

ROLE OF TROPOMYOSIN IN ACTIN FILAMENT FORMATION IN EMBRYONIC SALAMANDER HEART CELLS

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ABSTRACT

Recessive mutant gene *c* in *Ambystoma mexicanum* embryos causes a failure of the heart to function even though initial heart development appears normal. An analysis of the constituent proteins of normal and mutant hearts by SDS-polyacrylamide gel electrophoresis shows that actin (43,000 daltons) is present in almost normal amounts, while myosin heavy chain (200,000 daltons) is somewhat reduced in mutants. Both SDS-polyacrylamide gel electrophoresis and immunofluorescence studies reveal that tropomyosin is abundant in normal hearts but very much reduced in mutants. Electron microscope studies of normal hearts show numerous well-organized myofibrils. Although mutant cardiomyocytes contain a few 60- and 150-Å filaments, organized sarcomeres are absent. Instead, amorphous proteinaceous collections are prominent. Previously reported heavy meromyosin (HMM)-binding experiments on glycerinated hearts demonstrate that most of the actin is contained within the amorphous collections in a nonfilamentous state, and the addition of HMM causes polymerization into F actin (Lemanski et al., 1976, *J. Cell. Biol.* **68**:375-388). In the present study, glycerol-extracted hearts are incubated with tropomyosin, purified from rabbit or chicken skeletal muscle. This treatment causes the amorphous collections to disappear, and large numbers of distinct thin actin (60- to 80-Å) filaments are seen in their place. Negative staining experiments corroborate this observation. These results suggest that the nonfilamentous actin located in the amorphous collections of mutant heart cells is induced to form into filaments with the addition of tropomyosin.

KEY WORDS genetic mutation · heart · myofibrillogenesis · tropomyosin · actin

A naturally occurring recessive mutant gene, designated *c* for "cardiac lethal," has been discovered in an imported stock of Mexican axolotls, *Ambystoma mexicanum* (8). The affected embryos have hearts that fail to contract even though initial heart development appears unaltered. The mutation appears to exert its effect on heart development by way of abnormal inductive interactions from sur-

rounding tissues (22). Mutant (*c/c*) embryos are obtained from matings between heterozygous (+/*c*) adults and are first distinguishable from their normal (+/+ or +/*c*) siblings at stage 34 (30), when the heart first begins to beat rhythmically in normals. The mutant hearts at this stage upon gross examination appear structurally normal but fail to contract normally, and blood circulation is not established. In later development the heart becomes distended and remains thin-walled, and the embryo acquires ascites. The mutants live ~20

d beyond the heart-beat stage; they swim normally, indicating that gene *c* does not interfere with skeletal muscle development.

Structural and biochemical studies on normal and mutant embryonic hearts from stage 34 (heart-beat stage) through stage 41 (when mutant embryos die) have been reported. Studies with the electron microscope show that normal hearts have organized sarcomeres at stage 34. At stage 41, the normal myocardium is composed of highly differentiated myocytes containing numerous well-organized myofibrils and the ventricular heart wall displays extensive trabeculation (13, 14). The mutant myocardium remains a single cell layer thick throughout development with no indication of developing trabeculae. Mutant heart cells at stage 34 have few scattered thin (60-Å) and thick (150-Å) filaments along with an occasional Z body. A partial organization of myofibril components is noted in some mutant myocardial cells at more advanced stages; however, well-defined sarcomeres are absent. The mutant cells are characterized instead by containing amorphous proteinaceous collections at their cell peripheries where myofibrils would be expected to first organize in normal cells (15, 16). Thus, on the basis of these morphological data it is apparent that gene *c* causes a failure of normal myocyte differentiation in mutant hearts. The most obvious abnormality is an absence of organized sarcomeric myofibrils.

