Myofibrillogenesis in Living Cells Microinjected with Fluorescently Labeled Alpha-Actinin

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Abstract. Fluorescently labeled alpha-actinin, isolated from chicken gizzards, breast muscle, or calf brains, was microinjected into cultured embryonic myotubes and cardiac myocytes where it was incorporated into the Z-bands of myofibrils. The localization in injected, living cells was confirmed by reacting permeabilized myotubes and cardiac myocytes with fluorescent alpha-actinin. Both living and permeabilized cells incorporated the alpha-actinin regardless of whether the alpha-actinin was isolated from nonmuscle, skeletal, or smooth muscle, or whether it was labeled with different fluorescent dyes. The living muscle cells could beat up to 5 d after injection. Rest-length sarcomeres in beating myotubes and cardiac myocytes were ~1.9–2.4 μm long, as measured by the separation of fluorescent bands of alpha-actinin. There were areas in nearly all beating cells, however, where narrow bands of alpha-actinin, spaced 0.3–1.5 μm apart, were arranged in linear arrays giving the appearance of minisarcomeres. In myotubes, alpha-actinin was found exclusively in these closely spaced arrays for the first 2–3 d in culture. When the myotubes became contraction-competent, at ~day 4 to day 5 in culture, alpha-actinin was localized in Z-bands of fully formed sarcomeres, as well as in minisarcomeres. Video recordings of injected, spontaneously beating myotubes showed contracting myofibrils with 2.3 μm sarcomeres adjacent to noncontracting fibers with finely spaced periodicities of alpha-actinin. Time sequences of the same living myotube over a 24-h period revealed that the spacings between the minisarcomeres increased from 0.9–1.3 to 1.6–2.3 μm. Embryonic cardiac myocytes usually contained contractile networks of fully formed sarcomeres together with noncontractile minisarcomeres in peripheral areas of the cytoplasm. In some cells, individual myofibrils with 1.9–2.3 μm sarcomeres were connected in series with minisarcomeres. Double labeling of cardiac myocytes and myotubes with alpha-actinin and a monoclonal antibody directed against adult chicken skeletal myosin showed that all fibers that contained alpha-actinin also contained skeletal muscle myosin. This was true whether alpha-actinin was present in Z-bands of fully formed sarcomeres or present in the closely spaced beads of minisarcomeres. We propose that the closely spaced beads containing alpha-actinin are nascent Z-bands that grow apart and associate laterally with neighboring arrays containing alpha-actinin to form sarcomeres during myofibrillogenesis.

MYOFIBRIL formation in embryonic vertebrate skeletal and cardiac cells has been examined in a number of different ways. Biochemical data have shown there is coordinate synthesis of actin, myosin, tropomyosin, and alpha-actinin during vertebrate myogenesis (6). Immunocytochemical and electron microscopic data suggest that thick and thin filaments are formed at the same time in the cytoplasm and subsequently become organized into sarcomeric arrays (13, 18). The assumption in these studies (13, 18) is that the filaments are the same length as their counterparts in adult vertebrate muscle and that the sarcomeres they generate are also the same length as those in adult muscle. Studies of embryonic invertebrate muscle, however, show that sarcomeres and their constituent filaments lengthen during myogenesis (1, 2).

More than 20 yr ago, Aronson (1) demonstrated in an elegant study using polarizing microscopy that A-bands and sarcomeres in living mites increased in length during development. The first detectable sarcomeres were 2–2.5 μm and grew to 10 μm over 40 h. Aronson showed, moreover, that only after the sarcomeres had attained adult length did the muscle contract. Because adult sarcomere size in vertebrate muscle is only 2–2.5 μm, any prior growth occurring during myogenesis would be very difficult to detect (13, 18).

The use of fluorescently labeled contractile proteins provides the potential for following a particular protein that has been microinjected into living cells and thereby identifying changes that take place in the cytoplasmic structures in which the protein is localized. We have used fluorescently labeled alpha-actinin to visualize Z-bands (29) in living embryonic

cardiac myocytes and myotubes and in permeabilized models of the same cells. With these procedures we have been able to detect, in the same cell, both fully formed, actively contracting sarcomeric units and noncontracting linear arrays of closely spaced concentrations of alpha-actinin which we have termed minisarcomeres. Growth of minisarcomeres into fully formed sarcomeres was recorded by periodic observations of a single region of a myotube over many hours. We suggest that the beaded concentrations of alpha-actinin are nascent Z-bands and that the areas of the muscle cells in which they are found are areas where myofibril formation occurs. Growth of the minisarcomeres in a manner analogous to sarcomere growth in embryonic invertebrate muscle (1, 2) would give rise to definitive sarcomeres.

Materials and Methods

Preparation of Chick Muscle Cells

Cardiac muscle cells were isolated from 5–6-d-old chick embryos and grown in culture on glass coverslips as previously reported (33). The cells (plated at 100,000 cells/ml) were grown in Eagle's minimal medium supplemented with 10% fetal calf serum, 1% antibiotic–antimycotic solution (10,000 U penicillin, 10,000 μg streptomycin, 25 μg Fungizone), and 1.5% glutamine (all obtained from Gibco, G and Island, NY). Skeletal myoblasts were isolated from the breast muscle of 10–11-d-old chick embryo (14) (plated at 100,000 cells/ml) and grown in a medium containing 10% horse serum, 10% embryo extract, 1% antibiotic–antimycotic solution, and 1.5% glutamine.

Preparation of Cell Models

Chick muscle cells and fibroblasts were permeabilized in three different ways. (*a*) The medium in the culture dishes was removed and replaced with a cold solution (4°C) of 25% glycerol in standard salt solution (0.1 M KCl, 0.01 M phosphate buffer, 0.001 M $MgCl_2$, pH 7.0). After 2 h, the cells were washed with cold standard salt solution and exposed to labeled proteins. (*b*) The cells were placed in 0.02% Nonidet P-40 in standard salt solution for 1–5 min at room temperature (22°C), then washed with cold standard salt before being exposed to labeled proteins. (*c*) A coverslip with cells was plunged into cold acetone (−20°C) for 5–10 s and washed with cold standard salt solution (4°C) several times before staining. This latter procedure was our preferred method of permeabilizing cells because it was fast and very effective.

Preparation of Fluorescently Labeled Alpha-Actinin

Alpha-actinin was isolated from fresh or frozen chicken gizzards, from fresh chicken breast muscle (12), or from calf brain (8) and labeled with either lissamine rhodamine sulfonyl chloride (LR)[1] according to the method of Brandtzaeg (3), Lucifer Yellow VS (LY) (39), or with fluorescein isothiocyanate (FITC) (31, 32). The LR was purchased from Molecular Probes Inc. (Junction City, OR), the LY from Aldrich Chemical Co. (Milwaukee, WI), and the FITC from Sigma Chemical Co. (St. Louis, MO). Unbound dye was removed from the labeled protein preparations by passing the fluorescently labeled alpha-actinin through a G-25 Sephadex column (Pharmacia Inc., Piscataway, NJ). Subsequently, the labeled alpha-actinin was passed through a DE 52 column (Whatman Chemical Separation, Inc., Clifton, NJ) and eluted with a salt gradient to remove over-labeled and under-labeled protein (42, 43). Alpha-actinin–LR, alpha-actinin–LY, and alpha-actinin–FITC 0.5–1 mg/ml with one to two molecules dye/molecule of protein were used in these studies. The labeled alpha-actinin was stored in a 50% glycerol-standard salt solution and was passed through a Sephadex G-25 column equilibrated either with 1 mM $KHCO_3$ (pH 8.0) or standard salt to remove the glycerol storage solution. 1 mM $KHCO_3$ was used as the microinjection buffer and standard salt as the solvent when permeabilized cells were stained with fluorescently labeled alpha-actinin. The specificity of the labeled alpha-actinin was tested by using skeletal muscle myofibrils and permeabilized cardiac muscle cells as assay systems (34–36). The labeled alpha-actinin stained only Z-bands and intercalated disks (34–36). When we used labeled alpha-actinin that stained controls in this way, we rarely observed nuclear staining in our cell models and microinjected living cells.

1. *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; LR, lissamine rhodamine sulfonyl chloride; LY, Lucifer Yellow VS.

Microinjection of Alpha-Actinin into Living Chick Muscle Cells

The cells were grown on glass coverslips and microinjected with either alpha-actinin–LR, alpha-actinin–LY, or alpha-actinin–FITC (1 mg/ml) using techniques previously described (27, 30). Fresh medium was used to wash the injected cells, and after various time periods of incubation at 37°C in a CO_2 incubator, the coverslips were mounted in a few drops of medium and sealed with Vaseline on a glass slide. A heat curtain at 37°C was used during microscopic study of the injected cells.

For long-term observations cells were grown on round coverslips and placed in a Dvorak-Stotler chamber (Nicholson Precision Instruments, Inc., Gaithersburg, MD). Muscle medium at 37°C and 5% CO_2 was slowly perfused through the chamber using a peristaltic pump.

Monoclonal Antibody against Myosin

This antibody was a gift from the laboratory of Dr. Frank A. Pepe (University of Pennsylvania Medical School, Philadelphia, PA). It was prepared against myosin purified from adult chicken breast muscle, using procedures described earlier (41). The hybridoma supernatant was saturated 50% with solid ammonium sulfate and the precipitated antibody fraction dialyzed against phosphate-buffered saline (PBS) and stored at 4°C.

Immunoblotting determined the specificity of the antibody. Chick myotubes (7 d in culture) were solubilized in hot Laemmli's buffer (21) and electrophoresed on 10% polyacrylamide gel having 0.2% bis-acrylamide in a mini-gel apparatus. After the run, the gel was blotted on a nitrocellulose strip essentially using the method of Towbin et al. (40). After the unoccupied sites were blocked with bovine serum albumin (BSA), the blot was incubated with antimyosin (dilution 1:10) for 1 h at room temperature. The paper was washed and exposed to peroxidase-labeled rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA) for 45 min at room temperature. Visualization of peroxidase-labeled antibody was carried out by 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2. The blot processed in similar manner, except that myosin antibody was omitted in the first incubation, served as control.

The myosin antibody reacted with a band on the chick myotube extract corresponding to rabbit myosin heavy chain. However, the monoclonal antibody against chick myosin did not show any cross-reactivity with rabbit myosin (Fig. 1).

