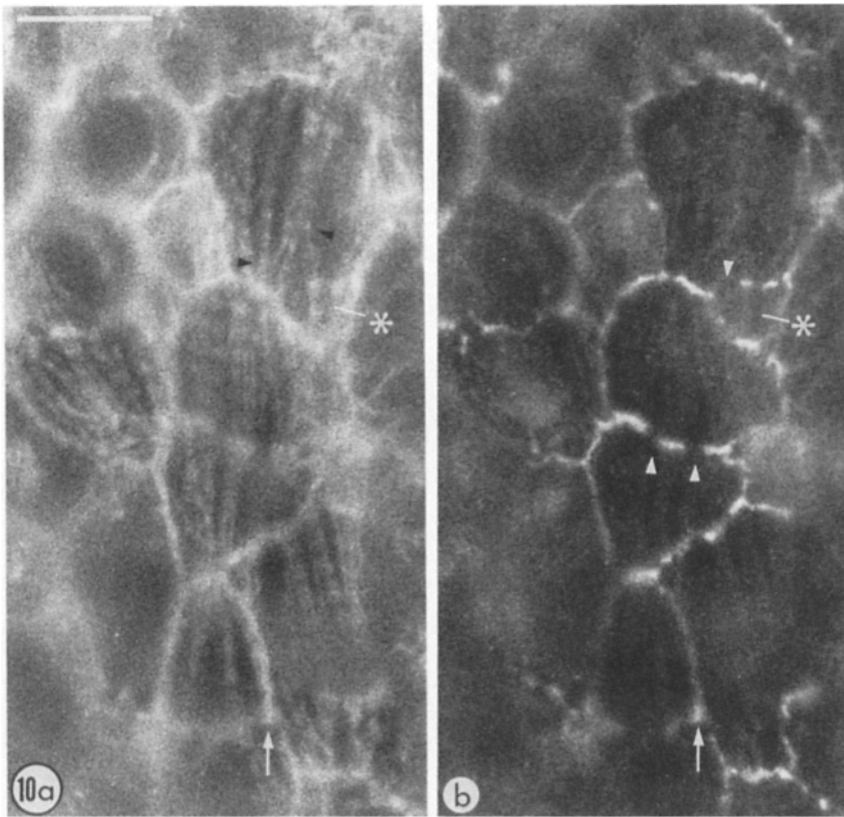
Figure 10. A 12-somite-stage heart double labeled for F-actin (*a*) and vinculin (*b*) and observed at a level near the myocardial outer surface. Myofibrils are seen to occupy nearly the entire width of the myocytes in *a*. Small areas of cell boundaries at which myofibrils do not terminate are found to be vinculin negative (white arrowheads in *b*; see corresponding areas in *a*). Branching of myofibrils is now clearly observed (black arrowheads in *a*). Some portions of the cell boundaries which are nearly parallel to the direction of the