Biochemical studies extend these basic morphological observations. SDS-polyacrylamide gel electrophoresis (21) and radioimmunoassay (17, 19) studies reveal that mutant hearts at stage 41 contain only ~50% as much myosin (200,000 daltons) as do the hearts of normal siblings; however, the quantity of myosin in mutant cells is significantly higher than in any of the nonmuscle tissues studied (i.e., liver, gut, and brain). SDS gel electrophoresis in combination with heavy meromyosin (HMM)-binding studies confirm the presence of substantial quantities of actin in mutant myocardial cells and strongly suggest that the protein is contained within the amorphous collections in a nonfilamentous state (17, 21, 38). Electrophoretic and immunohistochemical experiments suggest that tropomyosin (34,000 daltons) is substantially reduced from normal in mutant hearts.

The close structure-function relationship of tropomyosin with actin filaments in normally organized myofibrils is well known (5). Therefore, the present investigation was undertaken in an attempt to determine whether there might be a

causal relationship between the reduction of tropomyosin in mutant heart cells and their failure to form filamentous actin. The observations suggest that the addition of tropomyosin to glycerinated mutant hearts or to homogenates of mutant hearts results in the polymerization of amorphous actin into filaments. It is concluded that this simple gene mutation, by way of some kind of abnormal inductive processes (20, 22), results in an insufficiency of tropomyosin in mutant myocytes, which in turn causes a failure of the cells to form filamentous actin.

MATERIALS AND METHODS

Tissue Procurement

Heterozygous adult axolotls (+/*c*) were mated and the fertilized eggs were incubated at 16°–18°C in dilute Holtfreter's solution (29) until the embryos reached stage 41 (~30 d after fertilization). The staging system of Schreckenber and Jacobson (30) was used.

Tropomyosin and HMM Preparations

Tropomyosin preparations from both chicken and rabbit skeletal muscles were used in the study. The tropomyosin from chicken skeletal muscle was purified by following the methods of Bailey (1), except that several additional final ammonium sulfate re-precipitation steps were performed to achieve higher purity. The tropomyosin prepared from rabbit skeletal muscle was a generous gift from Dr. Yeu-Zu Yang of the National Heart, Lung, and Blood Institute, National Institutes of Health, and was prepared by published methods (3). SDS gel electrophoretic analysis of the tropomyosin preparations showed both to be highly purified with no minor contamination bands visible even after excessive overloading of the gels (Fig. 1). HMM was prepared by tryptic digestion of myosin (9, 32).

SDS-Polyacrylamide

Gel Electrophoresis

Hearts of stage 41 normal and mutant siblings were prepared for analysis by SDS gel electrophoresis by using methods detailed in an earlier paper (21). Briefly, the hearts were dissected from embryos and placed in a Steinberg's solution (29) at 0°C to which had been added the proteolytic inhibitor phenylmethylsulfonyl fluoride to a final concentration of 1.0 mM. 40 embryonic hearts were used for each gel; the total protein quantities of normal and mutant hearts were found to be comparable as determined by the Lowry method (23). In addition, myofibrils from adult axolotl heart and skeletal muscle, and chicken skeletal muscle were prepared for SDS gel electrophoresis. The myofibrils were made by homogenizing fresh muscle tissues in 50 mM KCl, 5 mM EGTA,

1 mM dithiothreitol, 1.0% Triton X-100, 10 mM imidazole, pH 7.1, and the myofibrils were collected by centrifugation. Molecular weight calibrations were made using the following as standards: (a) chicken and axolotl myosin heavy chain (200,000 daltons), (b) porcine α -actinin (100,000 daltons) (gifts from Dr. Judith Schollmeyer of the University of Minnesota and Dr. Darrel Goll of the University of Arizona) (6), (c) chicken and axolotl actin (43,000 daltons), (d) rabbit skeletal muscle tropomyosin (35,000 and 33,000 dalton subunits), and (e) chicken skeletal muscle tropomyosin (34,000 daltons). Standard methods were used to prepare the above samples for gel electrophoresis (31, 37). The samples were placed in 1.0% SDS, 1.5% β -mercaptoethanol, 1.0 mM phenylmethylsulfonyl fluoride, 10 mM phosphate, pH 6.8, heated at 100°C for 5 minutes, sonicated for an additional 5 min, and the protein concentrations were adjusted as necessary. Samples were run on 7.5% polyacrylamide gels containing 0.1% SDS and stained routinely with Coomassie Brilliant Blue R250 and for the densitometry studies with 1% acid fast green, the latter being well known for its linearity of staining for muscle proteins (7, 28). Graphic traces of the gels stained with acid fast green were made using a Gilford spectrophotometer model 4200 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a gel scanner attachment at an absorbance wavelength of 630 nm. The densitometry was performed to obtain information regarding the relative amounts of tropomyosin in normal and mutant hearts and to verify with traces visual judg-