Addition of Fluorescently Labeled Proteins to Permeabilized Cells

20 μl of labeled alpha-actinin in a standard salt solution was added to permeabilized cell models at a concentration of 0.5–1 mg/ml for 30–60 min at 4°C. The cell models were then washed in several changes of standard salt solution, fixed with buffered 3% paraformaldehyde (38), and mounted on a glass slide

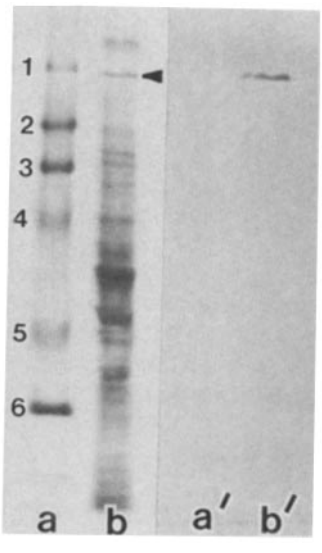

Figure 1. Nitrocellulose blots of (*a*) six protein standards transferred from a polyacrylamide gel and stained with Amido black. *1*, myosin heavy chain from rabbit skeletal muscle (200 kD); *2*, beta-galactosidase (116 kD); *3*, phosphorylase B (97 kD); *4*, BSA (66 kD); *5*, egg albumin (43 kD); *6*, carbonic anhydrase (29 kD). (*b*) Extract from chick myotube culture stained with Amido black. The myosin heavy chain band is indicated by an arrowhead. Lanes *a′* and *b′* are immunoblots corresponding to lanes *a* and *b*, respectively. Note that only the myosin heavy chain band in the chick myotube extract (*b′*) reacted with the monoclonal antibody and that there was no reaction of antibody with the rabbit myosin heavy chain (*a′*).

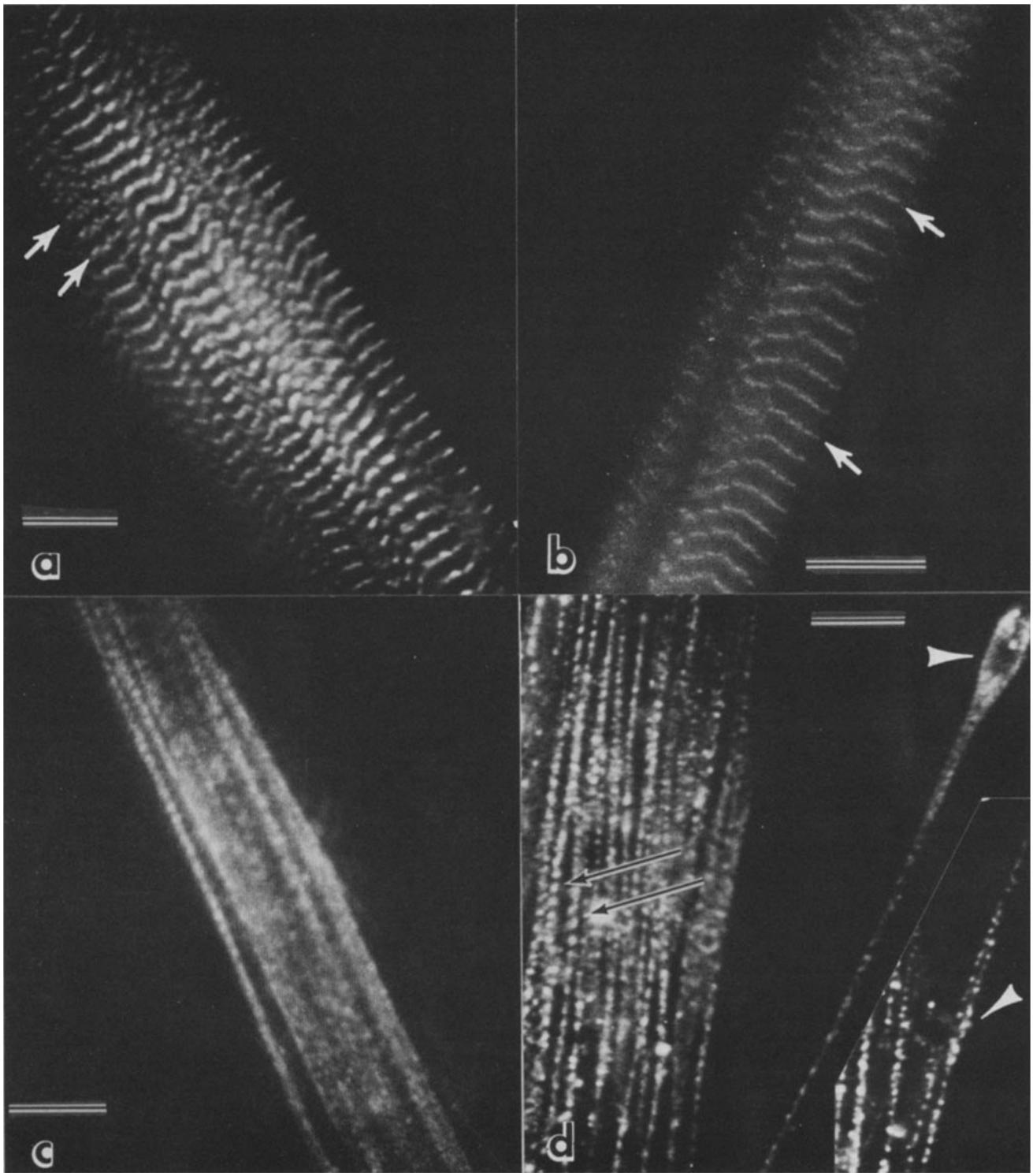

Figure 2. Living myotubes shown 2–3 h after injection with alpha-actinin–LR. (*a*) 10-d myotube viewed with an SIT camera shows incorporation of the protein in Z-bands which in some cases have a beaded substructure (*arrows*). (*b*) A myotube from the same culture incorporated enough alpha-actinin–LR to be directly photographed with a 35-mm camera. The increased resolution reveals that all the Z-bands have a beaded substructure. (*c*) A 3-d myotube incorporated alpha-actinin into fibrils in a finely striated pattern. (*d*) After 4 d in culture an increased number of fibrils have incorporated alpha-actinin–LR in a periodic pattern that varies from closely spaced beads of alpha-actinin to more widely spaced beads (*arrows*). The alph-actinin–LR diffused from the injection site in the myotube into a mononuclear cell (*arrowhead*) that had fused with the myotube. The inset shows the fibrils of the mononuclear cell fusing with those in the myotube (*arrowhead*). (*a, c,* and *d*) Bar, 10 μm; (*b*) bar, 10 μm.

in 25–50% glycerol in standard salt solution. The edges of the coverslip were sealed with clear nail polish. Cells that were to be labeled with both alpha-actinin and myosin antibody were first exposed to fluorescent alpha-actinin. This was done either (*a*) by permeabilizing the cells with acetone, exposing them to alpha-actinin, and then fixing them in 3% paraformaldehyde as described above, or (*b*) by microinjecting fluorescent alpha-actinin into living cells, and then fixing the cells with 3% paraformaldehyde and permeabilizing them in acetone. In both cases, the permeabilized cells were rinsed in standard salt containing 50 mM NH_4Cl and then rinsed in standard salt alone before exposure to the monoclonal myosin antibody for 30 min at 37°C. After several rinses with standard salt, the cells were stained with an FITC-conjugated rabbit anti-mouse antibody (Cappel Laboratories) for 30 min at 37°C, rinsed in standard salt, then rinsed in distilled water, and mounted in Elvanol, a water soluble mounting medium (Hoechst, Frankfurt, FRG).

Microscopy

Living and fixed cells were examined with either an Olympus Vanox photomicroscope or a Zeiss photomicroscope III equipped for epifluorescence. The objective used was a Zeiss Planapochromat 100× (1.3 numerical aperture). Images of permeabilized cells were recorded using Kodak Tri-X film developed with Acufine (Acufine, Inc., Chicago, IL) for an ASA rating of 1,000. Microinjected cells were viewed on a video monitor with a Dage-MTI, Inc. SIT camera (Wabash, MI), and the images photographed from the monitor on Kodak Plus X Film (ASA 125) with a 35-mm camera fitted with a 50-mm macro lens (27, 30). Spacings of nascent and fully formed myofibrils were measured on photomicrographs printed to give a final enlargement of 1,500×. The average center-to-center distances of mini-Z-bands ("Z bodies") and Z-bands were calculated by measuring a linear series of 6–10 sarcomeres and dividing by the number of sarcomeres. Five to ten measurements per cell were recorded for each time point.

An image processor (model 794, Hughes Aircraft Co., El Segundo, CA) was coupled to the SIT camera to process the fluorescent images of injected cells that were followed over extended periods of time. In this way, neutral density filters could be used to minimize the excitatory light, while images of the myofibrils were resolved and stored for comparison with future images of the same myofibrils.

Results

Microinjection of Alpha-Actinin–LR into Living Chick Myotubes

Alpha-actinin–LR became incorporated into Z-bands within 10 min after injection into 10-d-old contractile myotubes (Fig. 2*a*). In some myotubes the intensity of fluorescence was high enough to allow direct recording of the image on Tri-X film (developed for 1,000 ASA) without the need for video-image intensification. In these higher resolution images, the Z-bands appeared to be composed of fine beads of alpha-actinin (Fig. 2*b*). The beaded substructure of the Z-bands could also be seen in video images, but less clearly (Fig. 2*a*). The spacing between the fluorescent Z-bands was ~2.3 μm in quiescent myotubes. A small percentage of both injected and uninjected myotubes contracted spontaneously, during which time the fluorescent Z-bands delineated the alternately shortening and lengthening sarcomeres. Fluorescent alpha-actinin remained associated with Z-bands for as long as 4 d after injection, although the level of fluorescence declined with increasing time after injection.

