

Identical Distribution of Fluorescently Labeled Brain and Muscle Actins in Living Cardiac Fibroblasts and Myocytes

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ABSTRACT We have investigated whether living muscle and nonmuscle cells can discriminate between microinjected muscle and nonmuscle actins. Muscle actin purified from rabbit back and leg muscles and labeled with fluorescein isothiocyanate, and nonmuscle actin purified from lamb brain and labeled with lissamine rhodamine B sulfonyl chloride, were co-injected into chick embryonic cardiac myocytes and fibroblasts. When fluorescence images of the two actins were compared using filter sets selective for either fluorescein isothiocyanate or lissamine rhodamine B sulfonyl chloride, essentially identical patterns of distribution were detected in both muscle and nonmuscle cells. In particular, we found no structure that, at this level of resolution, shows preferential binding of muscle or nonmuscle actin. In fibroblasts, both actins are associated primarily with stress fibers and ruffles. In myocytes, both actins are localized in sarcomeres. In addition, the distribution of structures containing microinjected actins is similar to that of structure containing endogenous F-actin, as revealed by staining with fluorescent phalloidin or phalloidin. Our results suggest that, at least under these experimental conditions, actin-binding sites in muscle and nonmuscle cells do not discriminate among different forms of actins.

The actin family consists of multiple polypeptides that vary slightly in amino acid sequences (1). In muscle cells, the predominant isoform is α -actin; in nonmuscle cells, β and γ actins predominate (2, 3). These isoforms are similar biochemically (4). However, even across phylogenetically disparate species, the nonmuscle actins are more similar to each other than they are to α -actin (5, 6). It is, therefore, important to determine whether the different forms of actin have different functions in cellular processes.

One of the most direct ways to examine possible differences in function is to microinject fluorescently labeled muscle and nonmuscle actins into living cells (7). Muscle actin, which was the first cytoskeletal protein to be fluorescently labeled and microinjected (8), can be incorporated into normal actin-containing structures in many cell types, including gizzard cells (9), fibroblasts (10, 11), amoeba (7), and macrophages (12). Participation of injected actin in the assembly of *de novo* structures, and in the reorganization of existing structures had also been documented (13). Thus, labeled muscle actin is considered to be an accurate tracer of endogenous pools of actin (8–13). However, it is still not clear whether muscle actin actually behaves in a way identical to nonmuscle actin in nonmuscle cells. Nor is it known whether nonmuscle actins can be utilized by muscle cells.

The experiments described in this paper ask whether embryonic chick cardiac fibroblasts and myocytes can distinguish between co-injected muscle and nonmuscle actins. Since the muscle and nonmuscle actins are co-injected into the same cell, comparison of the fluorescent images should reveal the presence of structures that preferentially bind one or the other. In addition, we examine whether injected muscle and nonmuscle actins participate in all F actin-containing structures or whether either actin is excluded from some specific structures.

MATERIALS AND METHODS

Cell Culture, Microinjection, and Microscopy: Monolayer cultures of cardiac fibroblasts and myocytes were obtained by trypsinizing hearts of 7-d chick embryos (14). Cells were injected 24 h to 20 d after plating as described by Wang (13). Many myocytes continued to beat during and after injection. Approximately 5–20% of the cell volume were injected. Thus, the concentration of injected actin per cell was between 0.15 and 1.0 mg/ml. No variations in the distribution of actin were detected within this range. Injection volumes much smaller than 5% resulted in very faint structures that were difficult to resolve and photograph, and large volumes frequently caused cell damage. Phalloidin staining was performed as described by Amato et al. (12).

Fluorescent images of injected cells and phalloidin- or phalloidin-stained cells were observed using epi-illumination and a 63 \times oil immersion objective NA 1.25 or a 100 \times oil immersion objective NA 1.30. Filters with narrow bandwidths were used so that no crossover of fluorescence was observable in

experiments involving comparisons of different fluorophores. Images were detected by a Venus DV-2 image intensifier, and photographed off a video monitor.