FIGURE 1 SDS-polyacrylamide (5% acrylamide) gel electrophoresis pattern of chicken skeletal muscle tropomyosin prepared by a modified Bailey method (1). The gel shows a single 34,000-dalton band (arrow).

ments of tropomyosin on stained gels. I wish to stress that this semiquantitation is of the "more than" "less than" variety as opposed to "absolute" quantitation of tropomyosin. Radioimmunoassays (17, 19) currently in progress in our laboratory will be required to determine the absolute quantities of tropomyosin in normal and mutant hearts.

Preparation of the Antibodies

Antibodies against chicken skeletal muscle tropomyosin were prepared in young rabbits. Preimmune rabbits were carefully screened to eliminate nonspecific staining on the tissue samples to be used in the experiments. The antibodies were obtained by giving an initial subcutaneous injection of 4 mg of protein in Freund's complete adjuvant followed by three subcutaneous booster injections of 2 mg protein in Freund's incomplete adjuvant at 2-wk intervals. The rabbits were bled 8 d after the final injection and the gamma globulin fractions obtained by ammonium sulfate fractionation. The antibodies were stored at -70°C until ready for use. Purity of the antibodies was tested by double diffusion and immunoelectrophoresis against purified antigen and against crude heart muscle homogenates from chicken and salamander. Each antibody preparation gave a sharp single precipitin line indicative of a high specificity for tropomyosin (Fig. 2).

Immunohistochemical Staining

Tissues were fixed 4 h at 0°C in a periodate-lysine-paraformaldehyde solution (25), and 1- μ m frozen sections, prepared with a Sorvall FTS Frozen Thin Sectioning System (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) were affixed to glass slides. An indirect staining method was used. The primary antibodies in

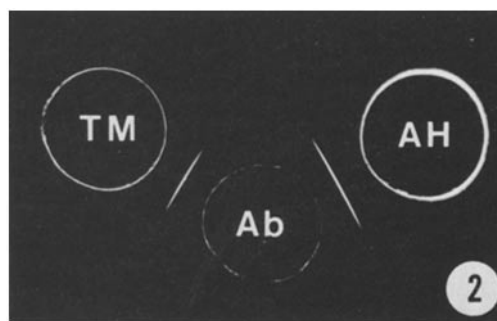


FIGURE 2 Double immunodiffusion of antitropomyosin antibodies. The center well contains antibodies directed against chicken skeletal muscle tropomyosin (*Ab*). The outer wells contain chicken skeletal muscle tropomyosin (*TM*) and axolotl adult crude heart homogenate (*AH*). Single sharp precipitin lines between antibody and antigens suggest a high level of antibody specificity.

phosphate-buffered saline (0.9% NaCl in 0.05 M phosphate buffer, pH 7.0) plus 25% glycerol (PBS-glycerol) were placed over the sections for 1 h at 37°C and after several washes in PBS-glycerol the sections were stained for 1 h at 37°C with the fluorescein isothiocyanate-labeled fraction of anti-rabbit IgG prepared in goat (Miles Laboratories, Inc., Kankakee, IL). Controls included: (a) staining with preimmune globulins from the same rabbit that produced the antibodies, (b) staining with antibodies which had been absorbed with excess purified antigen, (c) staining with the second antibody only. There was practically no background staining on the control slides.

The stained sections were viewed with a Zeiss Universal light microscope equipped with epifluorescence illumination and photographs were taken using 35-mm Kodak Plus-X film at 60-s exposure times.