Early myotubes injected on the second to third day in culture showed none of the fully formed sarcomeres that were present in older myotubes. Incorporation instead occurred in a finely striated pattern (0.5–1.2-μm spacings) along cytoplasmic fibrils (Fig. 2*c*). In some myotubes the cytoplasm was diffusely fluorescent with only one or two faintly fluorescent fibrils present, whereas in other myotubes, fibrils were uniformly spaced across the width of the myotube. By the fourth day in culture the periodic beads of alpha-actinin were more

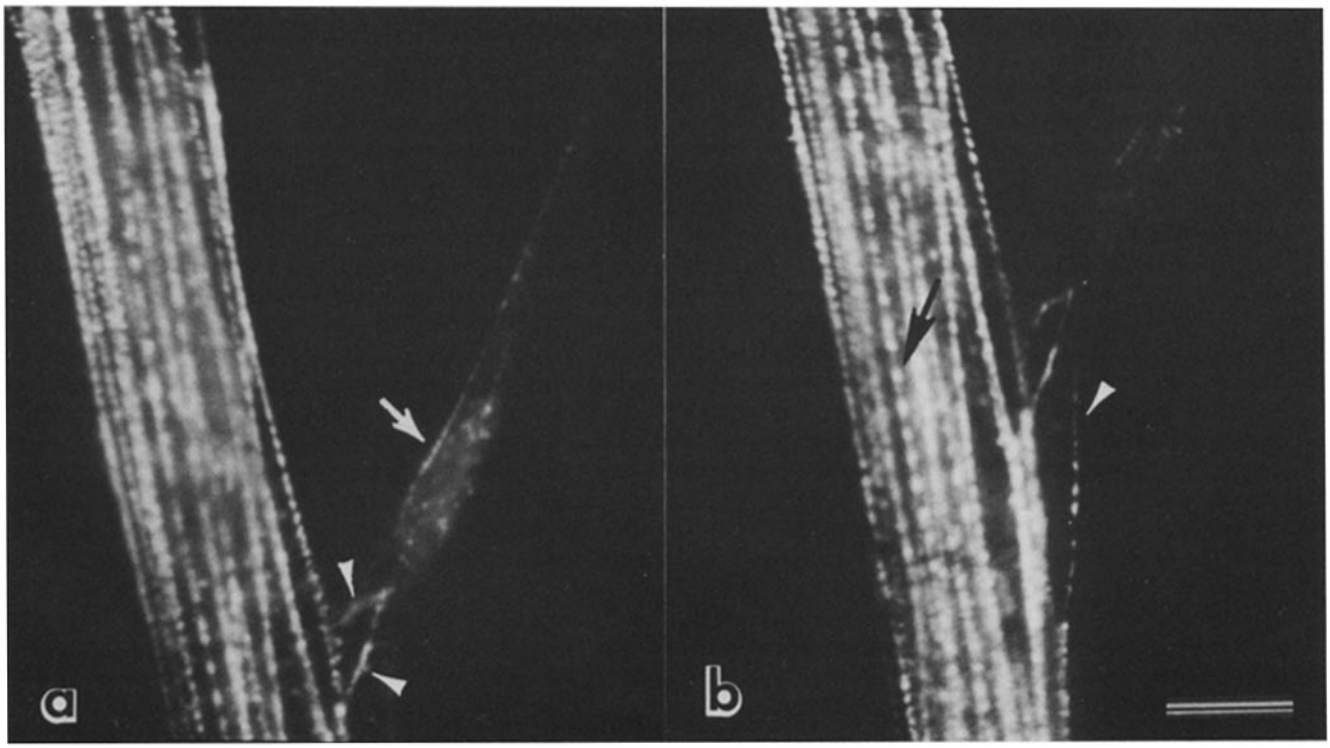

Figure 3. In a 4-d-old living myotube recorded at overlapping sites along the myotube and at two different focal planes, *a* and *b*, injected alpha-actinin–LR has been incorporated in a punctate pattern in fibrils of the myotube and fibrils of a mononuclear cell fused to it. In focus *a*, a single-beaded fibril (*arrow*) is seen adjacent to the nucleus of the mononuclear cell. Two fibrils (*arrowheads*) extend between the mononuclear cell and the myotube. In focus *b*, a third fibril (*arrowhead*) is visible extending between the myotube and the mononuclear cell. Most of the fibrils in this 4-d myotube have closely spaced concentrations of alpha-actinin, but in some the spacing is longer (arrow in *b*). (*a* and *b*) Bar, 10 μm.

clearly delineated along the fibrils (Fig. 2*d*). The center-to-center spacing of the beads of alpha-actinin remained 0.5–1.2 μm, with an occasional fibril exhibiting longer spacings of 1.2–1.9 μm (Figs. 2*d* and 3*b*). In cases where a mononucleated myocyte was fused with an injected myotube, fluorescent alpha-actinin diffused into the smaller cell, revealing finely striated fluorescent strands in the cytoplasm of the mononucleated cell (Figs. 2*d*, and 3, *a* and *b*). Often fibers of the mononucleated cell appeared to be continuous with fibrils of the larger myotube (Figs. 2*d* [*inset*] and 3*b*). Insertion of a microneedle into young myotubes such as those in Figs. 2, *c* and *d* and 3, *a* and *b* never elicited contractions as it invariably did in the older myotubes shown in Fig. 2, *a* and *b*, nor did injection of 1 mM $CaCl_2$ induce contraction in these myotubes as it did in older myotubes (5–12 d old) that contained fully formed myofibrils.

By the fifth day in culture, many injected cells had fibrils with bands of alpha-actinin that were spaced ~2.3 μm apart, and adjacent to them, fibrils with concentrations of alpha-actinin spaced 0.5–1.9 μm apart (Fig. 4). Video recordings of the 5-d-old myotube in Fig. 4, *a* and *b*, showed the two fibrils with full sarcomeric spacing (2.3 μm) of alpha-actinin rhythmically contracting (Fig. 4*a*) while the finely striated fibrils (0.5–1.5 μm) peripheral to the contracting fibrils were quiescent (Fig. 4*b*). Upon shifting focus from one plane (Fig. 4*a*) to a lower plane near the surface of the culture dish (Fig. 4*b*), we observed that the two myofibrils in the upper plane were joined in the lower plane into one common fiber that was pulled during contraction. In cultures of this age (4–5 d), some of the myotubes contracted when the microneedle penetrated the cell membrane and others did not. When 3-d cultures were injected with alpha-actinin–LR and returned to the incubator for 1–3 d before study, they were subsequently found to contain labeled Z-bands spaced 2.3 μm apart, some of which were present in spontaneously contracting myofibrils.

The distribution of alpha-actinin at the ends of myotubes also varied with the age of the cells. In young myotubes 2–5 d in culture, fibrils with closely spaced concentrations of alpha-actinin extended to within 2–10 μm of the end of a cell (Fig. 5, *a* and *b*). The extreme ends (ruffles) of these injected cells contained scattered punctate concentrations of alpha-actinin (Fig. 5, *a* and *b*). After 5–6 d in culture, myotubes that were filled with fully formed sarcomeres (Fig. 5, *d* and *f*) had ends that contained thin fibers with more closely spaced (0.5–1.9 μm) concentrations of alpha-actinin merging with randomly oriented beads of alpha-actinin at the tip of the myotube (Fig. 5*c*). Because of the thickness of myotubes of this age, it was not possible to determine whether the thin terminal fibers (Fig. 5*c*) were ligated with the mature myofibrils located further from the myotube end (Fig. 5*d*). In some mature myotubes, full sarcomeric spacing of alpha-actinin ran continuously from mid-myotube (Fig. 5*f*) to the tip (Fig. 5*e*).

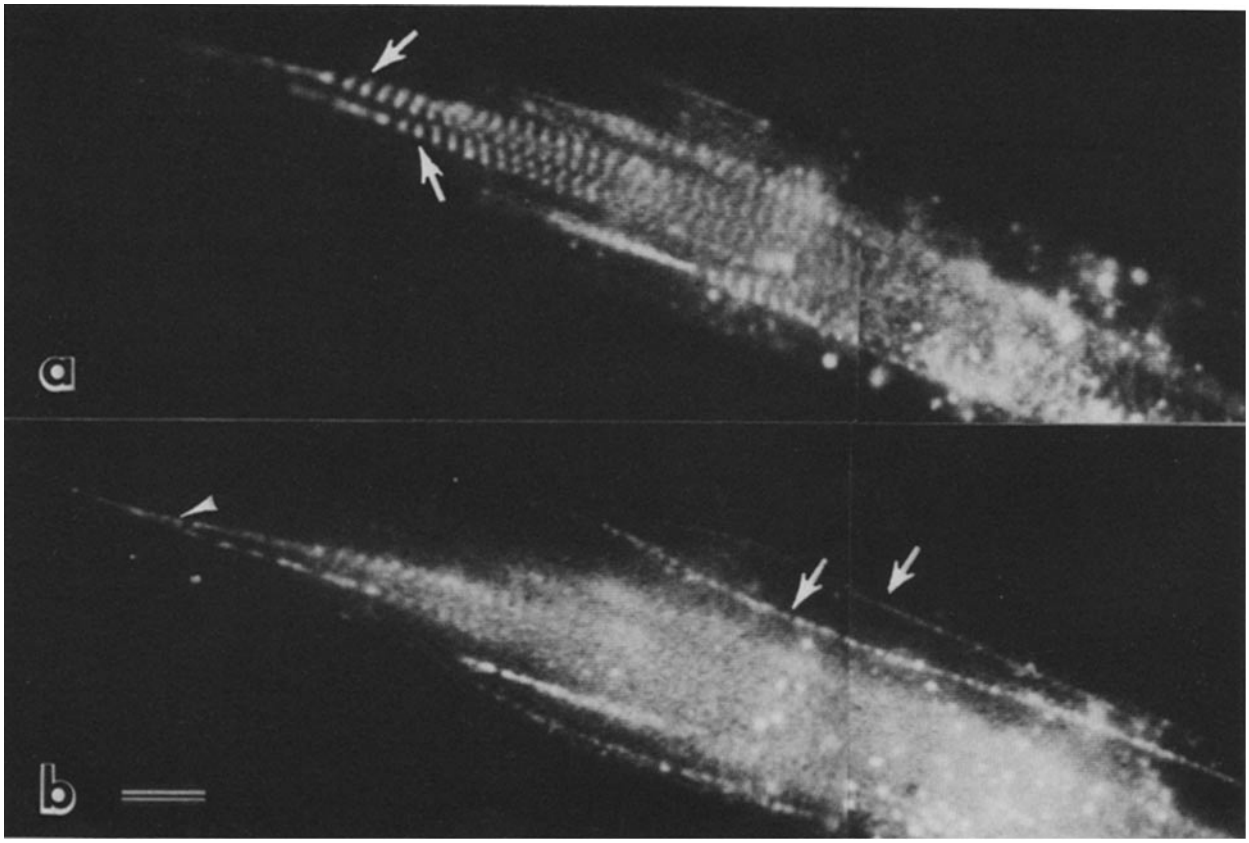

Figure 4. Sequences at two focal planes, *a* and *b*, from a videotape of a 5-d myotube beating in culture. (*a*) The fluorescent Z-bands of the myofibrils (*arrows*) move closer together during the rhythmic contractions. (*b*) A view closer to the ventral surface showed the two myofibrils fused (*arrowhead*) into one fiber that was pulled during contraction. Fibrils with small, closely spaced beads of alpha-actinin (*arrows*) showed no signs of contractile activity. (*a* and *b*) Bar, 10 μm.