Preparation of Fluorescent Analogues: Muscle actin was purified from rabbit back and leg muscles according to Spudich and Watt (15). Nonmuscle actin was purified from lamb brain according to Ruscha and Himes (16). Lissamine rhodamine B sulfonyl chloride (LRB¹; Molecular Probes, Junction City, OR) or fluorescein isothiocyanate (FITC; Research Organics, Cleveland, OH) was dissolved in 100 mM borate, 100 mM KCl, 0.4 mM MgCl₂, pH 8.8 (LRB), or pH 9.0 (FITC). An equal volume of F actin in 2 mM Tris, 100 mM KCl, 2 mM MgCl₂, 0.2 mM CaCl₂, 0.2 mM ATP, 0.05 mM dithiothreitol, pH 8.0 was added to obtain a final dye to protein mass ratio of 0.09 (LRB) or 0.9 (FITC). The LRB-actin solution was stirred at room temperature for 15 min; the FITC-actin solution was incubated 2 h at room temperature. Subsequent procedures were essentially as described previously (8). The final actin conjugate had a dye to protein molar ratio of 1.0 to 2.5 (LRB) or 0.7 (FITC), estimated using a molar extinction coefficient of 13,000 at 550 nm for LRB, and a molar extinction coefficient of 64,000 at 492 nm for FITC. The range of the final ratios did not affect our results. Before injection, the actin conjugates at a concentration of 3–5 mg/ml were dialyzed against 0.5 mM PIPES, 0.05 mM MgCl₂, 0.2 mM ATP, 0.1 mM dithiothreitol, pH 6.95, then clarified. Protein purity and absence of unbound dye were determined by gel electrophoresis. LRB-ovalbumin (Sigma Chemical Co., St. Louis, MO) was prepared as described by Amato et al (12).

RESULTS

Soon after injection with fluorescently labeled actin, cells are filled with diffuse cytoplasmic fluorescence. Within 30 min, this diffuse fluorescence diminishes and the fluorescent conjugate becomes associated with cellular structures.

Co-injection of LRB Brain and FITC Muscle Actins

When similar amounts of LRB brain actin and FITC muscle actin are co-injected into spread chick fibroblasts, both actins become associated with stress fibers to a similar extent and within the same time period (Fig. 1, *a* and *b*). The brightly labeled stress fibers and slight submembranous staining are typical for cells co-injected with both actins, or injected with either actin separately. A minor difference—the enhanced staining of the ends of stress fibers with LRB brain actin—is attributable to the presence of the fluorophore on the actin, not to the actin itself (discussed below).

Both LRB brain actin and FITC muscle actin, when co-injected into cardiac myocytes, localize along myofibrils in distinct bands (Fig. 1, *c* and *d*). These bands co-localize with the Z lines and I band regions of the sarcomere (described below). The width of the bands of injected actin varies: sometimes the fluorescence is localized in a thin line, sometimes in a wider band (Fig. 1, *c* and *d*; Fig. 5). The width of the bands along a single myofibril is usually similar, but myofibrils of both types may be present in a single cell (Fig. 5 *c*). In myofibrils where injected actins appear to be concentrated in thin lines, faintly stained bands can be detected on either side of the bright fluorescent line (Fig. 2). A nonfluorescent zone separates the faint bands from each other. Whatever the width of the fluorescent bands in a particular cell, co-injected brain and muscle actins always have identical distributions even up to 24 h after injection. In addition, neither protein seems to become associated with myofibrils more quickly or more completely than the other.