Electron Microscopy

Preparation of the embryonic hearts for routine electron microscope observation has been described in an earlier paper (14). Briefly, the embryos with pericardial cavities opened were placed in a glutaraldehyde-formaldehyde-picric acid-styphnic acid mixture, buffered to pH 7.4 with 0.10 M phosphate buffer (11) for 4 h at 2°C. After a brief rinse in buffer, the tissues were postfixed for 60 min at 0°C in 1.30% osmium tetroxide buffered to pH 7.4 with 0.10 M phosphate buffer. The hearts were dehydrated in ethanol and propylene oxide and embedded in Epon.

For the preparation of electron microscope sections of embryonic hearts incubated in tropomyosin, the methods developed by Ishikawa et al. (10) for HMM-binding experiments were followed. As a control in the present study, HMM-binding experiments also were done (10). The hearts were excised from embryos with glass dissecting needles and placed in 50% glycerol-standard salt solution (SSS) (0.01 M KCl, 0.005 M MgCl₂, and 0.006 M phosphate buffer, pH 7.0) for 12 h followed by 6 h each in 25% glycerol-SSS and 5% glycerol-SSS. The tissues were incubated for 18 h in tropomyosin or HMM dissolved in SSS plus 12.5% glycerol, pH 7.0. The final concentration of tropomyosin or HMM in solution was 6 mg/ml. Controls for these experiments included normal and mutant hearts processed as above but without tropomyosin or HMM in the final solution. These tissues were rinsed 1 h in 0.10 M KCl, fixed 8 h in 4% "Pure EM Grade" glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) buffered to pH 7.3 with 0.10 M phosphate buffer, and postfixed 1 h in 1% osmium tetroxide buffered to pH 7.2 with 0.10 M phosphate buffer. All procedures before dehydration were carried out at 2°C. After dehydration in graded ethanols and propylene oxide, the tissues were embedded in Epon.

In addition, negative staining experiments were performed. In a given negative staining experiment 10 normal and 10 mutant hearts were homogenized in separate vials, each containing 1.2 ml of SSS with 0.001 M EDTA

at 0°C. Homogenization was accomplished by rapidly drawing the hearts in and out of a disposable plastic syringe without a needle (10). Each homogenate was divided into three equal parts (0.4 ml each). To one vial an equal volume of tropomyosin solution (4 mg/ml) was added, to the second an equal volume of HMM solution (4 mg/ml), and to the third an equal volume of SSS. The vials were left at room temperature for 5, 15, or 30 min with gentle agitation. To negative stain, one drop of homogenate was placed on a Formvar-coated carbon-stabilized grid for 2 min, rinsed with a few drops of 0.02 M KCl or distilled water, and stained with 1% aqueous uranyl acetate.

Specimens were viewed on Philips 200 or AEI 801 electron microscopes at acceleration voltages of 60 or 80 kV. Negatives taken at magnifications ranging between 2,500 and 80,000 were photographically enlarged. Measurements of filament diameters were made on electron micrograph prints at $\times 100,000$ taken immediately after an accurate magnification calibration of the electron microscope had been performed using a carbon grating replica obtained from a commercial supplier.

RESULTS

Morphology

Detailed light and electron microscope studies comparing heart development in normal (+/+ or +/c) and cardiac lethal mutant (c/c) embryos from stage 34 (at which normal heartbeat initiates) through stage 41 (at which mutant embryos die) have been reported (13–15). The hearts of normal and mutant siblings become increasingly different with development and by stage 41 mutant abnormalities are striking. The normal hearts at this stage have extensively formed trabeculae and their myocytes contain numerous well-organized myofibrils (Fig. 3). Conversely, the mutant hearts show no indication of trabeculation and are composed of cells that lack organized myofibrils. Instead, mutant heart cells contain large collections of amorphous proteinaceous material in their peripheral cytoplasm (Fig. 4); this is the location where myofibrils first organize in normal cells (14, 15).