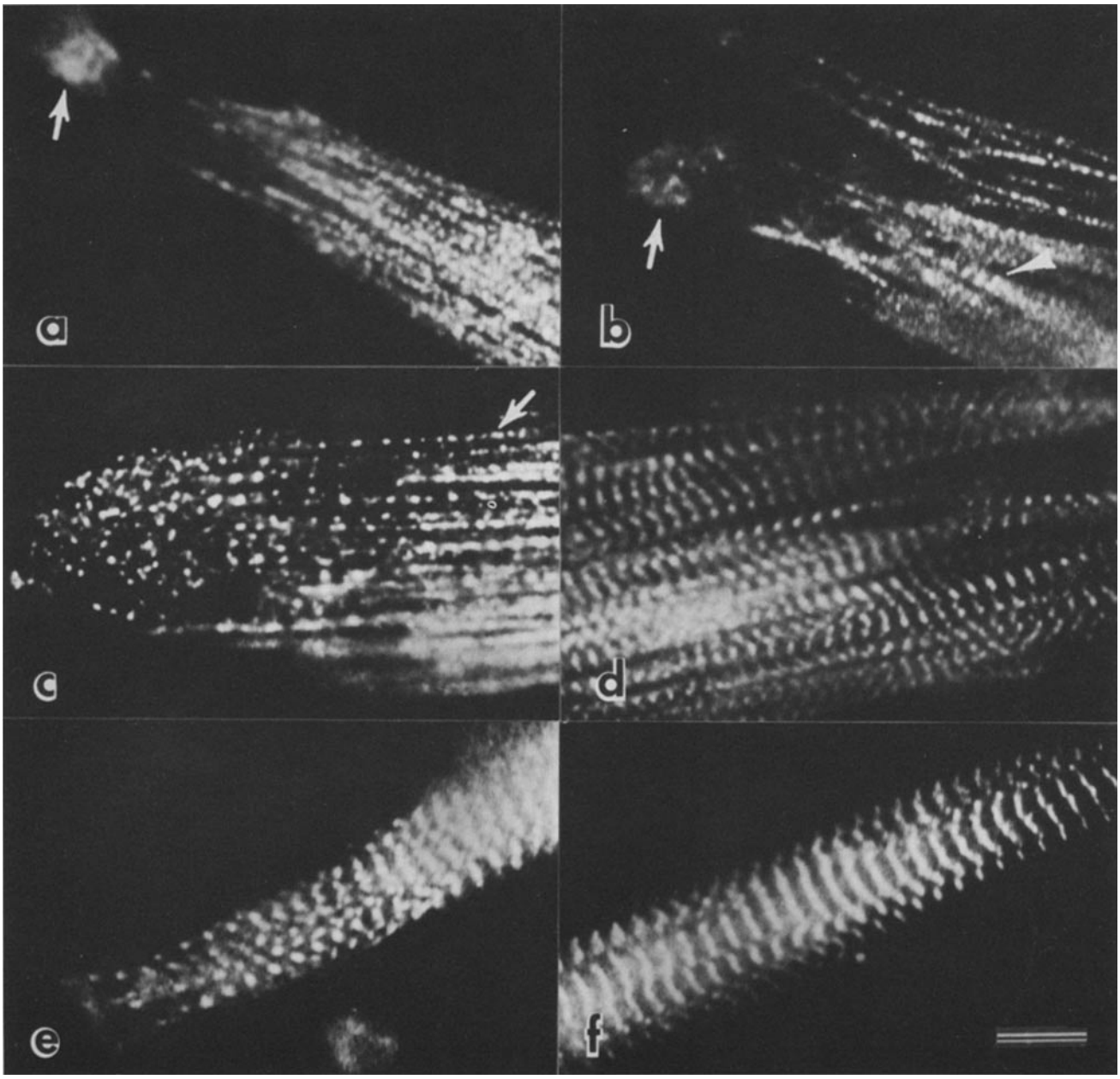

Figure 5. Incorporation of alpha-actinin–LR was diffuse in the ruffles commonly found at the ends of (*a*) 3-d myotubes (*arrow*) and (*b*) 4-d myotubes (*arrow*). Arrowhead in *b* denotes fibril with forming Z-bands amid fibrils with closely spaced beads of alpha-actinin. The distribution of alpha-actinin at the end of a 7-d-old myotube (*c*) was in small beads, in contrast to its distribution in Z-bands in the mid part of the same myotube (*d*). The arrow in *c* indicates a fibril with both long and short spacings between beads of alpha-actinin. At the end of other 7-d-old myotubes (*e*), beads of alpha-actinin were spaced the same distance apart as the Z-bands in the myofibrils in the middle of the same cell (*f*). (*a–f*) Bar, 10 μm.

Interaction of Permeabilized Myotubes with Alpha-Actinin–LR

When myotubes were permeabilized by brief exposure to acetone and incubated with alpha-actinin–LR, the patterns of localization of alpha-actinin mirrored those seen after microinjection of the same protein. In myotubes from cultures 6 d and older, alpha-actinin–LR associated with Z-bands (Fig. 6*a*) as it also does with isolated myofibrils (34, 35). Some of the Z-bands appeared beaded as was seen in injected myotubes (Fig. 2*b*). In myotubes 2 d in culture, fluorescence was faintly striated along the fibers (not shown). After 3 d, alpha-actinin was localized along fine fibers in discrete bands that were spaced 0.5–1.2 μm apart (Fig. 6*b*). Mononucleated cells that had apparently fused with myotubes contained the same type of periodic fibrils as those seen in injected myotubes in 3-d cultures (Figs. 2*d* and 3, *a* and *b*). The fibroblasts in these permeabilized cultures bound alpha-actinin–LR along their stress fibers in an aperiodic pattern typical of chick fibroblasts, but unlike most other stress fibers (35, 36).

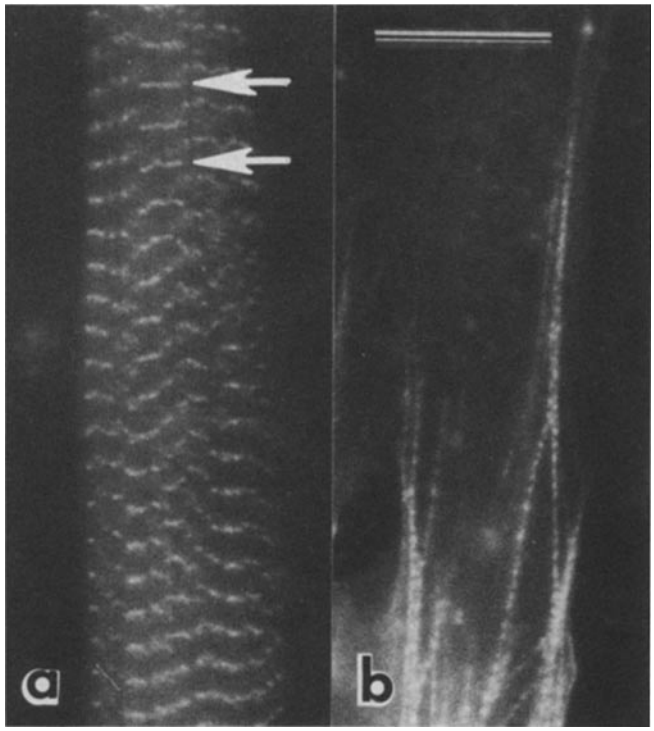

Figure 6. Permeabilized myotubes exposed to alpha-actinin–LR show (*a*) binding in a 10-d myotube to Z-bands (*arrows*) that were also visible with phase optics (not shown). The Z-bands were much more clearly resolved with the fluorescent probe and appeared beaded when labeled with the alpha-actinin–LR. (*b*) A 3-d myotube bound the protein in small beads along the fibrils at the terminus of the myotube. (*a* and *b*) Bar, 10 μm.

Incorporation of Different Types of Alpha-Actinin into Myotubes

To determine whether the source of alpha-actinin or the fluorescent label would affect incorporation into Z-bands, alpha-actinin was prepared from a smooth muscle source (chicken gizzard), from a skeletal muscle source (chicken breast), and from a nonmuscle source (calf brains) and labeled either with FITC, LY, or LR. Incorporation occurred in injected and permeabilized cells in both Z-bands and small periodicities, regardless of the type of alpha-actinin or the dye used to label it. Moreover, if any two of the three types of alpha-actinin (skeletal, smooth, or nonmuscle) were labeled with disparate dyes and co-injected into myotubes, both types of alpha-actinin were incorporated into Z-bands with apparently equal efficiency (Fig. 7, *a* and *b*).

Myofibrillar Growth in an Injected Myotube

Myotubes injected with alpha-actinin–LR on the fourth day in culture were placed in a Dvorak-Stotler chamber (Nicholson Precision Instruments, Inc.), mounted on the microscope stage, and observed intermittently for 5 d. Temperature was maintained at 37°C with a heat curtain and fresh muscle medium was constantly perfused through the chamber. A selected area of a single myotube was viewed for ~5 s every 6–12 h. Fig. 8 shows two micrographs of the same area of an injected myotube recorded 25 h apart. During this time the spacings between concentrations of alpha-actinins increased from 0.9–1.3 μm in the earlier time point to 1.6–2.3 μm in the later time point, resulting in the growth of minisarcomeres to fully formed sarcomeres. In addition to sarcomere growth in some myofibrils, other myofibrils appeared *de novo*. In Fig. 8 these new myofibrils are apparent at both sides of the myotube. The myofibrils in the interior of the myotube became progressively more aligned with one another during the course of the observations.