myofibrillar tracks are positive both for F-actin and vinculin (white arrows in *a* and *b*). Faint signs of cross-striations are often recognizable at such portions, suggesting that myofibrils are present and laterally associated with the cell membrane. The cell indicated with an asterisk in *a* shows a length of only 4–5 μm at this focal level, as measured in the direction of the myofibrils. Myofibrillar tracks as a whole are directed in the vertical direction that corresponds to the circumferential direction of the tubular heart. Bar, 10 μm.
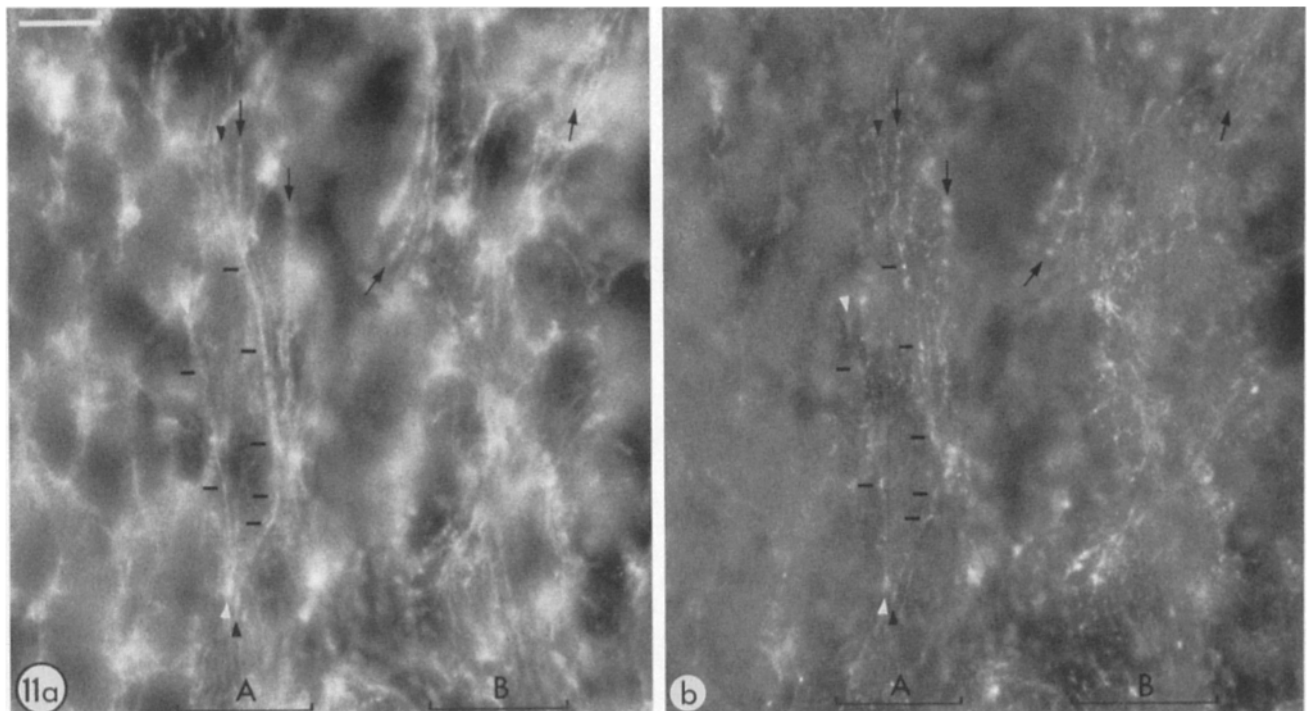