SDS-Polyacrylamide

Gel Electrophoresis

In evaluating the muscle proteins in the embryonic hearts it is necessary to make the following assumptions: (a) 34,000-dalton protein = tropomyosin, (b) 43,000-dalton protein = actin, (c) 200,000-dalton protein = myosin heavy chain. These assumptions are based on the widely ac-

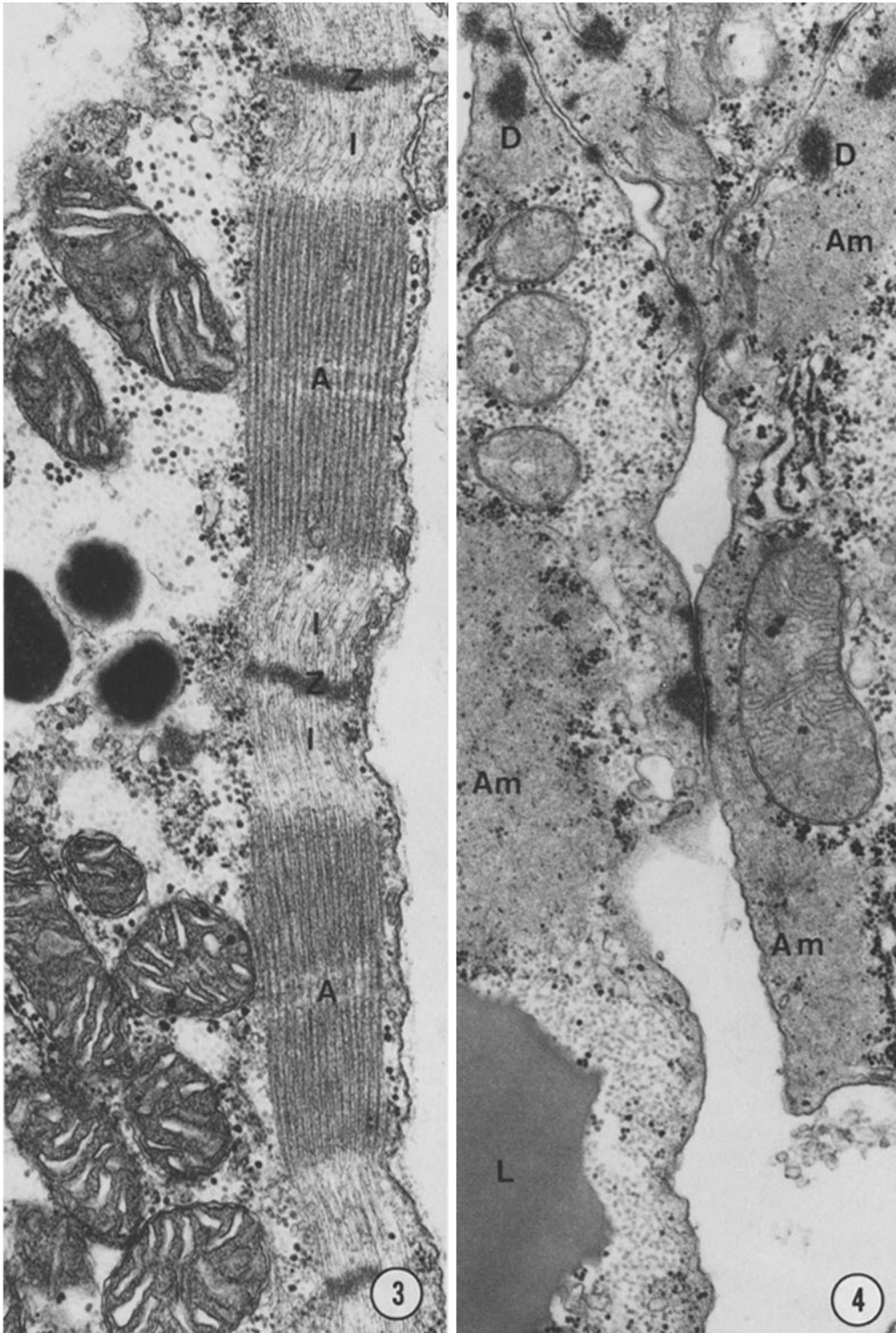


FIGURE 3 Electron micrograph showing portions of myocardial cells from a stage 41 normal embryo after preparation for conventional morphology. A well-differentiated myofibril is obvious in the peripheral area of the cell. *A*, A band; *I*, I band; *Z*, Z line. $\times 34,500$.