The myotubes were particularly sensitive to changes in the culture medium that occurred if fresh medium was not constantly perfused through the chamber. In one case when the peristaltic pump failed for 6 h, Z-bands in fully formed sarcomeres became disarrayed. After 6–12 h of resumed perfusion of fresh medium, the Z-bands became realigned and growth was detected in minisarcomeres.

Microinjections of Fluorescently Labeled Alpha-Actinin into Cardiac Myocytes

Injections of fluorescently labeled alpha-actinins into embryonic cardiac myocytes resulted in incorporation of the fluorescent protein primarily into Z-bands of the anastomosing myofibrils (Fig. 9). Mixtures of smooth muscle alpha-actinin–

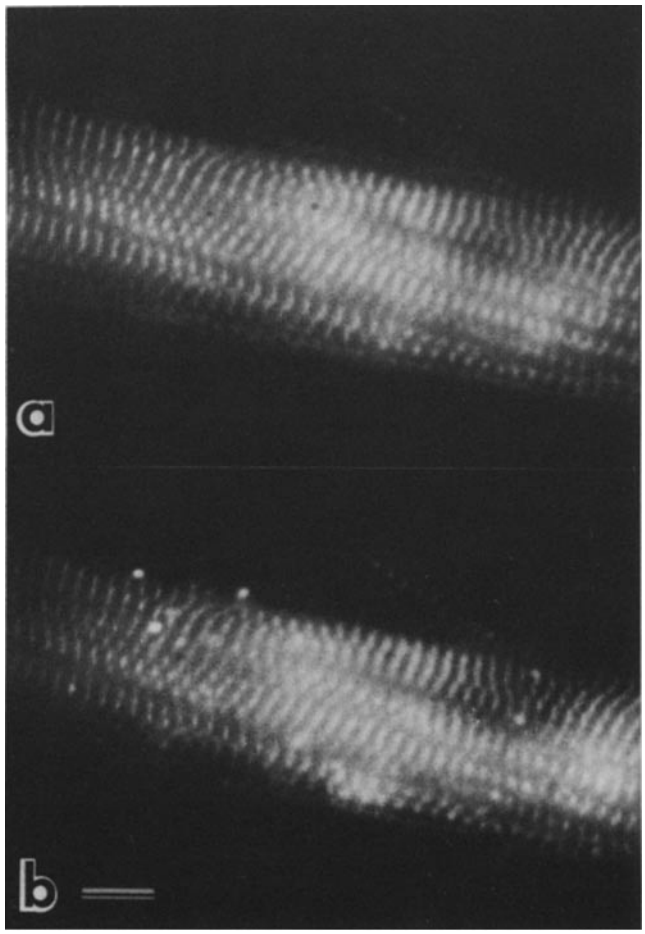

Figure 7. This living 5-d-old myotube recorded at two different wave lengths was injected the previous day with a mixture of (*a*) nonmuscle alpha-actinin–LR and (*b*) smooth muscle alpha-actinin–FITC. The patterns of incorporation of the two different proteins are identical. Notice the presence of several fluorescent granules, presumably lysosomes, in *b* but not *a*, demonstrating the absence of spillover between the two dyes. (*a* and *b*) Bar, 10 μm.

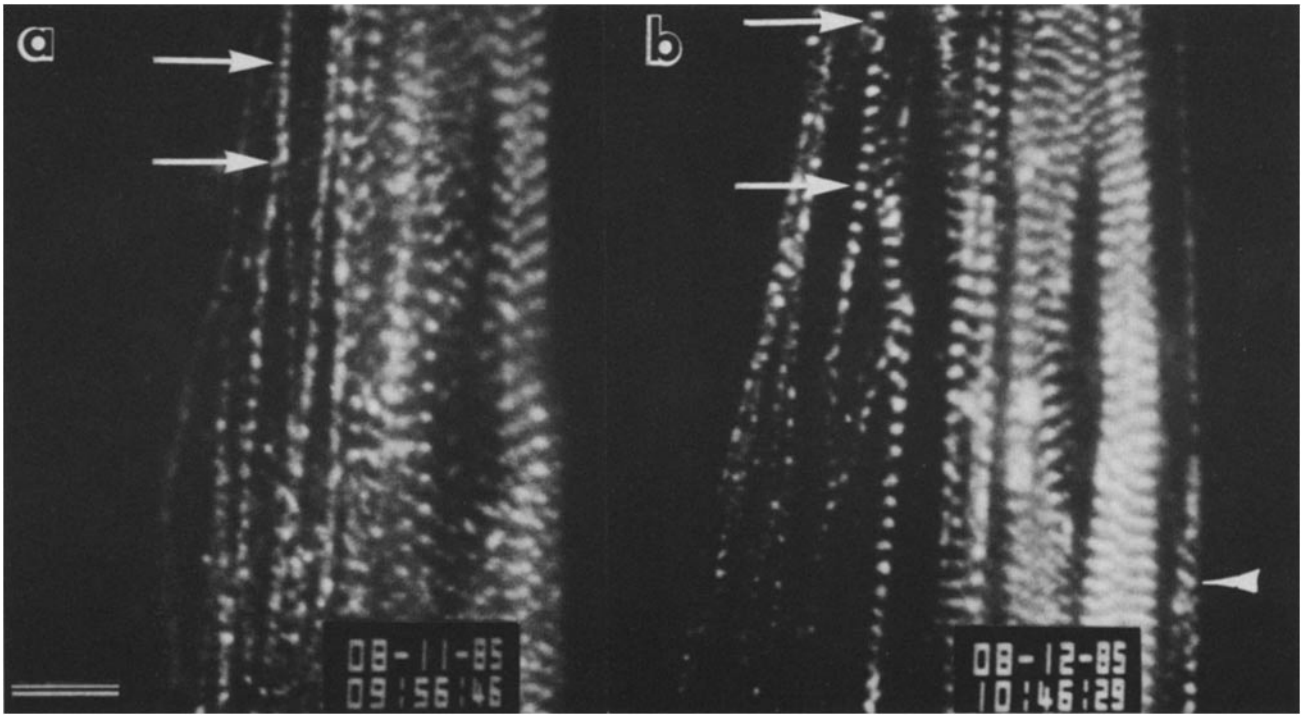

Figure 8. This living myotube was microinjected with rhodamine-labeled alpha-actinin and followed for 5 d. (*a*) Time point 1 d after the initial microinjection. Note the linear series of minisarcomeres (*arrows*). (*b*) Time point ~25 h later. Note the linear series of sarcomeres (*arrows*) that derived from the minisarcomeres. The myofibril on the right side of the myotube (*arrowhead*) has appeared *de novo*. (*a* and *b*) Bar, 10 μm.

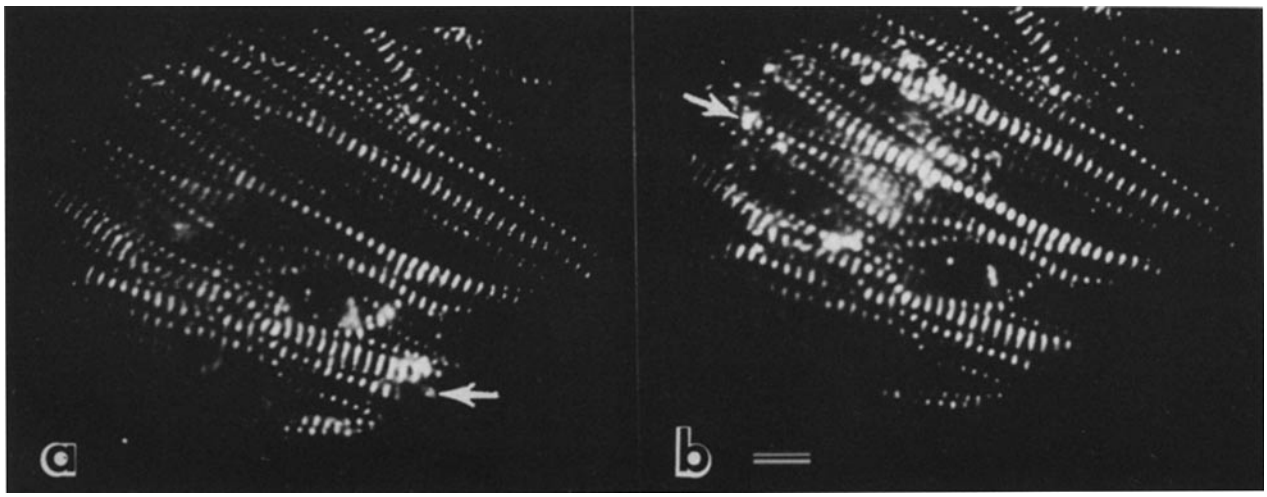

Figure 9. This living 5-d-old cardiac muscle cell recorded at two different wavelengths had been injected the previous day with a mixture of (*a*) skeletal muscle alpha-actinin–LR and (*b*) smooth muscle alpha-actinin–FITC. The patterns of incorporation of the two differently labeled proteins are the same. Note that a fluorescent (LR) granule (*arrow*) in *a* is not visible in *b*, nor is a fluorescent (FITC) granule (*arrow*) in *b* visible in *a*, demonstrating the absence of spillover between the two dyes. These granules are presumably lysosomes. (*a* and *b*) Bar, 10 μm.

