Visualization of Myosin in Living Cells

Balraj Mittal, Jean M. Sanger, and Joseph W. Sanger

Laboratory for Cell Motility Studies, Department of Anatomy and The Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6058

Abstract. Myosin light chains labeled with rhodamine are incorporated into myosin-containing structures when microinjected into live muscle and nonmuscle cells. A mixture of myosin light chains was prepared from chicken skeletal muscle, labeled with the fluorescent dye iodoacetamido rhodamine, and separated into individual labeled light chains, LC-1, LC-2, and LC-3. In isolated rabbit and insect myofibrils, the fluorescent light chains bound in a doublet pattern in the A bands with no binding in the cross-bridge–free region in the center of the A bands. When injected into living embryonic chick myotubes and cardiac myocytes, the fluorescent light chains were also incorporated along the complete length of the A band with the exception of the pseudo–H zone. In young myotubes (3–4 d old), myosin was localized in aperiodic as well as periodic fibers. The doublet A band pattern first appeared in 5-d-old myotubes, which also exhibited the first signs of contractility. In 6-d and older myotubes, A bands became increasingly more aligned, their edges sharper, and the separation between them (I bands) wider. In nonmuscle cells, the microinjected fluorescent light chains were incorporated in a striated pattern in stress fibers and were absent from foci and attachment plaques. When the stress fibers of live injected cells were disrupted with DMSO, fluorescently labeled myosin light chains were present in the cytoplasm but did not enter the nucleus. Removal of the DMSO led to the reformation of banded, fluorescent stress fibers within 45 min. In dividing cells, myosin light chains were concentrated in the cleavage furrow and became reincorporated in stress fibers after cytokinesis. Thus, injected nonmuscle cells can disassemble and reassemble contractile fibers using hybrid myosin molecules that contain muscle light chains and nonmuscle heavy chains. Our experiments demonstrate that fluorescently labeled myosin light chains from muscle can be readily incorporated into muscle and nonmuscle myosins and then used to follow the dynamics of myosin distribution in living cells.

THE most ordered of the actin-myosin–based contractile systems is found in striated muscle where arrays of myosin filaments interdigitate with thin filaments that are anchored at one end to Z bodies (18). This tripartite arrangement of myosin, thin filaments, and anchoring sites exists in the contractile apparatus of smooth muscle and nonmuscle cells as well (4, 23, 36). One approach to studying these systems in living cells has been to inject fluorescently labeled components of the contractile apparatus and to study their localization and changes therein as muscle and nonmuscle cells locomote, divide, and differentiate in culture (8, 21, 34, 51). Fluorescently labeled actin and tropomyosin have served as markers for thin filaments in injected cells (7, 21, 51, 54) and labeled alpha-actinin and filamin have served the same function for attachment sites (8, 27, 34, 35). The force-generating component of the contractile apparatus, myosin, has proved to be the most difficult of the contractile proteins to label fluorescently and to inject. Composed of a dimer of heavy chains (each has a molecular weight of 200,000) with four noncovalently bound light chains (with molecular weights ranging from 16,000 to 22,000), muscle myosin is 160 nm long and soluble in high salt (18, 50). The component light chains, however, are soluble in low salt (50). In this paper we report that the isolated light chains of myosin from chicken fast skeletal muscle can be labeled with the fluorescent dye iodoacetamido rhodamine (IAR)[1] and injected into living muscle and nonmuscle cells. There, the light chains become incorporated into myofibrils, stress fibers, and cleavage furrows, thus providing a probe for following changing patterns of myosin distribution in living cells.

A preliminary report of these results was presented at the General Scientific Meetings of the Marine Biological Laboratory (1986, *Biol. Bull. [Woods Hole]*, 171:470–471).

Materials and Methods

Purification of Myosin

Myosin was isolated from chicken breast muscle by the procedure described by Margossian and Lowey (24). Actin and phosphofructokinase were removed from crude myosin by ammonium sulfate precipitation. To remove C, M, and X proteins, myosin was further purified by chromatography on DEAE-Sephadex (28, 48).

1. *Abbreviations used in this paper*: IAR, iodoacetamido rhodamine; LC-1, LC-2, and LC-3, light chains 1, 2, and 3; S-1, myosin subfragment 1.

Isolation and Labeling of Myosin Light Chains and Alpha-Actinin

Mixed light chains were isolated from pure myosin by first denaturing the heavy chains with urea (50). Myosin was dissolved in 0.6 M KCl to a concentration of 20 mg/ml. After addition of an equal volume of 8 M urea (ultrapure; Schwartz-Mann Biotech, Cleveland, OH), 50 mM Tris-HCl, 10 mM EDTA, and 5 mM dithiothreitol (DTT) (pH 8.0), the solution was stirred gently for 1 h at room temperature. The myosin heavy chains were then precipitated by dilution with 10 vol of cold distilled water. All subsequent steps were carried out at 4°C unless otherwise noted. After centrifugation at 10,000 *g* for 30 min, the supernatant containing dissociated myosin light chains was loaded on a column (DE-52; Whatman Inc., Clifton, NJ) equilibrated with 50 mM Tris-HCl (pH 8.0). The bound light chains were eluted from the column with 1 M KCl in the Tris buffer, dialyzed overnight against 10 mM potassium phosphate (pH 6.7) containing 0.1 mM DTT, and subsequently lyophilized.

The mixed myosin light chains were labeled by a modification of the procedure by Marsh and Lowey (25). The light chains were dissolved in 7 M guanidine hydrochloride containing 0.5 M Tris-HCl, 1 mM DTT, and 5 mM EDTA (pH 8.0), and solid IAR (Research Organics, Cleveland, OH) was added to a final concentration of 19 mM. The mixture was stirred in the dark for 90 min at room temperature under a gentle stream of nitrogen. Labeling was terminated by addition of beta-mercaptoethanol to a concentration of 0.20 M. The free unreacted dye was largely removed by extensive dialysis against PBS (10 mM potassium phosphate, 0.1 M KCl, pH 7.0). The solution was then centrifuged at 10,000 *g* for 15 min to remove dye aggregates. The supernatant was passed through a column (G-25 Sephadex; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 50 mM imidazole-HCl buffer (pH 8.0). The colored fractions eluting in the void volume were loaded directly on a DE-52 column (1.6 × 15 cm) also equilibrated with imidazole-HCl buffer. The elution of bound proteins with a linear gradient of 0–0.35 M NaCl in the imidazole buffer (total vol 200 ml) resulted in separation of light chains into three fractions: light chain 1 (LC-1), light chain 2 (LC-2), and light chain 3 (LC-3), in that order. There was usually some overlap in the elution profile of LC-1 and LC-2 and LC-2 and LC-3. By judiciously avoiding the overlapping fractions, the three labeled light chains could be separated in a single step. All the fractions from the DE-52 column were pooled for mixed light chains. The pooled fractions were dialyzed against 10 mM potassium phosphate (pH 7.0) containing 0.1 mM DTT, lyophilized, and stored at −20°C.