FIGURE 4 Electron micrograph of portions of heart cells from stage 41 mutant embryo after preparation for conventional morphology. Unlike normal cells in which there are well-organized myofibrils, these cells lack myofibrils. Instead, amorphous proteinaceous collections (*Am*) containing a few dense bodies (*D*)

cepted premise that the molecular weights of the above myofibrillar proteins are well established and highly reproducible by gel electrophoresis (7, 28). In analyzing the densitometry of the gels, I make the additional assumption that given myofibrillar proteins of mutant and normal hearts bind the acid-fast green stain with identical stoichiometries.

In comparing the electrophoresis banding patterns and densitometry traces of contractile proteins in the normal and mutant hearts at stage 41, several differences are immediately obvious (Fig. 5). Myosin heavy chain (200,000 daltons) is clearly more abundant in normal hearts than mutant; however, mutant hearts contain significantly more 200,000-dalton protein than any nonmuscle tissue studied (brain, gut, liver) (21). Actin (43,000 daltons) appears to be only slightly reduced in the mutant hearts. Thus, mutant hearts at stage 41 apparently contain a substantial quantity of actin.

A protein band of 34,000 daltons is prominent in gels of normal hearts. This band most likely represents tropomyosin since it coelectrophoreses with chick breast muscle tropomyosin. The mutant hearts have only a very faint 34,000-dalton band, indicating that muscle tropomyosin is reduced significantly in mutant heart cells.

In view of recent reports in the literature (2, 4), the possibility that mutant hearts contain a nonmuscle type of tropomyosin (30,000 daltons) is not ruled out. Mutant gels have a prominent protein band at 30,000 daltons (Fig. 5) which could contain this protein. The bulk of the 30,000-dalton protein in mutant hearts is probably a yolk protein, however, since it appears only in gels of embryonic tissues that contain numerous yolk platelets (see discussion below).

Another very obvious band in mutant gels ranges from 90,000 to 150,000 daltons (center measurement = 130,000 daltons); this, too, is probably a yolk component. A comparable band is not prominent in normal hearts at stage 41, when virtually all of the yolk platelets have disappeared (13, 14). Yolk still remains in mutant hearts at this stage, however (15, 16). Furthermore, light and electron microscopy combined with electrophoresis studies of a variety of early normal tissues (heart, gut, brain, liver, myotomes, fertilized eggs, etc.) demonstrate that whenever yolk platelets are present, the 130,000-dalton band is present and whenever the cells lack platelets, the large band is absent (21). The same is true for the 30,000-dalton band described in the previous paragraph (21).