We have also co-injected LRB-labeled ovalbumin and FITC-labeled muscle actin to determine whether the fluores-

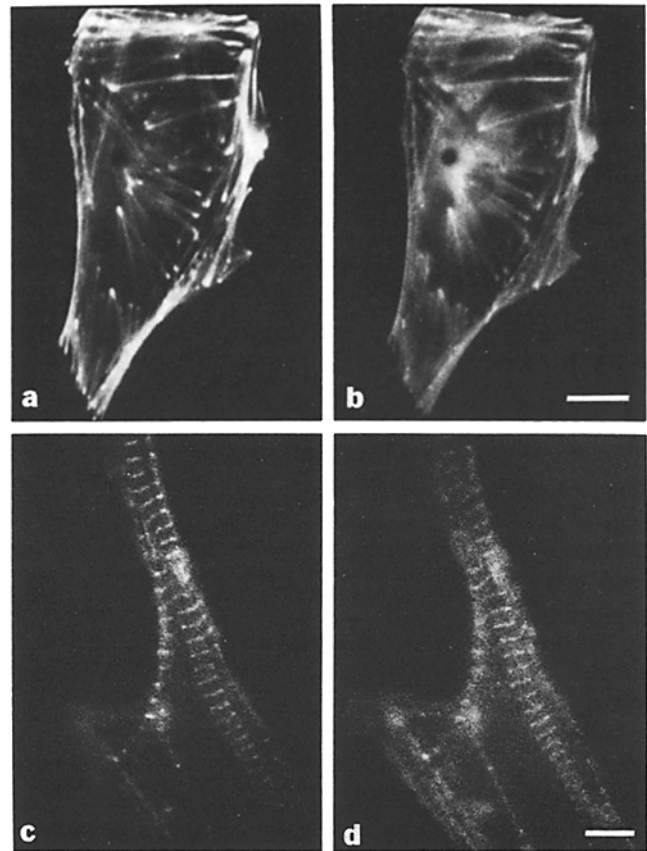


FIGURE 1 Fluorescent images of: LRB brain actin (*a*) and FITC muscle actin (*b*) co-injected into a chick cardiac fibroblast 2 h previously; and LRB brain actin (*c*) and FITC muscle actin (*d*) co-injected into a chick cardiac myocyte 1 h previously. Note that the termini of stress fibers in the LRB actin image (*a*) show a high level of fluorescence compared with the length of the stress fiber. Bars, 10 μ m (*a* and *b*); 5 μ m (*c* and *d*). \times 1,350 (*a* and *b*); \times 2,080 (*c* and *d*).

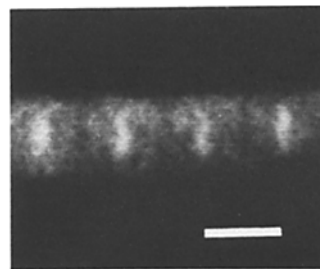


FIGURE 2 Enlargement of a myofibril with thin fluorescent lines flanked by adjacent faint bands. The cell was injected with LRB muscle actin 2 h previously. Narrow nonfluorescent bands separate the faintly labeled bands. Bar, 3 μ m; \times 3,130.

cent images obtained from actin injection reflect specific association of actins with cellular structures. Labeled ovalbumin, once it spreads throughout the cell, remains diffusely distributed even after 4 h. During the same time period, co-injected muscle actin becomes localized in stress fibers in fibroblasts and in myofibrils in muscle cells (not shown).

Co-injection of LRB Muscle and FITC Muscle Actins

As noted above, the fluorescence intensity of LRB actin is often higher at the ends of stress fibers than along the length of stress fibers. To determine whether this is due to the presence of the LRB fluorophore or to the properties of the brain actin, LRB muscle actin and FITC muscle actin were

¹Abbreviations used in this paper: FITC, fluorescein isothiocyanate; LRB, lissamine rhodamine B sulfonyl chloride; NBD, nitrobenzoxoadiazol.

co-injected into fibroblasts. The overall patterns of fluorescence are again similar, with the exception of the emphasis of the ends of stress fibers in the LRB image (Fig. 3, *a* and *b*). Thus, labeling with LRB seems to be responsible for this phenomenon. Labeled myofibrils in co-injected muscle cells do not show fluorophore-related differences in fluorescence intensity (not shown).