LC-2 (also called DTNB light chain) was also selectively dissociated from purified myosin, using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (50). DTNB was removed by extensive dialysis against Tris buffer with DTT. The LC-2 was then labeled with IAR as described for the mixed light chains. Any contamination with LC-1 and LC-3 was removed by chromatography on DEAE-cellulose in the imidazole buffer. The purity of isolated light chain preparations was determined by SDS-PAGE (22). The gel was fixed in methanol/acetic acid and photographed in UV light and then stained with Coomassie Blue (Fig. 1). No free dye was ever detected at the gel front. Myosin subfragment 1 (S-1) was prepared with chymotrypsin from chicken skeletal muscle (24).

Alpha-actinin was prepared from chicken gizzards and labeled with FITC or lissamine rhodamine sulfonyl chloride as described previously (44).

Reaction of Fluorescent Myosin Light Chains with Isolated Myofibrils and Synthetic Myosin Filaments

Myofibrils were prepared from glycerinated rabbit psoas muscle and insect (*Hyalophora cecropia*) flight muscles (43). They were rinsed with standard salt (0.1 M KCl, 0.01 M phosphate buffer, 0.001 M $MgCl_2$, pH 7.0), reacted with fluorescently labeled myosin light chains (0.5 mg/ml, 10 mM potassium phosphate, pH 7.0, 0.02% sodium azide, 1 mM DTT) either in suspension or after they had been attached to a coverslip and placed in a humidified chamber. Reaction with myosin light chain was carried out for periods ranging from 1 to 30 min at 4°C, followed by rinsing with standard salt to remove unbound light chains.

Synthetic myosin filaments were prepared from rabbit skeletal muscle by dialysis of the myosin (2 mg/ml) against 0.1 M KCl, 10 mM potassium phosphate (pH 7.0) as previously described (41). Two drops of the solution of synthetic filaments were added to a coverslip, resulting in the attachment of filaments to the coverslip. 20 μl of labeled myosin light chains (0.5 mg/ml in the same solution used for myofibrils) were added to the myosin filaments for 1–15 min in a humidified chamber in the cold (4°C). At the end of the reaction time, the coverslips were rinsed with cold standard salt and mounted as previously reported (46). The procedure for frozen sections of rat striated muscle was similar to that described for filamin or actin (7, 27).

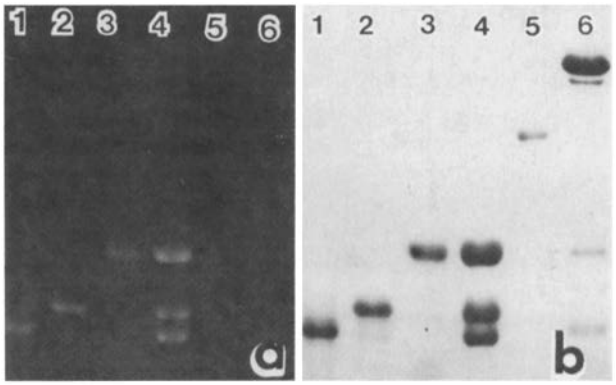

Figure 1. SDS-PAGE (12% acrylamide) of the IAR-labeled myosin light chains. (*a*) Fluorescence of the separated labeled light chains, LC-1, LC-2, and LC-3 in lanes *1*, *2*, and *3*, respectively, and the total labeled light chain fraction in lane *4*. (*b*) Coomassie Blue staining of the gel in *a* shows the labeled light chains in lanes *1–4*, unlabeled G-actin standard in lane *5*, and unlabeled myosin S-1 fraction in lane *6*. The S-1 was prepared by chymotrypsin digestion, which degrades LC-2 (24), but not LC-1 and LC-3, as can be seen in lane *6*.

Injection of Myosin Light Chains into Living Muscle and Nonmuscle Cells

All cells were grown on 22 mm^2 glass coverslips placed in 30 mm culture dishes. Gerbil fibroma and PtK_2 cells, an epithelial line, were obtained from American Type Culture Collection (Rockville, MD). Cardiac myocytes were isolated from 5–6-d chick embryo hearts (42) and skeletal myotubes, from 10-d chick embryo breast muscle (10). All cells were injected while in the culture dish using a pressure injection apparatus described previously (30, 39). The labeled myosin light chains were microinjected into cells at a concentration of 2 mg/ml (in 10 mM potassium phosphate, pH 7.0, 0.02% sodium azide, 1 mM DTT). After being injected, cells were returned to the CO_2 incubator for 5–12 h before being mounted on glass slides for microscopic examination. For experiments using DMSO, the medium was replaced with new medium containing DMSO (final concentration, 10%). Cells were exposed to this agent for 30–60 min and then returned to normal culture medium (47).

Microscopy

Myofibrils and injected cells were examined with epifluorescence using a Zeiss Planapochromat 100X, NA 1.3 phase objective and either a Zeiss Photomicroscope III or an Olympus Vanox microscope. Myofibrils were photographed directly from the microscope on Kodak Tri X film that was developed in Acufine for 1000 ASA. Injected cells were viewed with the aid of a SIT camera (Dage-MTI, Michigan City, IN) and a processing system (Image-I; Interactive Video Systems, Inc., Concord, MA). The images were summed for 128 frames and some were subjected to a convolution operation with a high pass filter to improve detection of the fluorescent signal (19). Images were photographed from the video monitor with a 35-mm camera fitted with a 50-mm Olympus macro lens and Kodak Plus X film, ASA 125 (30, 39).

Results

The total fraction of myosin light chains (LC-1, LC-2, LC-3) isolated from chicken skeletal muscle and labeled with IAR bound only to the A bands of glycerinated rabbit myofibrils and insect myofibrils (Fig. 2). Binding was in a doublet pattern with an absence of fluorescence in the cross-bridge–free region in the center of the A band. Within 1 min of exposure to the labeled light chains, myofibrils exhibited the A band