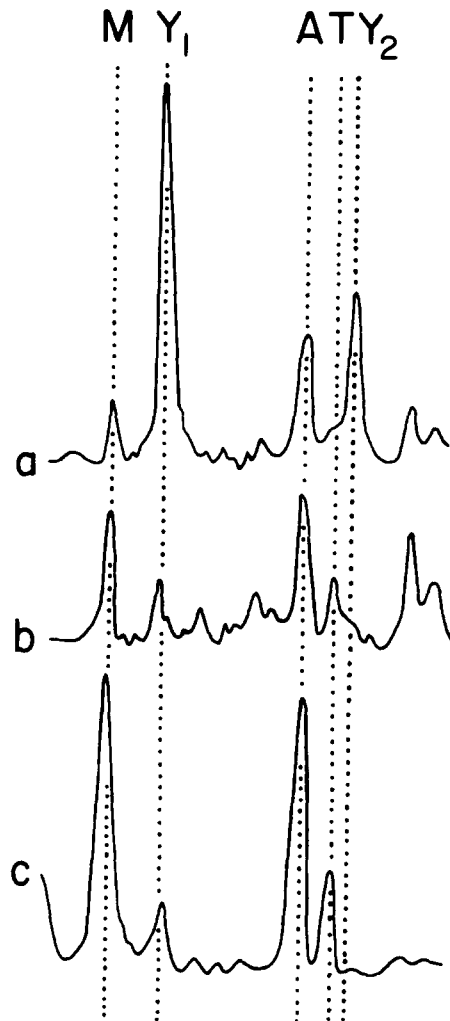


FIGURE 5 Densitometer traces of SDS-polyacrylamide (7.5% acrylamide) gels stained with acid fast green: (a) stage 41 mutant heart; (b) stage 41 normal heart; (c) chicken skeletal muscle myofibrils. *M*, myosin heavy chain (200,000); *A*, actin (43,000); *T*, tropomyosin (34,000); *Y*₁, probably a combination of yolk and α -actinin in Fig. 5 *a* and *b*, but α -actinin only in Fig. 5 *c* (90,000–150,000; center measurement = 130,000); *Y*₂, probably mostly yolk protein but possibly contains some nonmuscle type of tropomyosin (30,000).

The normal hearts have several electrophoretic bands that fall within the 90,000- to 150,000-dalton range, and some of these bands may represent different contractile proteins (e.g., α -actinin at 100,000 (6); C protein at 92,000 (26), etc.). Further studies will be required to determine if comparable bands are present in mutant hearts, but masked by