Phallotoxin Staining of Actin-injected Cells

To determine whether injected actin is accessible to all detectable F actin-containing structures, the distribution of injected actins was compared with the distribution of total filamentous actin by staining injected cells with rh-phalloidin (for cells injected with FITC muscle actin) or nitrobenzoxadiazol (NBD)-phalloidin (for cells injected with LRB muscle actin or LRB brain actin). Photographs of the fluorescent images of injected actin were taken after fixation; images before fixation were comparable except for slightly higher diffuse fluorescence. Fig. 4, *a* and *b* show that there is essentially no difference between the fluorescent patterns of injected FITC muscle actin and rh-phalloidin in a fixed fibroblast. The second pair of photographs (Fig. 4, *c* and *d*), which show injected LRB muscle actin and NBD-phalloidin images, indicate that the enhanced labeling of the ends of stress fibers characteristic of LRB actin is not present in phalloidin staining.

In cardiac muscle cells, comparison of the distribution of injected actins with that of phalloidin or phalloidin again demonstrates great similarity. Fig. 5, *a* and *b* show an image of FITC muscle actin fluorescence and its phalloidin stained counterpart. No significant difference is detectable. In Fig. 5, *c* and *d*, LRB brain actin fluorescence is distributed in a very similar pattern to that revealed by phalloidin staining. In this cell, both a myofibril with thin lines of fluorescence, and a myofibril with wider bands can be discerned (arrows, Fig. 5, *c* and *d*). By superimposing aligned photographs such as Fig. 5, *c* and *d*, we can demonstrate that both the thin lines and the wide bands co-localize with the phallotoxin stained bands. In addition, as Fig. 6, *a* and *b* show, phallotoxin-stained bands co-localize with the Z lines and I bands of sarcomeres. The localization of phallotoxin fluorescence is the same in both injected and uninjected muscle cells.

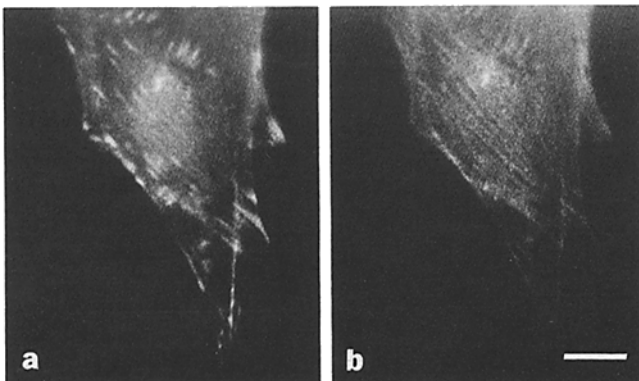


FIGURE 3 Comparison of LRB muscle actin (*a*) and FITC muscle actin (*b*) fluorescent images. The LRB muscle actin image of a co-injected fibroblast shows relatively high fluorescence at the ends of stress fibers, whereas the FITC muscle actin image does not. Bar, 10 μm ; $\times 1,350$.