Figure 11. A 9-somite-stage heart double labeled for F-actin (*a*) and vinculin (*b*) and observed at a level very close to the myocardial inner surface. The areas in which myofibrils or myofibrillar tracks are located coincide with those in which vinculin spots are seen (compare regions indicated with *A* and *B* brackets in *a* with those in *b*). Series of vinculin spots are found exactly at the locations of myofibrils or myofibrillar tracks (arrows in *a* and *b*). Along long myofibrils (pair of black arrowheads and pair of white ones), vinculin spots are found in variable intervals (line segments in *a* and *b*). Myofibrillar tracks are directed approximately in the vertical direction that corresponds to the circumferential direction of the tubular heart. This area and that of Fig. 4 belong to the same heart. Bar, 10 μm.
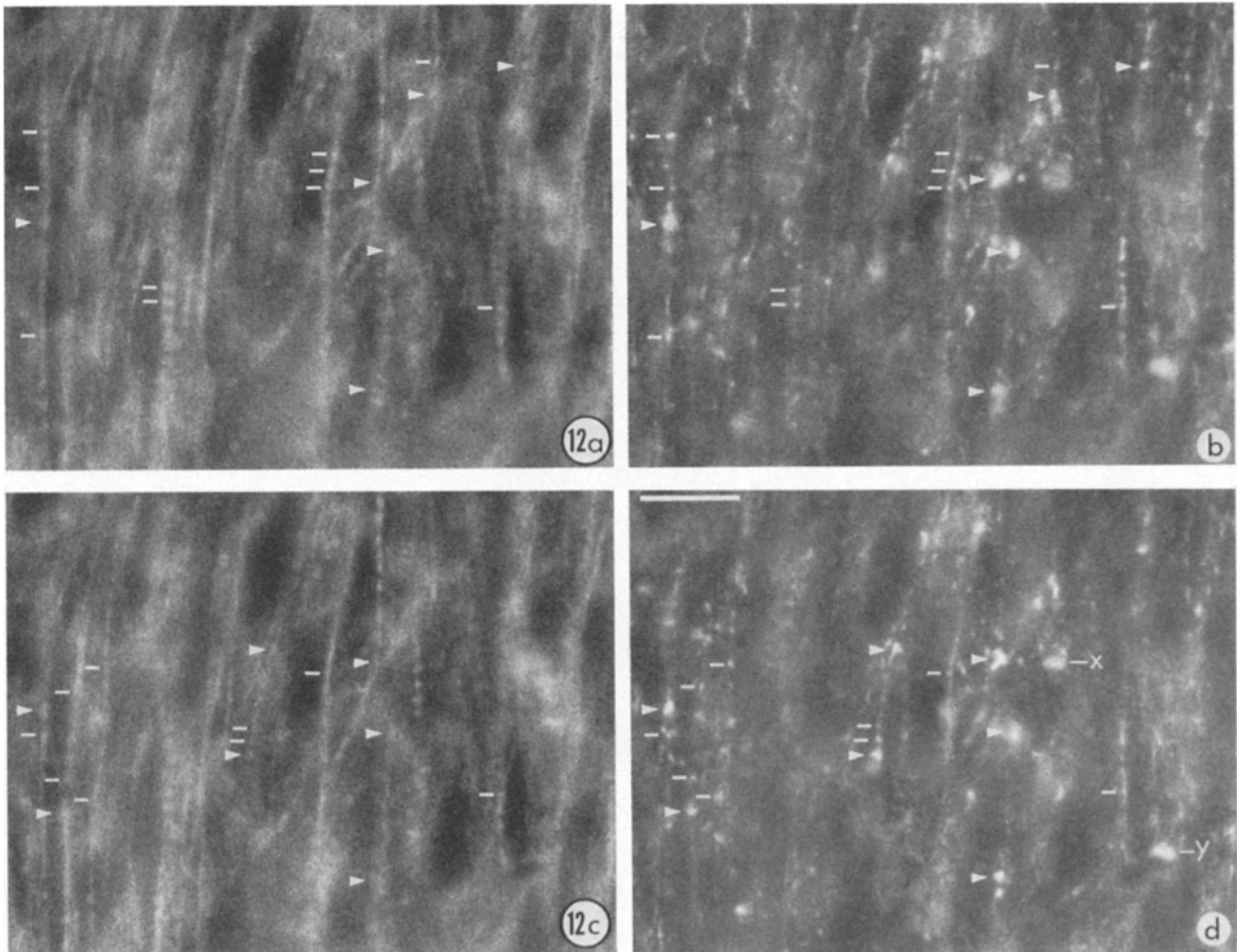
Figure 12. A 12-somite-stage heart double labeled for F-actin (*a* and *c*) and vinculin (*b* and *d*) and observed at two focal levels very close to the inner surface of the myocardial wall. *a* and *b*, and *c* and *d* are at the same focal levels. The level of *c* and *d* is deeper into the wall than that of *a* and *b* by 1 μm. Vinculin spots (small and large spots indicated with short line segments and arrowheads, respectively, in *b* and *d*) are found along myofibrils (corresponding markings in *a* and *c*). Two large spots indicated with arrows in *b* are apparently unaccompanied by a myofibrillar counterpart in *a* but as they are more closely focused in *d*, a myofibril becomes recognizable in *c* (*arrows*). The myofibrillar counterparts of two vaguely defined, large spots (*x* and *y*) are not discernible in *a* or *c* but become recognizable at a level 1.5 μm deeper than the level of *c* and *d* (not shown). Myofibrillar tracks are directed in the vertical direction that corresponds to the circumferential direction of the tubular heart. Bar, 10 μm.