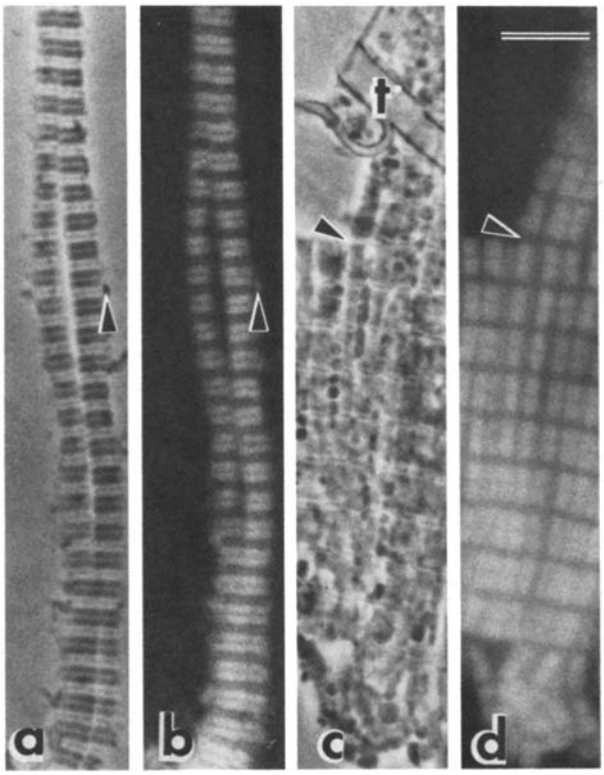

Figure 2. Isolated myofibrils attached to coverslips and exposed to the IAR-labeled mixed light chain fraction. (*a* and *b*) Phase and fluorescence of rabbit psoas myofibrils. Arrowheads point to a dirt speck adjacent to one of the Z bands. (*c* and *d*) Phase and fluorescence of myofibrils of insect flight muscle. Arrowheads point to a row of Z bands. A tracheid (*t*) visible in the phase does not bind the fluorescent light chains. In both types of muscle there is a doublet staining pattern in the A band. The nonstaining band in the center of the A band appears to be wider in the rabbit myofibrils than in the insect. All micrographs are the same magnification in order to emphasize the different A band sizes in a vertebrate versus an invertebrate muscle (1.6 vs. 3.3 μm). Bar, 10 μm.

fluorescence. If the myofibrils were pretreated with unlabeled myosin light chains and then exposed to the IAR light chains, the same pattern of fluorescence was observed. Myofibrils exposed to individual light chains (LC-1, LC-2, or LC-3) exhibited the same doublet A band binding as obtained with the total fraction of the myosin light chains. The light chains did not bind to nuclei, mitochondria, or tracheids in the insect muscle (Fig. 2, *c* and *d*). Frozen sections of rat skeletal muscle also bound the light chains in patterns identical to those of isolated glycerinated myofibrils. The labeled light chains bound in a continuous pattern to synthetic myosin filaments after as short an exposure as 1 min.

When labeled myosin light chains were injected into contractile embryonic myotubes, incorporation also occurred in a doublet along the full length of the A bands (Fig. 3 *a*). Injection of each of the individual light chains, LC-1, LC-2, and LC-3, produced similar patterns of incorporation (Fig. 3, *b*, *c*, and *d*). There was no binding of labeled myosin light chains to anything other than the A bands. Co-injection of fluorescein-labeled alpha-actinin and rhodamine-labeled myosin light chains into myotubes produced complimentary patterns of incorporation (Fig. 4, *a* and *b*); FITC–alpha-actinin in the Z bands and IAR light chains in the A bands. Embryonic cardiac myocytes incorporated labeled light chains in the same pattern as that observed in microinjected myotubes (Fig. 4, *c* and *d*). Spontaneous contractions were observed in both types of muscle cells after incorporation of injected myosin light chains into the A bands. The fluorescent A bands did not lose their labeled light chains during contractions.

When fluorescent myosin light chains were injected into myotubes on the third day in culture, no contractile response occurred during injection nor were the myotubes observed to contract spontaneously. The fluorescent light chains were localized both in an aperiodic pattern and in small periodic bands spaced about 0.8 μm apart in fibers adjacent to the myotube membrane (Fig. 5 *a*). In addition, round aggregates of myosin light chains were dispersed in the cytoplasm and

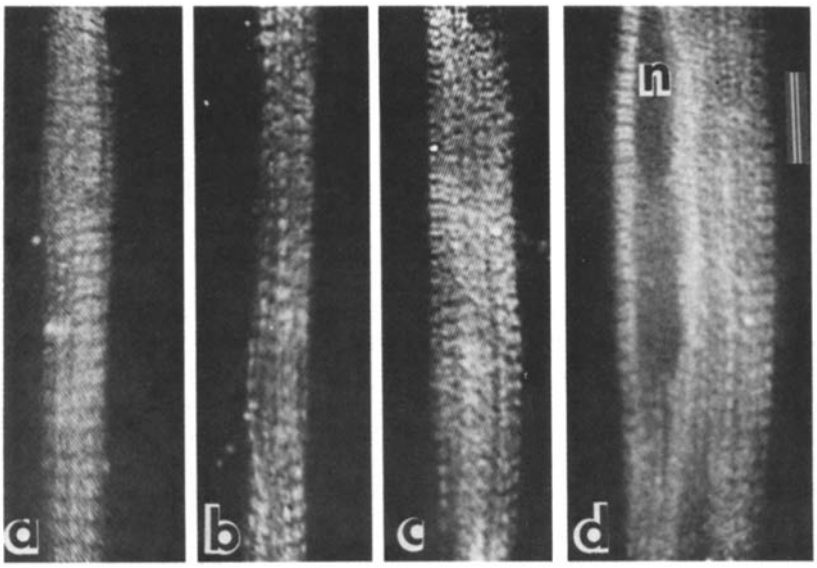

Figure 3. Living embryonic chick myotubes injected with IAR light chains after 7 d in culture. (*a*) Mixture of all three light chains; (*b*) LC-1; (*c*) LC-2; and (*d*) LC-3. In each case, the light chains are incorporated along the full length of the A band, with the exception of the center, where cross-bridges are absent. Spontaneous contractions were observed in many of the injected myotubes. No label was found in the nuclei (*n* in *d*). Bar, 10 μm.

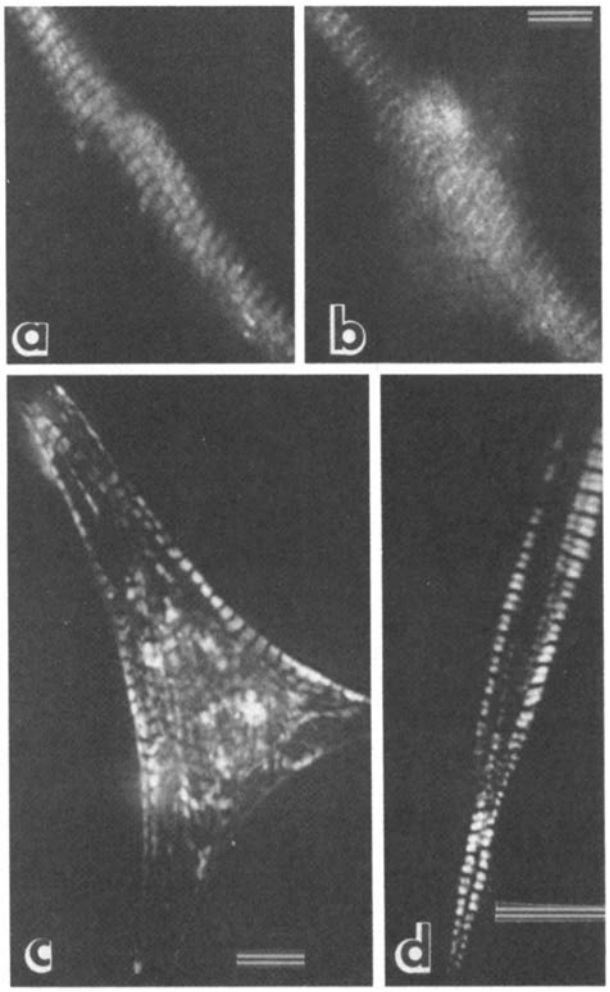

Figure 4. Living embryonic chick myotube co-injected with IAR light chains (*a*) and FITC–alpha-actinin (*b*). Fluorescent bands show complimentary patterns of localization as expected from the A and Z band localization of the two proteins. Living embryonic cardiac myocytes injected with IAR myosin light chains (*c* and *d*). At higher magnification (*d*) A band doublet incorporation is clearly discernable. Bars, 10 μm.