FITC and skeletal muscle alpha-actinin–LR were incorporated by the myocytes in identical patterns (Fig. 9), as were mixtures of brain and muscle alpha-actinin. As in myotubes, the myofibrils of injected cells could contract and the distance between fluorescent Z-bands was ~2.3 μm at rest. Most injected myocytes contained, in addition, punctate concentrations of alpha-actinin–LR randomly arrayed in areas of cytoplasm devoid of mature myofibrils (Fig. 9) or aligned in linear arrays (0.5–1.5-μm spacings) (Fig. 10*a*) reminiscent of the fibrils seen in injected myotubes 2–4 d in culture. In spontaneously beating, injected myocytes, only those areas of cytoplasm containing myofibrils with sarcomeres 1.9–2.5 μm long were contractile. Regions of cytoplasm with punctate distributions of alpha-actinin were quiescent and were invariably localized at the periphery of the myocytes. In some cardiac myocytes, fibrils with small particles of alpha-actinin (0.5–1.2 μm spacings) appeared to be directly connected (ligated) to myofibrils with fluorescent Z-bands (Fig. 10, *a* and *b*). Other similar fibrils appeared to be unligated (Fig. 10*a*). In a video recording, the myofibrils in Fig. 10*b* could

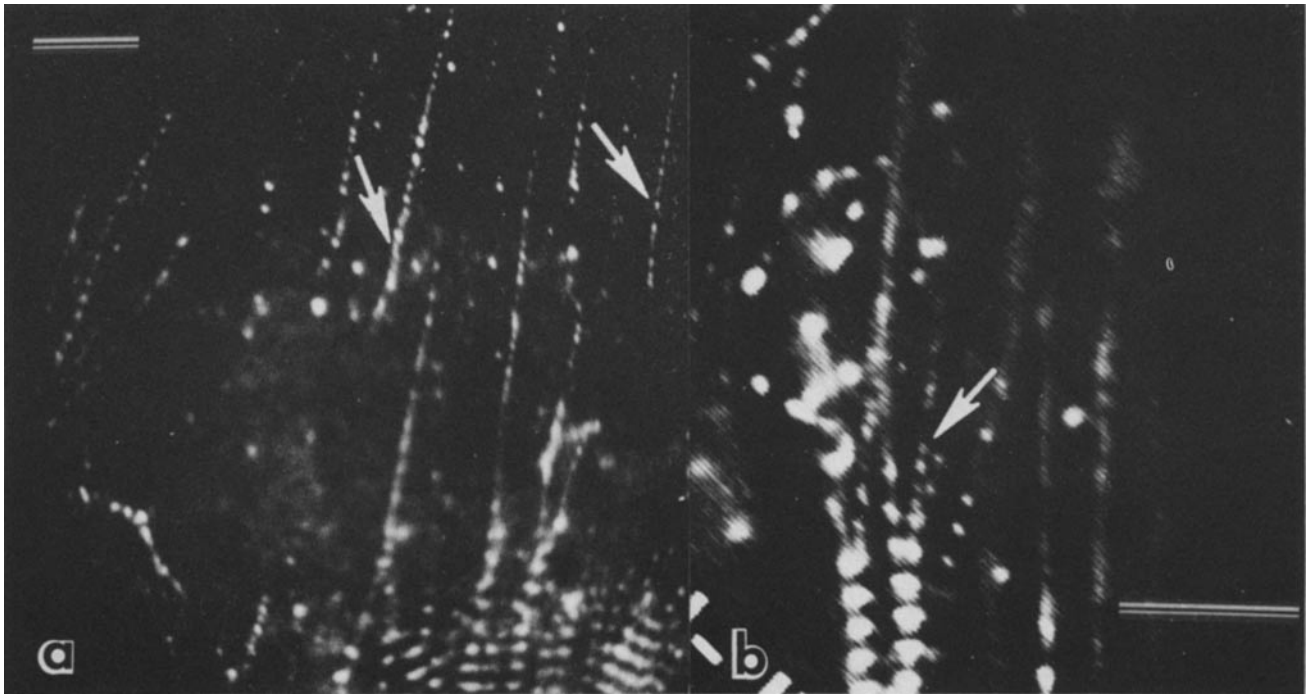

Figure 10. Stills of two different injected cardiac myocytes that were contracting during video recording are seen in *a* and *b*. (*a*) The finely beaded fibrils, some of which were unattached (*arrows*) to the myofibrils seen in the lower part of the figure, were quiescent when the myofibrils were beating. (*b*) The finely beaded fibril (*arrow*) ligated to a myofibril was pulled during contractions of the myofibril. The fluorescent Z-bands could be observed to move closer to one another at each contraction; however, no change could be detected in the relative positions of the small beads of alpha-actinin. (*a*) Bar, 10 μm. (*b*) Bar, 10 μm.

be seen contracting and pulling the fine fibrils that extended from their ends. The fluorescent particles were 0.5–1.2 μm apart along the fibrils and it was not possible to detect whether this distance shortened during contraction as did the distance between the Z-bands of the myofibrils.

Interaction of Alpha-Actinin–LR with Permeabilized Cardiac Myocytes

The association of fluorescently labeled alpha-actin-(LR, FITC, or LY) with permeabilized cardiac myocytes matched that seen when the protein was injected into living cells. Because the cells were extracted and photographed directly on 35-mm film, however, finer detail could be seen in the distribution of alpha-actinin in the permeabilized myocytes (Fig. 11). Fibrils with closely spaced beads of alpha-actinin (0.4–1.5-μm spacings) were concentrated in the peripheral areas of myocytes, aligned parallel to groups of myofibrils (Fig. 11 *a*) and also extending in series from the ends of myofibrils (Fig. 11 *b*). In some cases, particularly behind an extending cell process, the beaded fibrils (0.4–1.5-μm spacings) were unconnected to myofibrils (Fig. 11 *a*). The Z-bands localized in the center of myocytes were usually uniformly fluorescent, whereas more distally positioned Z-bands in the same myofibrils were beaded. Myocytes with up to five ruffles have also been seen with finely periodic fibrils behind each ruffle.

Staining of Myotubes and Cardiac Myocytes with Skeletal Muscle Myosin Antibody

The monoclonal myosin antibody raised against chick skeletal muscle myosin stained the A-bands of isolated adult chicken myofibrils, embryonic cardiac myocytes, and myotubes that had been grown in culture for 5–10 d (Fig. 12). No fibroblasts in either the myotube cultures or the cardiac muscle cultures reacted with the myosin antibody (Fig. 13). Not only did the antibody stain A-bands, it also stained aperiodic fibers in both myotubes and cardiac myocytes. Double labels with myosin antibody and fluorescent alpha-actinin showed that in 2–3-d myotubes, fibers that consisted of closely spaced beads of alpha-actinin (0.5–1.2-μm spacings) (Fig. 12 *a*) also reacted positively in an aperiodic pattern with the muscle myosin antibody (Fig. 12 *b*). In these young myotubes, there was nearly total correspondence between the finely periodic alpha-actinin–positive fibers and the aperiodic myosin-positive fibers. Only occasionally were isolated mononuclear myoblasts found to stain positively with the myosin antibody. Mononuclear cells, however, that had apparently fused with a myotube (e.g., a cell such as Fig. 1 *d*) were often seen with one to two myosin-positive fibers.

Cardiac myocytes showed the same relationship between alpha-actinin–positive fibers and myosin-positive fibers. In areas of the myocytes where alpha-actinin was present in Z-bands (Fig. 12 *c*), myosin antibody stained the A-bands of the myofibrils (Fig. 12 *d*). Where alpha-actinin was present as small beads in the myocyte, the myosin antibody staining was finely filamentous with no discernible periodicity (Fig. 12, *c* and *d*).

Discussion

The microinjection of fluorescently labeled alpha-actinin into living muscle cells has made it possible to observe the growth of myofibrils and to correlate myofibril formation with con-

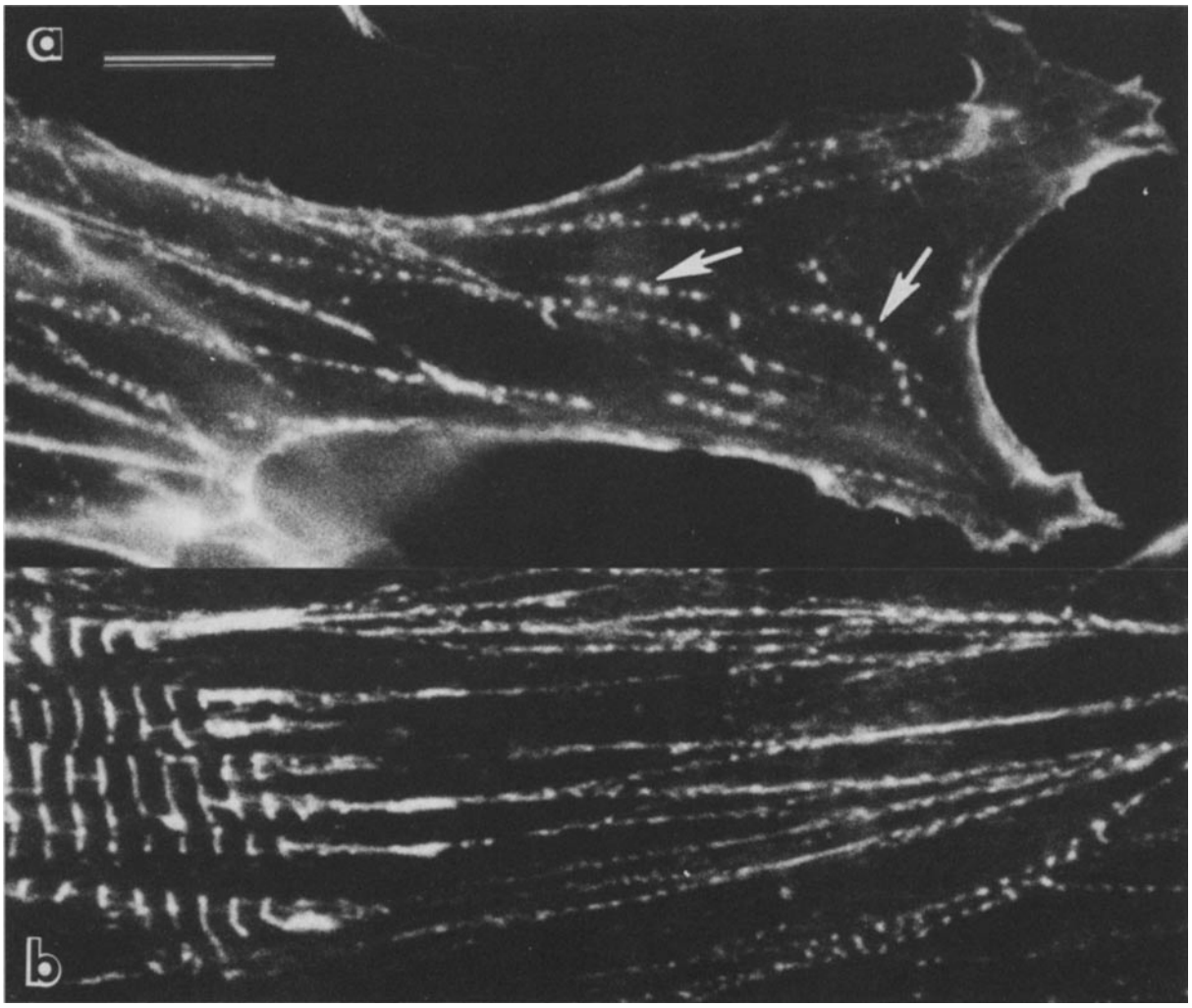

Figure 11. Peripheral regions of two permeabilized myocytes that were exposed to alpha-actinin–LR show that some of the finely beaded fibrils found in these areas are unligated (*arrows* in *a*), whereas others appear to be directly linked to myofibrils containing full-sized sarcomeres (left side of *b*). (*a* and *b*) Bar, 10 μm.