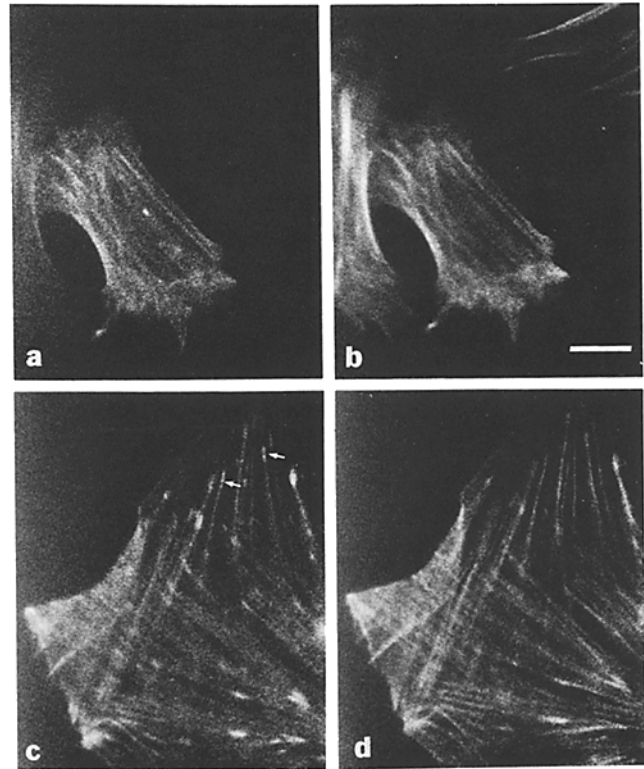


FIGURE 4 Distribution of injected fluorescent actins compared with the distribution of total F actin as revealed by phallotoxin staining in fibroblasts. The pattern of fluorescence of a fibroblast injected with FITC muscle actin (*a*) is identical to that of rh-phalloidin (*b*). The upper right hand corner of *b* includes the phalloidin image of an uninjected cell. The enhanced fluorescence of stress fiber termini (arrows) observed in the LRB muscle actin image (*c*) is not present in its NBD-phalloidin stained counterpart (*d*). Bar, 10 μm ; $\times 1,350$.

DISCUSSION

These results demonstrate that injected muscle and brain actins are not distinguished by either chick fibroblasts or cardiac muscle cells. Fibroblasts, which synthesize only trace amounts, if any, of α actin (17, 18), can use injected muscle and nonmuscle actins to the same extent in stress fibers and other actin-containing structures. Similarly, injected brain actin becomes localized in cardiac myofibrils just as readily as does muscle actin. Our data, therefore, suggest that, at this level of resolution, neither actin has a detectably greater affinity for available sites in any structure in these cell types. Furthermore, the results of the phallotoxin staining experiments indicate that all F actin-containing structures can associate with injected, fluorescently labeled actin from either source.

Localization in Fibroblasts

Although all fibroblast structures that stain with phallotoxin also label with injected fluorescent actin, the intensity of the LRB fluorescence at the ends of some stress fibers is relatively high. This phenomenon does not change over an 8-h period, as would be expected if it is due to a very high rate of exchange of actin at these sites. Nor can this enhanced fluorescence be attributed to the fluorophore's response to local conditions,