there was a diffuse distribution of myosin light chain fluorescence throughout the myotube. On day 4 in culture only rarely did an injected myotube contract when injected and none were observed to contract spontaneously. These myotubes incorporated the fluorescent light chains into fibers that were sometimes aperiodic but often appeared banded (Fig. 5 *b*). Bright round aggregates of myosin light chains and a diffuse cytoplasmic fluorescence were also characteristic of injected myotubes at this stage. By day 5 in culture most myotubes contracted when injected, and most of the light chain fluorescence was present in fibers by 5 h after injection (Fig. 5 *c*). There was little diffuse background fluorescence and few granules. A banded pattern was resolvable in nearly all fibers, with most of the bands composed of doublets of fluorescence identical to the A bands of older myotubes (Fig. 3). The doublet pattern was first seen most clearly in the 5-d myotubes when the video image, obtained by summing 128 frames, was exposed to a high pass filter to sharpen the image (Fig. 5 *c*). In such images small singlet bands of fluorescence were also seen along some fibers, similar to those seen in 3- and 4-d myotubes. Fluorescent myosin light chains were also localized in the actively moving ruffles at the ends of young myotubes (Fig. 5 *c*). In injected myotubes from 6 d onward, A band doublets were easily resolved (Fig. 3). The A bands also became increasingly more aligned, their edges sharper, and the separation between them (I bands) wider (cf. Fig. 3 *d* and Fig. 5 *c*). Bright granules and background fluorescence were absent, and strong spontaneous contractions were often observed, during which the fluorescent A bands moved closer together.

The labeled myosin light chains isolated from skeletal muscle were also incorporated in a banded pattern in stress fibers of injected nonmuscle cells (Fig. 6, *a* and *b*). The periodic bands of fluorescence were spaced closer together in PtK_2 cells, an epithelial line, than they were in gerbil fibroma cells. As in myofibrils, the individual light chains, LC-1, LC-2, and LC-3, gave the same pattern of incorpora-

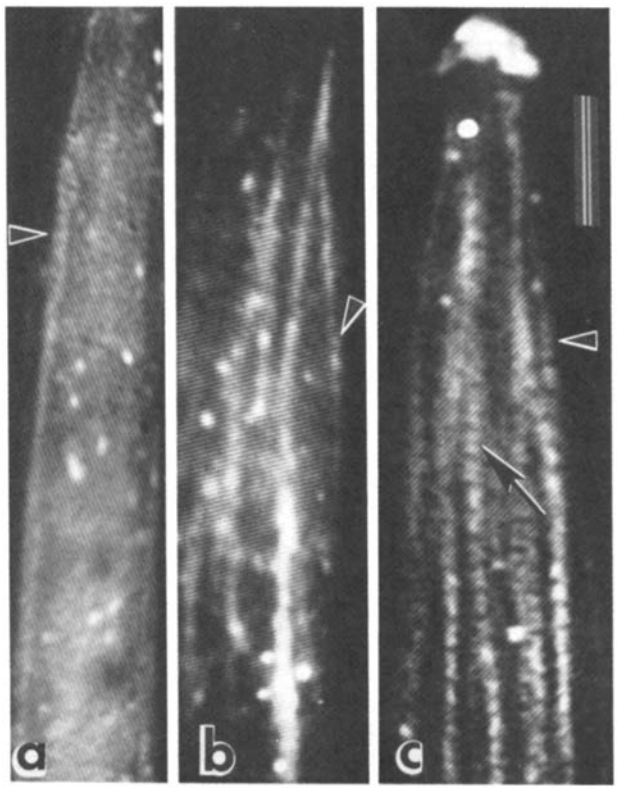

Figure 5. Living chick embryonic myotubes injected with IAR myosin light chains. (*a*) In myotubes injected on day 3 in culture, myosin light chains are incorporated in small periodic bands (*arrowhead*) in fibers near myotube membrane. There is also a high background of fluorescence in the cytoplasm and bright aggregates. (*b*) In myotubes injected on day 4 in culture there is a greater number of fibers containing periodic bands of fluorescence (*arrowhead*). Fluorescent aggregates and diffuse myotube fluorescence are also present in these myotubes. (*c*) In myotubes injected on day 5 in culture, most of the fluorescence is present in periodic bands of ~1.6 μm. In *c* spatial filtration (see Materials and Methods) was carried out on a previously summed image. The doublet composition of the fluorescent bands is seen more easily in this sharpened image (*arrow* in *c*). An arrowhead in *c* points to a region of one fiber where small bands of fluorescence similar to those seen in younger myotubes (*a* and *b*) are visible. A concentration of myosin fluorescence is apparent in the ruffle at the end of the myotube. Bar, 10 μm.