tractility in embryonic myotubes and cardiac myocytes. By following myotube formation day by day, we observed that alpha-actinin initially was incorporated into fibrils in a beaded or punctate pattern of Z-bodies (10) that had a periodicity of 0.3–1.2 μm. Over 4–5 d, the periodicity increased (0.3–2.5 μm) and was accompanied by lateral association of the beaded fibrils resulting in the production of myofibrils with beaded Z-bands spaced 2.0–2.3 μm apart. Contraction was observed only in fibrils with Z-band periodicities of 2.0–2.5 μm. If such myofibrils were present in the same myotube with fibrils having Z-bodies of shorter periodicity, contraction was limited to the myofibrils with fully formed Z-band spacings (Fig. 4). Although fibrils with sarcomeric spacing of alpha-actinin appeared in myotubes after 4–5 d in culture and were present in increasing numbers from this time onward, there were always other fibrils with shorter periodicities present in the same myotubes. These fibrils decreased in number with time in culture and were concentrated predominantly at the ends of the myotubes. Observations of the same microinjected living cell over time revealed the growth of minisarcomeres into fully formed sarcomeres and the *de novo* appearance of myofibrils at the periphery of the myotubes.

In embryonic cardiac myocytes, there was not the transition from noncontractile cell to contractile cell that occurred in myotubes. Rather, peripheral regions of most myocytes contained fibrils with short Z-body periodicities that were not observed to contract and in addition, contained more centrally located contractile myofibrils with full Z-band spacing. We propose that in both the cardiac myocytes and the young myotubes, the noncontractile fibrils with closely spaced Z-bodies are nascent myofibrils whose forming sarcomeres undergo concurrent growth in width (through lateral association of Z-bodies; Figs. 2 *b* and 12) and length (seen as increases in alpha-actinin periodicity; Figs. 5 and 11) to form contractile myofibrils. The ability of alpha-actinin to self-associate in solution (24) as well as to bind to Z-bands of isolated myofibrils (34, 36) may account for the lateral alignment of Z-bodies and the eventual growth in width of Z-bands. An increase in length could occur if the interstitial thick and thin filaments grow longer as they do in insect muscle (1, 2).

The increase in alpha-actinin spacings that we observed in chick myotubes resembles the more dramatic growth in sarcomere size documented over 20 yr ago by Aronson (1) in developing mite muscle. Polarization microscopy was used to

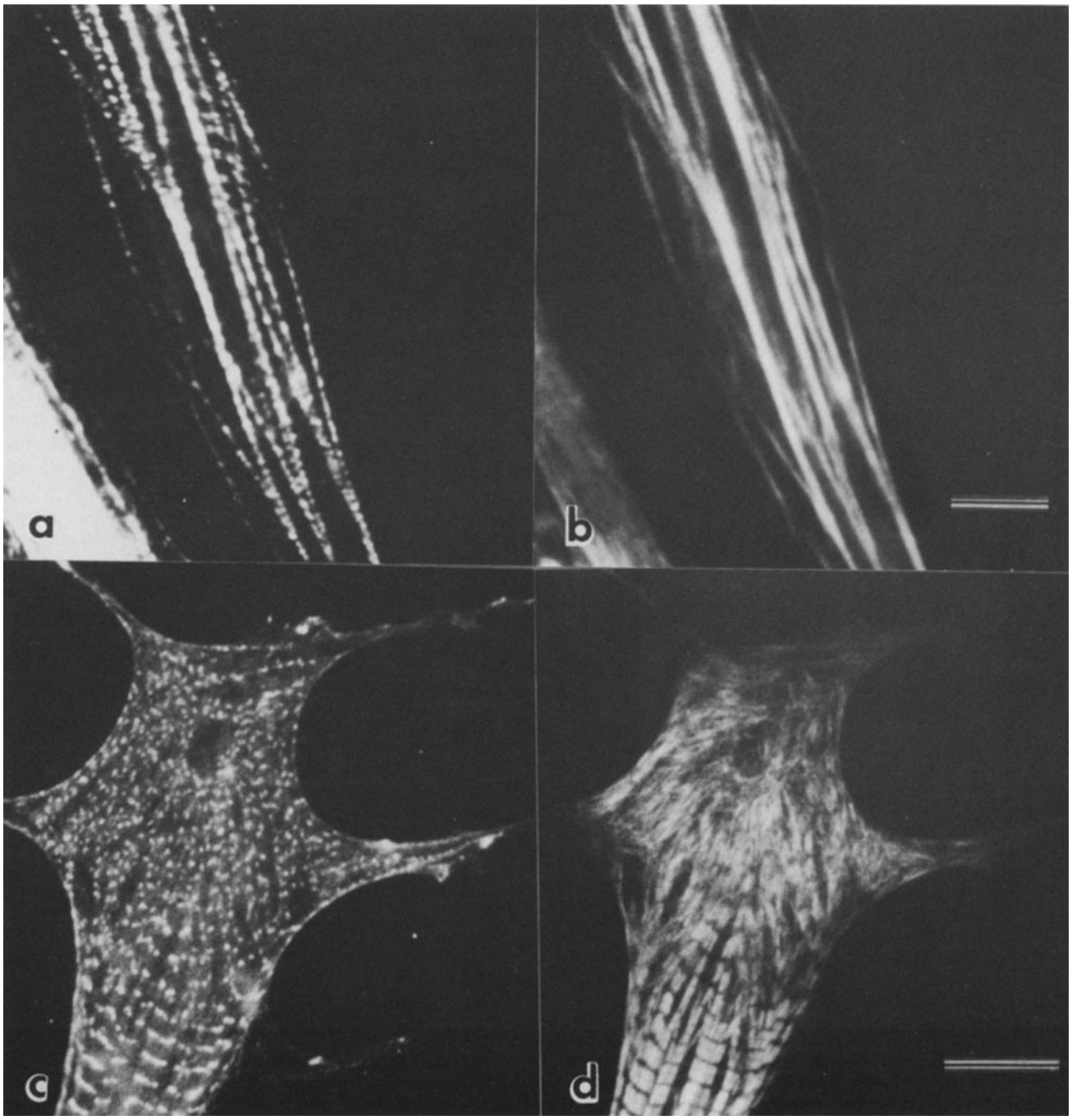

Figure 12. Coordinate localization of alpha-actinin and myosin in myotubes (*a* and *b*) and cardiac myocytes (*c* and *d*). (*a*) 3-d-old myotubes that were microinjected with alpha-actin–LR and then 3 h later, were permeabilized, show incorporation of alpha-actinin in fine beads typical of this state of myotube development. (*b*) The permeabilized myotubes in *a* were incubated with a monoclonal antibody against skeletal myosin (second antibody having FITC label). All the fibrils with a beaded pattern of alpha-actinin–LR stained in a continuous pattern with the myosin antibody. (*c*) A 4-d-old cardiac myocyte was permeabilized and stained with alpha-actinin–LR has fully formed sarcomeres in the lower part of the micrograph and a distribution of fine beads of alpha-actinin in the top part of the cell. (*d*) The same cell as in *c* after staining with the skeletal myosin antibody (FITC label) reveals A-band staining in the lower part of the cell and staining in the top part of the cell that appears to be in fibers coincident with the beaded arrays of alpha-actinin–LR seen in the top part of the cell in (*c*). (*a* and *b*) Bar, 10 μm; (*c* and *d*) Bar, 10 μm.

record A-bands and sarcomere length over a 40-h period in larval muscle in situ. During this time, the sarcomere length increased from 2.5 μm to 10 μm with the A-band growing to three times its initially observed length (1.3–4.6 μm) and the I-band growing approximately five times. Only when the sarcomeres reached their final length of 10 μm did contractions occur. Auber (2), in an ultrastructural study of embryonic insect muscle (*Calliphora erythrocephala*), reported a twofold growth in sarcomere length over 5 d (1.8 μm on day 5, 3.7 μm on day 10). In this time period the A-band grew

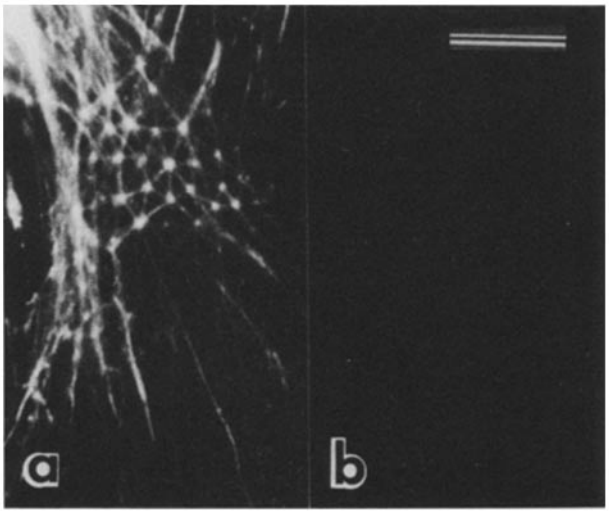

Figure 13. Cardiac fibroblast, permeabilized and doubly stained with (*a*) fluorescein-labeled alpha-actinin and (*b*) a monoclonal antibody against skeletal myosin (rhodamine-labeled rabbit anti-mouse antibody). The muscle myosin antibody does not stain the cytloskeleton in the nonmuscle cell. (*a* and *b*) Bar, 10 μm.

from 1.6 to 3.0 μm while the thin filaments grew from 0.9 to 1.5 μm.