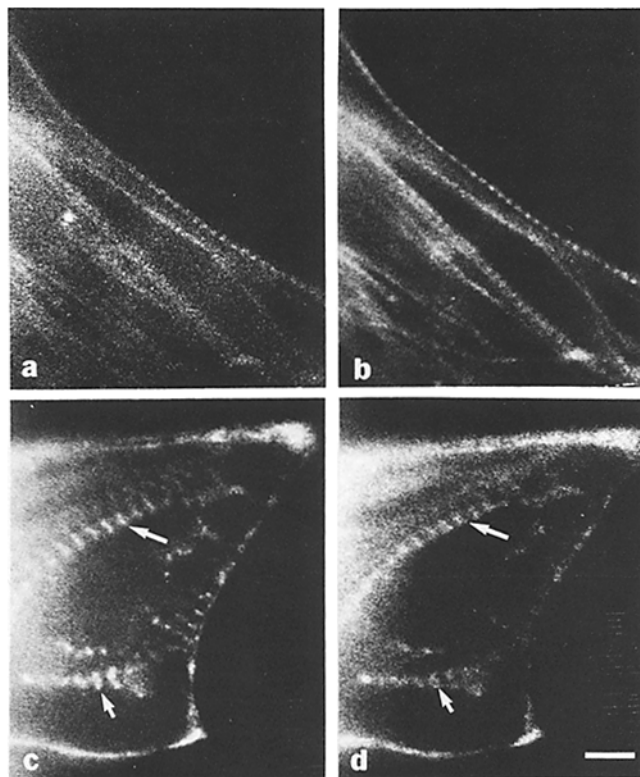


FIGURE 5 Distribution of injected fluorescent actins in muscle cells compared with that of total F actin as revealed by phalloidin staining. The patterns of fluorescence for both injected FITC muscle actin (a) and rh-phalloidin staining (b) are identical in this fixed myocyte. LRB brain actin (c) is also distributed identically to NBD-phalloidin staining (d). The width of some of the myofibril bands is comparable for both injected LRB actin and NBD-phalloidin (short arrows). Other bands, however, are slightly narrower in the injected LRB actin image compared with the NBD-phalloidin image (long arrows). Bar, 5 μm ; $\times 2,080$.

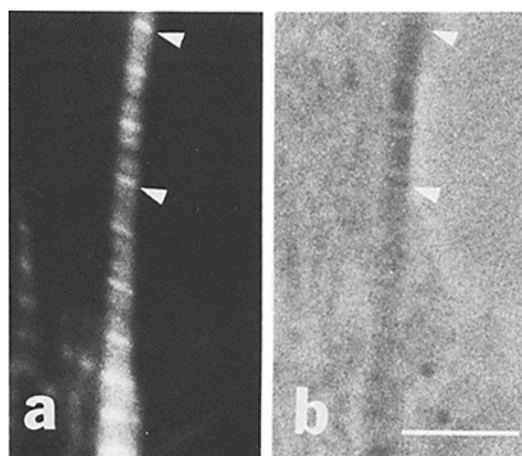


FIGURE 6 Co-localization of bands stained by rh-phalloidin (a) with the Z lines and I bands visible in the corresponding phase photograph (b) of this myofibril from an uninjected muscle cell. Arrowheads indicate points of alignment. The fluorescence image is a direct photograph. Bar, 5 μm $\times 3,200$.

such as pH or ionic strength (7), since the relative intensity does not change after fixation and permeabilization. Kreis et al. have also described this labeling pattern for the termini of stress fibers in LRB actin injected gizzard fibroblasts (9).

Although these authors do not discuss this particular observation, they do point out that the mobility of injected actin at the end of stress fibers (focal contacts) is similar to that along the length of stress fibers. Possibly, actin molecules at the ends of stress fibers are associated with different accessory proteins, or assume a different conformation, compared with those along the length of stress fibers. The resulting difference in microenvironment could affect the fluorophore's intensity (7). Alternatively, if different domains of the cell incorporate actin by different mechanisms, then actin incorporation into these domains could be affected to different extents by the presence of LRB on actin molecules. We have observed that actin labeled with 5-iodoacetamidofluorescein, when microinjected into muscle cells, associates to a lesser extent with myofibrils compared with actins labeled with FITC or LRB (unpublished results). However, the distribution of muscle or nonmuscle actins is always identical for each fluorescent probe.

Localization in Myofibrils

Injected fluorescent actin becomes associated with the sarcomere as either a bright band or a bright line flanked by two slightly less bright bands. Comparison of these patterns with phalloidin-stained images and phase photographs indicates that the injected actin becomes localized in the α -actinin-containing Z lines and the adjacent I bands. The dark region between the I bands is probably the H zone. The width of I band fluorescence may be related to differences in the metabolism or maturity of individual myofibrils. The intensity of the Z line labeling may be attributable to actin- α -actinin interactions. The Z line has often been proposed as an organizing center for myofibrils (19, 20). Furthermore, Sanger et al. (21) have suggested that newly synthesized α -actinin may create attachment sites for the insertion of actin into existing myofibrils. Thus, the Z line may contain large numbers of high affinity-binding sites that are easily accessible to injected actins. Phalloidin staining suggests that actin may be associated with the Z line even in uninjected cells.