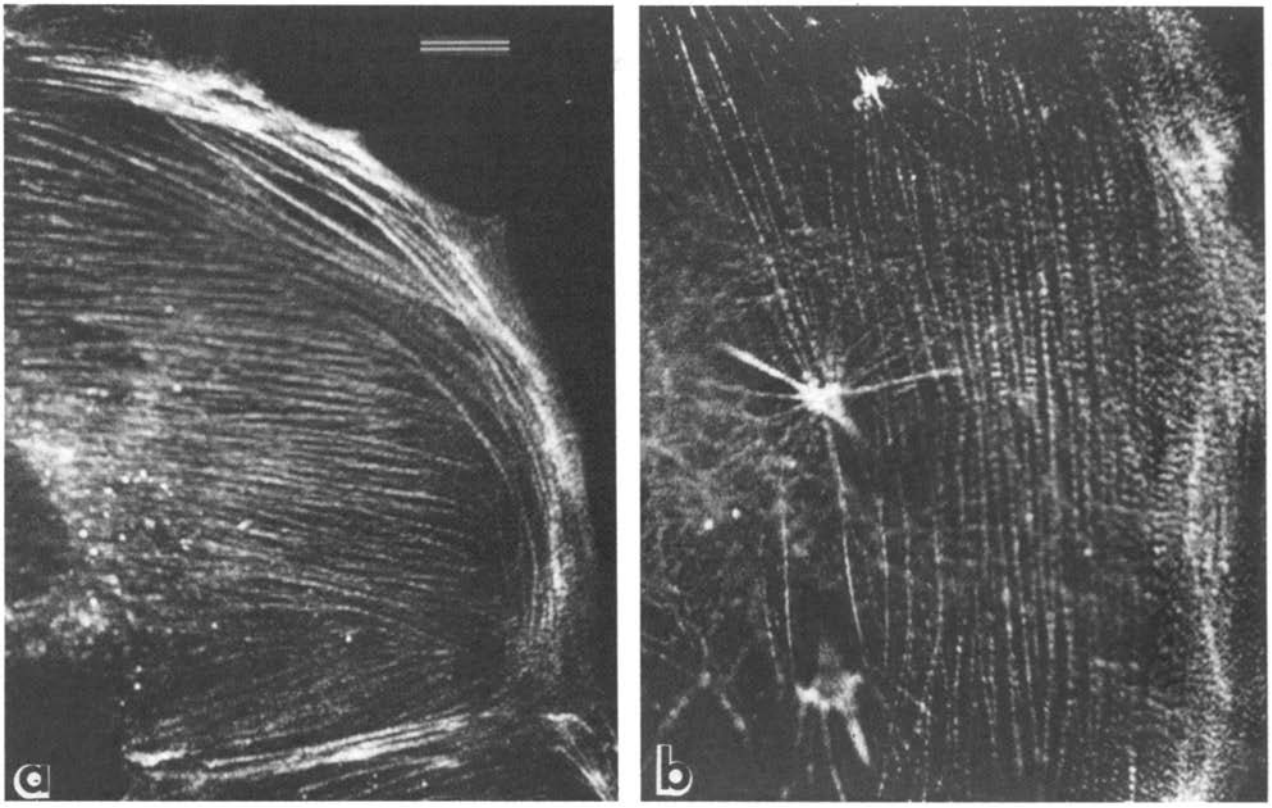

Figure 6. Living nonmuscle cells injected with fluorescent light chains. (*a*) PtK_2 cell showing striated pattern of myosin localization along the stress fibers. (*b*) Gerbil fibroma cell in which periodicity of myosin light chain fluorescence can be seen to be longer than in the epithelial PtK_2 shown at the same magnification in *a*. Bar, 10 μm.

tion when injected individually as did the total light chain fraction. A doublet pattern of fluorescence was not evident in stress fibers as it was in myofibrils. In cells having a polygonal network arrangement of stress fibers, the injected myosin light chains were absent from the foci of the networks but present in a striated pattern in the fibers connecting the foci. In contrast to these results, microinjection of fluorescently labeled alpha-actinin into gerbil fibroma cells resulted in the incorporation of alpha-actinin into the foci and attachment plaques.

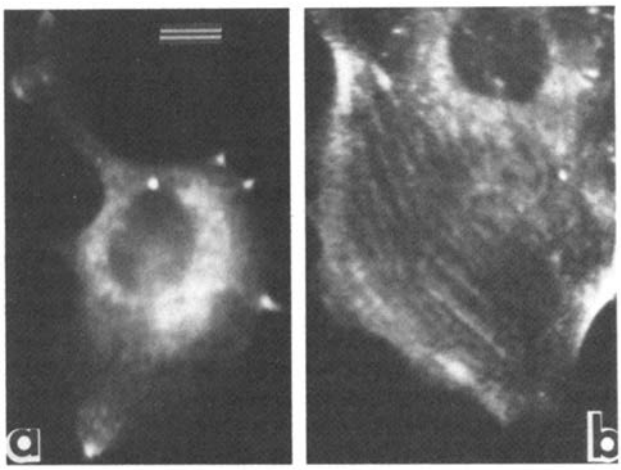

Figure 7. Two PtK_2 cells that were injected with IAR light chains and 5 h later exposed to 10% DMSO, a disrupter of stress fibers. (*a*) After 60 min in DMSO, myosin light chain fluorescence can be seen in aggregates in the cytoplasm but is absent from the nucleus. (*b*) 60 min after removal of DMSO myosin light chain fluorescence is found in a banded pattern in the reformed stress fibers. Bar, 10 μm.

Exposure of nonmuscle cells 5 h after injection with fluorescent light chains to 10% DMSO caused breakdown of stress fibers and a redistribution of the fluorescent myosin light chains into several aggregates at the cell periphery and a more diffuse disposition in the cytoplasm (Fig. 7 *a*). Fluorescent myosin light chains did not enter the nuclei of the treated cells. Approximately 30 min after removal of DMSO from the culture medium, the stress fibers that reformed contained periodic bands of fluorescent myosin light chains (Fig. 7 *b*).

Stress fiber breakdown was also observed in injected gerbil cells at the onset of prophase, leading to a diffuse distribution of fluorescence in the cytoplasm. Myosin light chain fluorescence was initially dispersed uniformly in the mitotic cell, becoming concentrated between the separating chromosomes in anaphase (Fig. 8 *a*). Before furrowing was apparent, a bright band of myosin fluorescence was visible beneath the membrane where the cleavage furrow would form (Fig. 8, *b* and *c*). This submembrane band of myosin fluorescence persisted during cytokinesis (Fig. 8, *d–f*) and was seen as a ring in images focused near the furrow membrane (Fig. 8 *e*). At the end of cytokinesis, fluorescent periodic stress fibers reformed in the daughter cells.

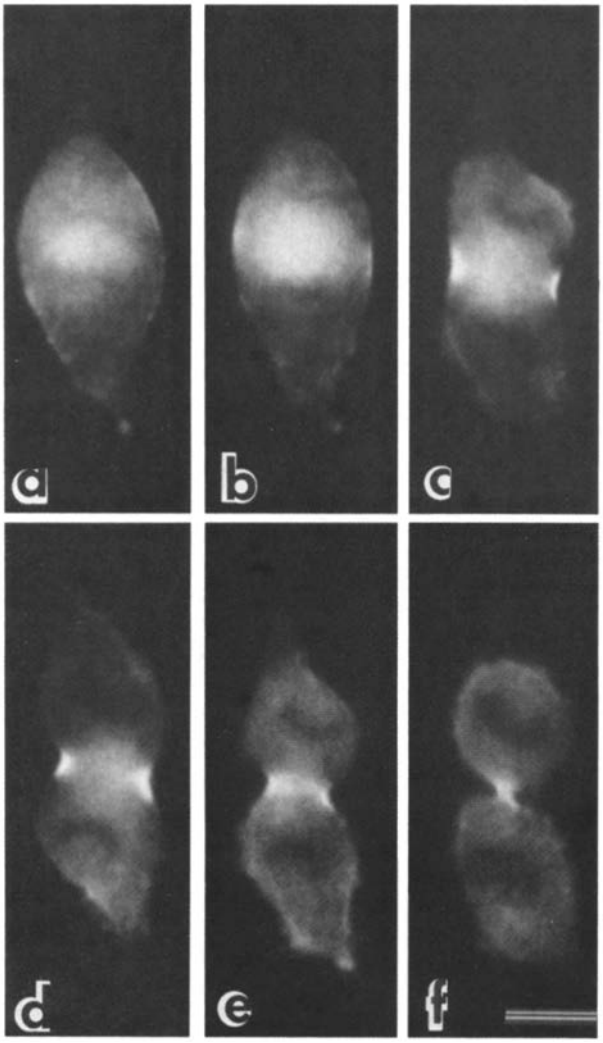

Figure 8. Gerbil fibroma cell injected 6 h before with IAR myosin light chains and recorded intermittantly while undergoing mitosis. During anaphase, myosin light chain fluorescence is brightest between the separating chromosomes. When the chromosomes have moved to the poles (*b*) a bright line of fluorescence is visible beneath the membrane where the cleavage furrow will form. As cytokinesis progresses (*c–f*), bright bands of myosin fluorescence remain beneath the furrow membrane, forming a ring that is best seen when the ring grows smaller and the focal plane is close to the membrane. The elapsed time between micrographs is approximately 2 min. Bar, 10 μm.