26). Recently, we observed the presence of CMs in the postmitotic skeletal myoblasts in vitro (Colley, N. J., K. T. Tokuyasu, and S. J. Singer, manuscript in preparation).

In some myocytes of the 9–10 somite stage, the earliest myofibrils which do not show cross-striations are often not only unidirectionally oriented within the cytoplasm of individual myocytes but are bound to the cell membrane at both their ends (Fig. 6). It is possible that the myofibrils are initially formed in association with the cell membrane and laterally proliferate by a process similar to the mechanism known in skeletal muscles (8). However, it is also possible that short myofibrils are initially formed in a directed manner in the cytoplasm and then elongate through a mechanism such as that proposed by Dulgosz et al. (1) or Sanger et al. (21). To investigate whether or not stress fiber–like structures such as described by Dulgosz et al. are present in the 9–12-

somite-stage hearts, we took advantage of the fact that titin is a protein specific to myofibrils of striated muscles (27). Our double-labeling studies of whole-mount preparations of the hearts with rhodamine–phalloidin and anti–titin antibodies have so far failed to reveal filamentous structures that are F-actin positive but titin negative. Also, the shortest sarcomeres that we were able to observe in the 9–10-somite-stage hearts were ~1.5 μm (25), much longer than the "minisarcomeres" that were reported by Sanger et al. It should be noted that the cultured cells investigated in these studies were much larger than the cardiac myocytes of the 9–12-somite-stage chick embryos. In the early stage embryonic hearts, the cellular lengths, which are often equal to the myofibrillar length measured from FA to FA, is commonly only 10–20 μm and can be as short as 5 μm (Fig. 10), whereas cultured cardiac myocytes often reach 150 μm in length and 50 μm

in width (1). More importantly, embryonic cardiac myocytes closely associate with each other, whereas cardiac myocytes cultured on dishes are typically single flat cells attached to the substrate. In other words, the intercellular boundaries which are an essential feature of cardiac myocytes in vivo are not present in cardiac myocytes cultured on dishes. Thus, many features of cardiac myofibrillogenesis in vivo might not be meaningfully studied by observing myofibrillogenesis in vitro.

In the mature heart, two myofibrils belonging to adjacent myocytes terminate on a common FA from opposite sides so that they form a colinear alignment through the FA. In the 9–10-somite-stage embryonic hearts, however, some myocytes that contain myofibrils terminating on the cell borders are seen to be surrounded by myocytes without myofibrils (Fig. 6). At the boundaries of these two types of myocytes, the nascent FAs are associated with myofibrils from one side but not from the other. An interesting question is whether or not FAs in the myocytes containing myofibrils affect the pattern of myofibril formation in adjoining myocytes that do not contain myofibrils.

As demonstrated above, in the myocardial outer cell layer, portions of the preexisting ZAs are converted to FAs at the sites at which the myofibrillar actin filaments terminate to the cell membrane in an end-on fashion. This was detected by observing an increase in the intensity of vinculin labeling at the termination sites. The fact that vinculin is newly added only to the termination sites of nascent myofibrils suggests that the termination sites have unique features. It will be important to identify these features not only for understanding the process of FA formation but, more generally, the mechanism by which a specific protein is deposited to limited areas.

In typical epithelial cells, a bundle of microfilaments laterally associates with ZAs (12), whereas in cardiac muscle the myofibrils appear to terminate at FAs in an end-on fashion (2). An interesting question is whether a basic alteration occurs in the relationship between actin-containing filaments and the cell membrane at the time of the conversion of ZAs to FAs or whether the termination of the myofibrils at the cell membrane occurs in a double-back fashion so that the contact of the myofibrils with the cell membrane actually remains tangential.

A widely accepted proposal as to the mechanism for the alignment of nascent myofibrils in the circumferential direction is that myofibrils become bound to the cell membranes at both their ends only after they are circumferentially aligned by an external force created during heart looping (17). The present findings do not support the basic assumption of this proposal that myofibrils are initially free from, or bound only at one end to, the cell membranes in the earliest stages of myofibril formation. Nevertheless, it is still quite possible that heart looping imposes important effects upon the development of the myofibrillar networks or vice versa.

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