Sanger et al. have also shown that when fluorescently-labeled actin is added to glycerinated myofibrils, it does not bind anywhere along myofibrils in the presence of ATP, and, in the absence of ATP, binds only to the myosin heads within the H zone (21). Since ATP is available in living cells, it is expected that microinjected actin should not form a stable association with myosin heads *in vivo*. The lack of actin binding to the Z lines and I bands of glycerinated myofibrils may be attributable to the extraction process, which may remove available binding sites from the myofibrils or may inactivate mechanisms that control actin localization in living cells.

Recently, Glacy reported that actin labeled with iodoacetamido tetramethylrhodamine is incorporated into the I bands and the M lines, but not into the Z lines, of cultured cardiac myocytes as rapidly as 5 min after injection (22). The assignment of localization was based on comparison of the fluorescence pattern of injected actin to reports of anti-actin antibody staining of myofibrils. We have not observed this pattern of fluorescence in our LRB or FITC actin injected cells or phalloidin-stained cells, nor have we observed such rapid association of injected actin with myofibrils. However, we have obtained a pattern of incorporation that is apparently similar to Glacy's by injecting actin prepared in buffer that

has been aged for a week or in which ADP has been substituted for ATP (in preparation).

Isoforms of Actin

There is considerable evidence that actin isoforms *in vitro* can co-polymerize and activate myosin ATPase (23, 24). However, isoforms vary in their interactions with some actin regulating proteins (25), in their sensitivities to parameters affecting polymerization, and in their activations of myosin ATPase (4, 23, 24). *In vivo*, the isoforms of actin, like those of other proteins (26–28), seem to be separated either through differential timing of protein synthesis or through spatial localization. Both cardiac (2, 3, 29, 30) and skeletal muscle cells synthesize β and γ actins early in development. As development proceeds, an increasingly larger percentage of actin synthesis is composed of α actin, until, finally, α actin becomes the only biochemically detectable isoform in mature cells. It has also been reported that cultured skeletal muscle cells preferentially release nonmuscle actins into the culture medium (31). In mouse diaphragm where only α actin can be detected biochemically, polyclonal antibody staining suggests that γ actin is present in small amounts and that it is sequestered near mitochondria (32). It has also been reported that in rat muscle fibers, cytoplasmic actin can be detected only in or near the postsynaptic membrane of neuromuscular junctions (33, 34).

On the other hand, there are indications that actin isoforms can be utilized to the same extent in various different structures. For example, immunofluorescence studies using antibodies specific for muscle or nonmuscle actins indicate that both muscle and nonmuscle actins can participate in early sarcomerogenesis (35). In addition, Gunning et al. have shown that when a cloned cardiac α actin gene is introduced into mouse L cells, α actin is synthesized and incorporated into the Triton-X insoluble cytoskeleton to the same extent as is endogenous β actin, suggesting that L cells may not discriminate between α and β actins (36).

As these observations and our results suggest, it is likely that actin isoforms, when present simultaneously, can substitute for one another in cellular structures. If embryonic chick cardiac cells do make use of the subtle differences among different actins for fine regulation, then control must be achieved through the timing of synthesis, the localization of synthesis, and/or selective degradation, such that a particular form of actin may be enriched or depleted. It is equally possible that the different forms of actin do not have different functional roles. Perhaps, as Fryberg et al. (37) have suggested, isoforms are expressed in differentiating cells only because their respective structural genes have become associated with specific regulatory elements.

The authors thank Dr. P. A. Amato for the generous gift of rhodamine-phalloidin, and Dr. D. L. Taylor for helpful discussions.

This study was supported by grants from Muscular Dystrophy Association, National Science Foundation PCM-8306971, and American Cancer Society CD-200 to Y.-L. Wang.

Received for publication 23 April 1984, and in revised form 31 August 1984.

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