Discussion

The ability to detect rapid exchange of fluorescently labeled myosin light chains with the native light chains of isolated myofibrils is impressive in view of the fact that in vitro studies with intact myosin molecules have shown exchange of light chains only at 37°C in the presence of 10 mM ATP (5, 29). However, Pastra-Landis and Lowey (29) detected alkali light chain exchange at 37°C and 10 mM ATP only if LC-2 was first removed from the myosin molecule. Our studies using fluorescence as a probe indicate that exchange of all three light chains can occur in intact myofibrils in the cold and in the absence of ATP. The doublet pattern of myosin light chain binding in the A band is a reflection of the alignment of the bipolar thick filaments (18). The nonfluorescent zone in the middle of the A bands results from the lack of cross-bridges and, hence, light chain binding sites there (18). The nonstaining region of the A band in rabbit myofibrils (1.6 μm long) (18) appears wider than that of the moth myofibril (3.3 μm long) (41) (Fig. 2). This variation may result from a different packing of the overlapping myosin molecules in the two different muscle types.

The A band binding of the labeled light chains that we observed appears to result from an exchange with native myosin light chains for several reasons. If it were due to replacement of native light chains lost during myofibril preparation, pretreatment of the myofibrils with unlabeled light chains should have blocked the subsequent addition of fluorescent light chains and it did not. The fluorescent light chains also bound in A band doublets in frozen sections of skeletal muscle where loss of native light chains should be minimal. Light chains, furthermore, are not known to self-associate on the myosin molecule nor are more than two light chains known to associate with each myosin heavy chain (49, 50). In living myotubes and cardiac myocytes injected with labeled light chains, fluorescent A bands could not be seen for 3–5 h after injection. We assume that this is the time required for enough injected light chains to become incorporated so that the fluorescent signal in the A band can be resolved over the background fluorescence of unincorporated light chains.

Further support for our conclusion that the fluorescent light chains exchange with endogenous light chains to become incorporated into the cells' myosin molecules comes from experiments with injected cells and DMSO. When this drug was used to induce the breakdown of stress fibers in injected PtK_2 cells, fluorescent light chains remained in the cytoplasm (Fig. 7 *a*). Molecules <45,000 in molecular weight, such as actin, have been shown to enter the nuclei after DMSO treatment, whereas larger molecules, such as tropomyosin, alpha-actinin, and myosin do not (47). Light chains with molecular weights in the range of 16,000–22,000 should have entered the nuclei of DMSO-treated cells, were they not associated with myosin heavy chains. In addition, we have noticed that in the first hour after injection of fluorescent myosin light chains, there is fluorescence present in nuclei but there is no evidence of nuclear fluorescence several hours later.

The labeled, individual light chains associated with the A bands of isolated myofibrils and living muscle and with nonmuscle cells in the same way as did the total light chain fraction. The endogenous light chains of rabbit myofibrils are identical to those from chicken skeletal muscle, the source of labeled light chains for this study. Thus, the light chain exchange that produced A band fluorescence in the rabbit myofibrils may have arisen exclusively from exchange between homologous light chains of rabbit and chicken origin. In the insect myofibrils, the embryonic muscle cells, and the nonmuscle cells, endogenous light chains differ from those in adult chicken skeletal muscle (3, 50). Thus, exchange between heterologous light chains must have occurred after microinjection of these cells with labeled chicken light chains (or in the case of insect myofibrils, after incubation with the labeled light chains). In solution, hybrid myosin molecules have been formed by the exchange of light chains at 10–16-fold molar excess with myosin or S-1 (3, 51). Assuming an injection volume of 10^{-14}–10^{-13} liters (15), we inject

0.6×10^6–6×10^6 myosin light chain molecules/cell. Based on an actin concentration of 10 mg/ml in nonmuscle cells (20) and a myosin/actin ratio of 1:100 (31), there are $\sim 2.4 \times 10^6$ myosin molecules or 9.6×10^6 myosin light chain molecules/cell. Thus, the number of fluorescent light chains introduced is 6–60% of a nonmuscle cell's endogenous light chains. In embryonic myotubes and cardiac myocytes the endogenous myosin concentration is probably 100-fold greater (31) and thus in these cells the injected light chains represent tracer amounts. Nevertheless, in injected cells, both muscle and nonmuscle, the incorporation of heterologous, fluorescent light chains into myosin molecules permitted qualitatively normal contractile activity: ruffling, cytokinesis, sarcomere contraction.

The ability of disparate light chain isoforms to become incorporated into intact stress fibers and myofibrils lends further support to the suggestion that the switch from one contractile protein isoform to another during development (3) can occur without the need for disassembly and subsequent reassembly of existing contractile units (34, 35). The fact that labeled light chains exchange so readily also suggests an alternative interpretation for other experiments carried out with fluorescently labeled myosin molecules (33). In those studies, fluorescence energy transfer between disparately labeled synthetic myosin filaments was cited as evidence that myosin molecules exchanged between synthetic thick filaments. Based on our results, it is likely that light chain exchanges may have contributed to the observed fluorescence energy transfer.

It has long been noted in studies of myofibrillogenesis that the initial fibers are aperiodic and located near the cell surface (9, 13, 16, 17, 53). The pattern of myosin light chain incorporation that we have observed during myofibril development in myotubes follows a progression from predominantly aperiodic on days 3 and 4 in culture (Fig. 5, *a* and *b*) to definitively banded with doublet substructure by day 6 in culture (Fig. 3). Evidence of a banded localization of the myosin in the injected myotubes can be seen as early as day 3, however. These first bands are much closer together (0.8 μm) along the fibers than A bands and show no evidence of a doublet substructure. By day 5 (Fig. 5 *c*), broader doublet bands coexist with smaller bands (arrow in Fig. 5 *c*). Clearly defined fluorescent doublets that are coincident with phase-dense A bands are found in the majority of myotubes injected after 5 d in culture. It appears that the progression in A band formation involves an increasing alignment of myosin filaments until the pseudo–H zones (the mid region of myosin filaments that lack light chains) are in register and can be visualized as a dark line in the middle of the doublets of myosin light chain fluorescence. Accompanying the alignment of myosin filaments in the A bands is an increase in I band length, a growth in width of the individual myofibrils, and an increase in the alignment of adjacent myofibrils with one another.