The sarcomeres of adult vertebrate muscle are only 2.3 μm long and the A-bands 1.5 μm long; thus if A-band and sarcomere growth occur in these muscles, it would be very difficult to detect. Reports that the appearance of striated muscle in vertebrates is preceded by aperiodic myofibrils date from 1903 (reviewed in 13, 17). The short periodicites we have detected by injecting alpha-actinin–LR or exposing permeabilized cells to the fluorescent protein cannot be detected by phase microscopy. Electron microscopy of thin sections through nascent myofibrils have revealed Z-bodies in a number of vertebrate systems (13), but no previous studies have shown a progressive increase in spacing between longitudinal arrays of Z-bodies during vertebrate myogenesis. The time sequence studies of microinjected myotubes in our studies have revealed the growth of minisarcomeres to fully formed sarcomeres over a 24-h period..

In some respects, the punctate alpha-actinin localization we have seen in myotubes in the first few days in culture and in the peripheral cytoplasm of cardiac myocytes resembles that seen in stress fibers (38). In cultured rat myotubes, Jockusch and Jockusch (19) described a "stress fiber-like" pattern of alpha-actinin antibody staining in 3-d-old myotubes and at the end of a 7-d-old myotube. Gard and Lazarides (15) showed similar antibody staining in a 3-d chick myotube doubly stained with desmin and alpha-actinin antibodies. Based on electron microscopic evidence (26) and immunofluorescence-staining results (7, 11, 20), a number of investigators have proposed that stress fibers provide a template on which myofibrils form (7, 11, 20, 26). We do not believe that the fibrils present in young myotubes (e.g., Fig. 2, *c* and *d*), at the ends of older myotubes (Fig. 4*c*), and near the periphery of cardiac myocytes (e.g., Fig. 12) are stress fibers serving as templates for myofibril formation, but rather that they are nascent myofibrils. In the first place, the periodicity of alpha-actinin along the fibrils in the myotubes grows longer with time of development. Alpha-actinin periodicities in stress fibers can vary between the fibers in an individual cell, but there is a characteristic range of periodicities for all cells of a given cell type (38). The temporal progression in length of alpha-actinin periodicity in myotubes is unlike anything reported in stress fibers.

Secondly, the monoclonal antibody to skeletal muscle myosin used in this study reacted with the same fibrils in young myotubes and in the peripheral cytoplasm of cardiac myocytes that also contain closely spaced beads of alpha-actinin (Fig. 12). This monoclonal antibody did not stain any of the stress fibers in fibroblasts in the same cultures but did stain the A-bands in fully formed myofibrils in both myotubes and cardiac myocytes (Fig. 12). These antibody results are in agreement with the finding of Kulikowski and Manasek (20) that polyclonal skeletal myosin antibody stained solid fibrils in the peripheral cytoplasm of spreading cardiac myocytes, as well as A-bands of myofibrils that formed subsequently in the cells. Fallon and Nachmias (11) and Dlugosz et al. (7), however, reported two separate populations of fibrils in developing myotubes (11) and cardiac myocytes (7), one of which reacted with nonmuscle myosin antibody and the other of which reacted with skeletal myosin antibody. Occasionally there was a small region of overlap in staining by the two antibodies, but in the main, the nonmuscle antibody stained only mononucleated cells and fibrils predominantly in the ends of the myotubes (11) and fibrils termed "stress fiber-like" in the peripheral cytoplasm of cardiac myocytes (7). Antibody against skeletal myosin stained only centrally localized fibrils that often exhibited sarcomeric subunits in myotubes (11), as well as the A-bands of fully formed myofibrils in cardiac myocytes (7).

It is surprising that the polyclonal skeletal myosin antibodies in these studies (7, 11) reacted with only a fraction of the fibrils in the embryonic muscle cells, whereas the monoclonal antibody in this study reacted with all the fibrils. If either of the polyclonal antibodies reacted only weakly with some fibrils, this may have been recorded as an absence of binding. The skeletal myosin antibody of Dlugosz et al. (7), for example, did not stain broad A-bands in some cases (see upper left myofibril in their Fig. 3, *e* and *f*). By localizing both alpha-actinin and myosin in the same myotube or cardiac myocyte (Fig. 12), we have shown that all alpha-actinin–containing fibrils also contain skeletal muscle myosin. Although we believe that both stress fibers and myofibrils are organized in a sarcomeric pattern (38), we do not think that the definition of stress fiber includes fibers that contain muscle myosin. A possibility that we cannot address from our results is that all fibrils contain both a nonmuscle myosin as well as skeletal muscle myosin. If this were the case, the fibrils in these embryonic muscle cells would be stress fiber-myofibril hybrids. In any case, our results do not support the theory that stress fibers serve as transitory templates along which myofibrils form (7). The model of Dlugosz et al. for cardiac myocytes (7) requires that the alpha-actinin spacing along the stress fiber template be the same as the myofibril Z-band spacing (2.0–2.3 μm), yet much smaller alpha-actinin periodicities are found along the peripheral myocyte fibrils (0.3–1.5 μm) that are the proposed stress fiber templates. The model also requires that myofibrils form along preexisting stress fibers, but if such were the case, there should be some fibrils in the myocyte peripheral cytoplasm that do not react positively with skeletal myosin antibody, i.e., templates along

which nascent myofibrils have not yet begun to form. Instead we find that all fibrils containing alpha-actinin also react with monoclonal antibody against skeletal muscle myosin. A further problem with stress fibers serving as templates for myofibrils is the question of what provides the template for the sarcomeric organization of the stress fibers.

Alpha-actinins from skeletal muscle, smooth muscle, and brain are similar in molecular weight and subunit composition and in their cross-reactivity with antibodies against skeletal muscle alpha-actinin (4, 9, 10). They all differ, however, in amino acid composition and peptide maps. Further, brain and other nonmuscle alpha-actinins, unlike the muscle alpha-actinins, exhibit diminished ability to bind to F-actin in the presence of calcium (8).

Despite these differences in alpha-actinins, both living, microinjected cells and permeabilized cells are indiscriminate in their incorporation of different forms of alpha-actinin. Injected nonmuscle cells incorporate smooth muscle alpha-actinin into stress fibers (29, 37) and also incorporate nonmuscle and striated muscle alpha-actinins in identical patterns (unpublished observations). In living cells injected with the smooth muscle isoform, fluorescent alpha-actinin cycles from stress fiber to cleavage furrow and back to stress fibers during mitosis, and remains incorporated in cells for at least 11 d (37). All three types of muscle—cardiac, skeletal, and smooth—incorporate injected smooth muscle alpha-actinin into myofibrils (29) and, as shown in this paper, skeletal and cardiac myocytes also use nonmuscle and skeletal muscle alpha-actinins with apparently equal efficiency. The same patterns of incorporation of smooth muscle alpha-actinin occur if the nonmuscle (35), smooth (29), cardiac, or skeletal muscle (this study) cells are permeabilized rather than injected. Nonmuscle and skeletal muscle alpha-actinins also give the same results with permeabilized muscle and nonmuscle cells (data not shown), extending previous observations (16, 34–36) that fluorescently labeled alpha-actinin binds to all sites in permeabilized cells where antibodies indicate that endogenous alpha-actinin is localized. Thus, although different cells synthesize distinct isoforms of alpha-actinin, they are capable, whether living or permeabilized, of utilizing either the nonmuscle, smooth muscle, or the skeletal muscle form. These results parallel those of McKenna et al. (25) who showed that cardiac myocytes and fibroblasts incorporated microinjected actin in myofibrils and stress fibers, respectively, regardless of whether the labeled actin was prepared from a muscle or nonmuscle source.

Despite the ability of living muscle cells to incorporate nonmuscle forms of alpha-actinin and actin into myofibrils, it appears from immunofluorescence studies that nonmuscle isoforms of these proteins are absent from myofibrils but present along the membrane of the muscle cells (10, 22). Endo and Masaki (10) found that skeletal muscle-specific alpha-actinin antibody stained Z-bands in 5-d chick myotubes, and in younger fused myoblasts, stained smaller Z-bodies that were spaced the same distance apart as the Z-bands, i.e., 2.5 μm. These Z-bodies, however, did not stain with their smooth muscle alpha-actinin antibody, whereas structures along the myotube membrane and fibroblast stress fibers in the same culture did.

The discrepancy between the immunofluorescence findings and the microinjection experiments suggests that perhaps the controls used by muscle cells to compartmentalize isoforms of alpha-actinin or actin are in some way bypassed when an exogenous supply of protein is introduced via microinjection. On the other hand, the microinjection studies also suggest that if the synthesis of one contractile protein isoform is replaced during development by another (as is the case with myosin [28]), the muscle cell would be able to incorporate the new isoform into exisiting myofibrils without the need to break them down and build new ones. If this occurs it should be possible to detect, at certain stages of development, two isoforms of protein within a single myofibril.

In addition to the postulated growth in sarcomere length and the increase in myofibril width, there may also be ligation of fibrils in myotubes and cardiac myocytes. When alpha-actinin–LR was injected into early myotubes, it sometimes diffused into mononucleated cells that had fused with the injected myotube (Figs. 2*d* and 3). Nascent myofibrils appeared to run directly between the mononuclear cells and the myotube, suggesting that a nascent myofibril from the fused mononuclear cell had become ligated to a myofibril in the myotube. Equally possible is that preexisting fibrils in the myotube branched and extended into the recently fused cell. Capers (5), in fact, demonstrated in a cinè study that when a mononuclear cell fused with a myotube, the thin mononuclear cell process expanded in thickness over a period of 3 h, as if cytoplasm from the myotube moved into the newly fused cell. In peripheral areas of cardiac myocytes, unligated fibrils are also seen in both injected (Fig. 10*a*) and permeabilized (Fig. 11*a*) cells. Microinjection studies that include monitoring injected cardiac myocytes and myotubes intermittently over many hours should make it possible to observe directly such processes as ligation of fibrils, growth of sarcomeres, and myofibril formation.

Two courses, one headed by Dr. Shinya Inouè, Marine Biological Laboratory, on light microscopic techniques, and the other, headed by Dr. Adolf Grässmann, Frei Universität Berlin, on microinjection techniques, were of great value in carrying out aspects of this work. We thank Theresa Grigsby for her expert and patient help in the word processing of this manuscript. We also acknowledge John M. Sanger and Neil R. Bigioni for their capable photographic assistance during the course of this work.

This research was supported by grants from the National Institutes of Health (HL-15835 to the Pennsylvania Muscle Institute and GM-25653 to J. W. Sanger and J. M. Sanger) and the National Science Foundation (DMB 82-19920).

Received for publication 15 April 1985, and in revised form 25 February 1986.

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