Our previous studies of myofibrillogenesis, combining injection of fluorescent alpha-actinin with myosin antibody labeling, led us to conclude that sarcomeres, as measured by alpha-actinin patterns, grow in length during their development into fully formed contractile units (34, 35, 38, 45). When short nascent sarcomeres were seen after injection of fluorescent alpha-actinin, myosin antibody localization indicated that there was a continuous distribution of muscle myosin along the length of these 3- and 4-d-old noncontractile myofibrils. We assumed that thick filaments of myosin overlapped one another along these myofibrils and we had no evidence from the antibody labeling that shorter than adult-sized A bands might be present. In developing insect muscles, Aronson (1) and Auber (2) showed that A band growth is an integral part of myofibrillogenesis. The small bands of myosin fluorescence seen in some fibers of 3-, 4-, and 5-d-old injected myotubes (Fig. 5) may be short A bands, and the aperiodic fluorescence may be due to an overlapping of nascent A bands. More work is necessary with co-injections of alpha-actinin and myosin coupled to electron microscopy of these cells in order to resolve this issue.

The differences we have recorded in the myosin band patterns in injected epithelial and fibroblastic cells (Fig. 6, *a* and *b*) is compatible with other evidence, on both fixed and injected cells, that stress fibers have a sarcomeric arrangement of contractile proteins (14, 35, 36, 40, 52) whose spacings are longer in fibroblastic cells than in epithelial cells (35, 36, 46). The incorporation of myosin light chains in discrete bands in stress fibers of living cells also suggests that stress fiber myosin is localized in the form of filaments such as those detected by electron microscopy using special fixation techniques (23, 40). The absence of fluorescent light chains in the foci of polygonal networks is also in agreement with myosin antibody studies showing an absence of myosin from these areas (32). In neither gerbil fibroma nor PtK_2 cells were we able to discern a doublet pattern in the myosin light chain fluorescence, although Langanger et al. (23) have demonstrated convincingly with immunoelectron microscopy that the myosin filaments in stress fibers are bipolar, and furthermore, with immunofluorescence these authors could detect a doublet pattern in the myosin bands. Our inability to detect doublet substructure is probably due to the decreased resolution obtained with a SIT video camera at very low light levels.

The localization of myosin in a previously injected, cleaving cell (Fig. 8) is similar to patterns found in cells fixed and exposed to myosin antibodies (11). Myosin-like filaments, among oppositely polarized actin filaments (40), have been demonstrated ultrastructurally in the cleavage furrow of tissue culture cells (26). Following an individual microinjected cell through mitosis shows that when furrow contraction begins asymetrically, myosin fluorescence is brighter on the edge that begins to contract first. A similar bright band appears when the other side begins to contract. As the cleavage furrow pinches down, the width of the band of myosin fluorescence becomes narrower (Fig. 8, *b–f*). Fluorescent alpha-actinin, ovalbumin, and BSA show similar interzonal spindle localization (Fig. 8 *a*) in injected cells in culture (37, 51), perhaps because this area of the spindle is large in volume and excludes most cell organelles. Unlike ovalbumin and BSA, injected alpha-actinin remains concentrated in the cleavage furrow during cytokinesis (37) as does myosin (Fig. 8, *b–f*), and both proteins become reincorporated into stress fibers after cytokinesis (37). Thus the injected myosin light chains prepared from chicken skeletal muscle do not inhibit contraction in these nonmuscle cells, and, as in the case of DMSO-treated cells (Fig. 7), the hybrid myosin molecule can be reincorporated into stress fibers that have disassembled and subsequently reassembled.

In summary, our results indicate that the position of myo-

sin in living cells can be followed by the injection of fluorescently labeled muscle myosin light chains. With this development, the force-producing component of actin-myosin–based contractile systems can now be traced in living cells and its dynamic distribution correlated with those of thin filaments (using labeled actin or tropomyosin) (21, 54) and the molecules that serve to anchor or cross-link them (labeled alpha-actinin, filamin, talin, or vinculin) (6, 8, 12, 27).

The authors thank Ms. Judy Jackson for her efficient and cheerful formatting of this manuscript from the handwritten draft to the finished typed form.

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

Received for publication 29 April 1987, and in revised form 20 May 1987.

References

1. Aronson, J. 1961. Sarcomere size in developing muscles of a tarsonemid mite. *J. Biophys. Biochem. Cytol.* 11:147–156.
2. Auber, J. 1969. La myofibrillogènese du muscle strié. I. Insectes. *J. Microsc. (Paris).* 8:197–232.
3. Bandman, E. 1985. Myosin isoenzyme transitions in muscle development. *Int. Rev. Cytol.* 97:97–131.
4. Bond, M., and A. V. Somlyo. 1962. Dense bodies and actin polarity in vertebrate smooth muscle. *J. Cell Biol.* 95:403–413.
5. Burke, M., and M. Sivaramakrishnan. 1981. Subunit interactions of skeletal muscle myosin and myosin subfragment 1. Formation and properties of thermal hybrids. *Biochemistry.* 20:5908–5913.
6. Burridge, K., and L. Connell. 1983. A new protein of adhesion plaques and ruffling membranes. *J. Cell Biol.* 97:359–367.
7. Dome, J. S., B. Mittal, M. B. Pochapin, J. M. Sanger, and J. W. Sanger. 1987. Incorporation of fluorescently labeled actin and tropomyosin into muscle cells. *Cell Differ.* In press.
8. Feramisco, J. R. 1979. Microinjection of fluorescently labeled alpha-actinin into living fibroblasts. *Proc. Natl. Acad. Sci. USA.* 76:3967–3971.
9. Fischman, D. A. 1972. Development of striated muscle. *In* The Structure and Function of Muscle. Vol. I. G. H. Bourne, editor. Academic Press Inc., New York. 75–148.
10. Fishbach, G. D. 1972. Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* 28:407–429.
11. Fujiwara, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage, and mitotic spindle of human cells. *J. Cell Biol.* 71:848–875.
12. Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18: 193–205.
13. Godlewski, E. 1902. Die Entwicklung des Skelet- und Herzmuskelgewebes der Saugethiere. *Arch. Mikr. Anat. Entwicklungsmechanik.* 60:111–156.
14. Gordon, W. E. 1978. Immunofluorescent and ultrastructural studies of "sarcomeric" units in stress fibers of cultured non-muscle cells. *Exp. Cell Res.* 117:253–260.
15. Grässmann, A., M. Grässmann, and C. Müller. 1980. Microinjection of early SV40 DNA fragments and T antigen. *Methods Enzymol.* 65:816–825.
16. Heidenhain, M. 1913. Über die Entstehung der Querqestreiften Muskelsubstanz bei der Forelle. *Arch. Mikr. Anat.* 83:427–447.
17. Holtzer, H., J. M. Marshall, and H. Finck. 1957. An analysis of myogenesis by the use of fluorescent antimyosin. *J. Biophys. Biochem. Cytol.* 3: 705–724.
18. Huxley, H. E. 1983. Molecular basis of contraction in cross-striated muscles and relevance to motile mechanisms in other cells. *In* Muscle and Nonmuscle Motility. A. Stracher, editor. Academic Press, Inc., New York. 1–104.
19. Inoué, S. 1986. Video Microscopy. Plenum Publishing Corp., New York. 1–584.
20. Kreis, T., and W. Birchmeier. 1982. Microinjection of fluorescently labeled proteins into living cells with emphasis on cytoskeletal proteins. *Int. Rev. Cytol.* 75:209–227.
21. Kreis, T. E., K. H. Winterhalter, and W. Birchmeier. 1979. *In vivo* distribution and turnover of fluorescently labeled actin microinjected into human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 76:3814–3818.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.
23. Langanger, G., M. Moeremans, G. Daneels, A. Sobieszek, M. DeBrabander, and J. DeMey. 1986. The molecular organization of myosin in stress fibers of cultured cells. *J. Cell Biol.* 102:200–209.
24. Margossian, S. S., and S. Lowey. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85(Part B): 55–71.
25. Marsh, D. J., and S. Lowey. 1980. Fluorescence energy transfer in myosin subfragment-1. *Biochemistry.* 19:774–784.
26. Maupin, P., and T. D. Pollard. 1986. Arrangement of actin filaments and myosin-like filaments in the contractile ring and of actin-like filaments in the mitotic spindle of dividing HeLa cells. *J. Ultrastruc. Mol. Struc. Res.* 94:92–103.
27. Mittal, B., J. M. Sanger, and J. W. Sanger. 1987. Binding and distribution of fluorescently labeled filamin in permeabilized and living cells. *Cell Motil. Cytoskeleton.* In press.
28. Offer, G., C. Moos, and R. Starr. 1973. A new protein of the thick filaments of vertebrate skeletal myofibrils: extraction, purification and characterization. *J. Mol. Biol.* 74:653–676.
29. Pastra-Landis, S. C., and S. Lowey. 1986. Myosin subunit interactions. Properties of the 19,000 dalton light chain-deficient myosin. *J. Biol. Chem.* 261:14811–14816.
30. Pochapin, M., J. M. Sanger, and J. W. Sanger. 1983. Microinjection of lucifer yellow CH into sea urchin eggs and embryos. *Cell Tissue Res.* 234:309–318.
31. Pollard, T. D. 1981. Cytoplasmic contractile proteins. *J. Cell Biol.* 91: 156s–165s.
32. Rathke, P. C., M. Osborn, and K. Weber. 1979. Immunological and ultrastructural characterization of microfilament bundles: polygonal nets and stress fibers in an established cell line. *Eur. J. Cell Biol.* 19:40–48.
33. Saad, A. D., J. D. Pardee, and D. A. Fischman. 1986. Dynamic exchange of myosin molecules between thick filaments. *Proc. Natl. Acad. Sci. USA.* 83:9483–9487.
34. Sanger, J. M., B. Mittal, M. B. Pochapin, and J. W. Sanger. 1986. Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. *J. Cell Biol.* 102:2053–2066.
35. Sanger, J. M., B. Mittal, M. Pochapin, and J. W. Sanger. 1986. Observations of microfilament bundles in living cells microinjected with fluorescently labeled contractile proteins. *J. Cell Sci.* 5(Suppl.):17–44.
36. Sanger, J. M., B. Mittal, and J. W. Sanger. 1985. Structure and assembly of microfilament bundles. *In* Cell Motility: Mechanism and Regulation. H. Ishikawa, H. Sato, and S. Hatano, editors. University of Tokyo Press, Tokyo. 461–476.
37. Sanger, J. M., B. Mittal, M. B. Pochapin, and J. W. Sanger. 1987. Stress fiber and cleavage furrow formation in living cells microinjected with fluorescently labeled alpha-actinin. *Cell Motil. Cytoskeleton.* 7:209–220.
38. Sanger, J. M., B. Mittal, A. Wegner, B. M. Jockusch, and J. W. Sanger. 1987. Differential response of stress fibers and myofibrils to gelsolin. *Eur. J. Cell Biol.* 43:421–428.
39. Sanger, J. M., M. B. Pochapin, and J. W. Sanger. 1985. Midbody sealing after cytokinesis in embryos of the sea urchin *Arbacia punctulata*. *Cell Tissue Res.* 240:287–292.
40. Sanger, J. M., and J. W. Sanger. 1980. Banding and polarity of actin filaments in interphase and cleaving cells. *J. Cell Biol.* 86:568–575.
41. Sanger, J. W. 1971. Formation of synthetic myosin filaments: influence of pH, ionic strength, cation substitution, dielectric constant and method of preparation. *Cytobiologie.* 4:450–466.
42. Sanger, J. W. 1977. Mitosis in beating cardiac myoblasts treated with cytochalasin-B. *J. Exp. Zool.* 201:463–469.
43. Sanger, J. W., B. Mittal, and J. M. Sanger. 1984. Analysis of myofibrillar structure and assembly using fluorescently labeled contractile proteins. *J. Cell Biol.* 98:825–833.
44. Sanger, J. W., B. Mittal, and J. M. Sanger. 1984. Interaction of fluorescently labeled contractile proteins with the cytoskeleton in cell models. *J. Cell Biol.* 99:918–928.
45. Sanger, J. W., B. Mittal, and J. M. Sanger. 1984. Formation of myofibrils in spreading chick cardiac myocytes. *Cell Motil.* 4:405–416.
46. Sanger, J. W., J. M. Sanger, and B. M. Jockusch. 1983. Differences in the stress fibers between fibroblasts and epithelial cells. *J. Cell Biol.* 96:961–969.
47. Sanger, J. W., J. M. Sanger, T. E. Kreis, and B. M. Jockusch. 1980. Reversible translocation of cytoplasmic actin into the nucleus caused by dimethyl sulfoxide. *Proc. Natl. Acad. Sci. USA.* 77:5268–5272.
48. Starr, R., and G. Offer. 1982. Preparation of C-protein, H-protein, X-protein, and phosphofructokinase. *Methods Enzymol.* 85(Part B):130–138.
49. Szent-Gyorgyi, A. G., E. M. Szentkiralyi, and J. Kendrick-Jones. 1973. The light chains of scallop myosin as regulatory subunits. *J. Mol. Biol.* 74:179–203.
50. Wagner, P. 1982. Preparation and fractionation of myosin light chains and exchange of the essential light chains. *Methods Enzymol.* 85(Part B): 72–81.
51. Wang, Y.-L., and D. L. Taylor. 1979. Distribution of fluorescently labeled actin in living sea urchin eggs during early development. *J. Cell Biol.* 82: 672–679.
52. Weber, K., and U. Groeschel-Stewart. 1974. Antibody to myosin. The specific visualization of myosin containing filaments in non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 71:4561–4563.
53. Weed, I. G. 1936. Cytological studies of developing muscle with special reference to myofibrils, mitochondria, golgi material and nuclei. *Z. Zellforsch. Mikrosk. Anat.* 25:516–540.
54. Wehland, J., and K. Weber. 1980. Distribution of fluorescently labeled actin and tropomyosin after microinjection in living culture cells observed with TV image intensification. *Exp. Cell Res.* 127:397–408.