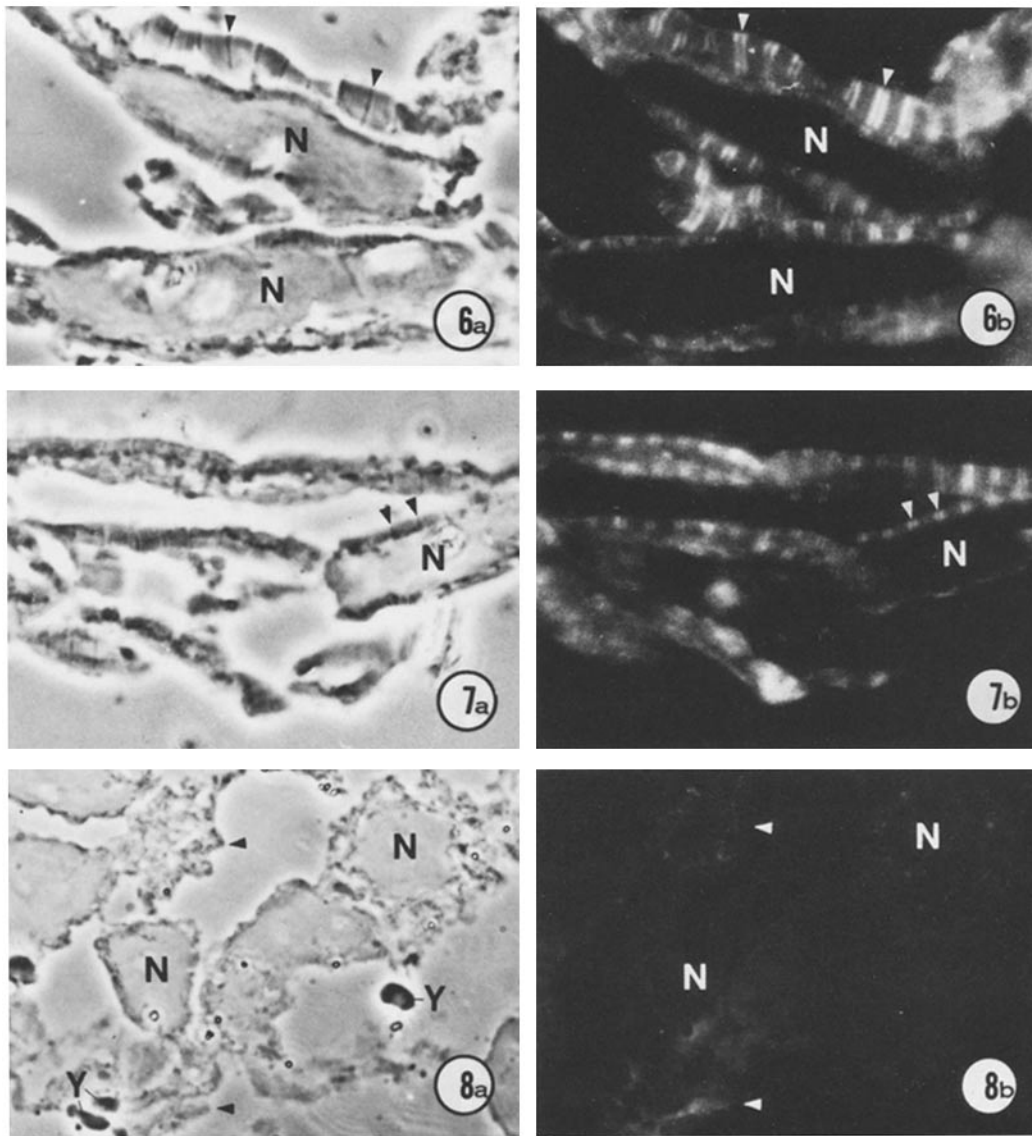


FIGURE 6 Phase-contrast (a) and fluorescence (b) micrographs of 6-mo-old juvenile axolotl heart sections after staining with antibody directed against chicken skeletal muscle tropomyosin using an indirect technique. The antitropomyosin very specifically stains the I bands of organized myofibrils. Arrows indicate locations of corresponding Z lines. *N*, nucleus. $\times 1,025$.

FIGURE 7 Phase-contrast (a) and fluorescence (b) micrographs of stage 41 normal heart sections after staining with antitropomyosin (same antibody as above) using an indirect technique. The antibody specifically stains the I bands of myofibrils. Arrows indicate locations of corresponding Z lines. *N*, nucleus. $\times 1,025$.

FIGURE 8 Phase-contrast (a) and fluorescence (b) micrographs of stage 41 cardiac lethal mutant heart sections after staining with antitropomyosin (same antibody as above) using an indirect technique. The fluorescence staining is faint and diffuse; there is no obvious striated pattern. Arrows indicate corresponding areas. *N*, nucleus; *Y*, yolk platelet. $\times 1,025$.

yolk. What is evident, however, is that yolk platelets are still abundant in mutant cells long after their disappearance from normal cells. This, in itself, suggests that mutant cells have failed to differentiate normally.

Immunohistochemistry

Antitropomyosin antibodies very specifically stain the I bands of organized myofibrils in fixed frozen sections of juvenile or adult heart tissue (Fig. 6) and of stage 41 normal embryonic hearts (Fig. 7). The stage 41 normal cardiomyocytes contain numerous myofibrils that exhibit staining as might be expected. If preimmune globulins or antitropomyosin absorbed with an excess of purified tropomyosin are used to stain the sections, no banding can be seen and the resulting fluorescence is too low to record on film. Mutant hearts stained with antitropomyosin show little fluorescence staining when compared to the normal cells; furthermore, mutant cells exhibit no obvious striations (Fig. 8). Therefore, these immunohistochemical studies corroborate the SDS gel data and further suggest that the normal hearts contain substantial amounts of tropomyosin by stage 41, while mutant hearts contain little.

Incubation with Tropomyosin or HMM

The general morphology of mutant myocardial cells processed through glycerol solutions is not very well preserved. Nevertheless, when the mutant hearts are processed through a graded series of glycerol and SSS without tropomyosin or HMM, amorphous proteinaceous collections are

distinguishable in the peripheral cytoplasm of the cells (Fig. 9). Occasionally, a few thin (60-Å) filaments may emanate from these amorphous areas, although this is somewhat unusual. After glycerol extraction and incubation for 18 h a solution containing 6 mg/ml of tropomyosin, the amorphous collections totally disappear and large numbers of 60-Å filaments become obvious (Figs. 10-11). The filaments usually appear random in arrangement (Fig. 10) but, on occasion, cells have filaments that exhibit a semblance of order (Fig. 11). Very clearly the number of visible thin filaments drastically increases in cells treated with tropomyosin and this increase seems to occur at the expense of amorphous collections. HMM essentially mimicks tropomyosin in forming actin filaments (21, 38) in the mutant hearts, except of course, that the filaments are "decorated" when HMM is used (Fig. 12). Negatively stained mutant heart homogenates corroborate the observations of sectioned specimens. Without adding tropomyosin or HMM to the mutant heart preparations before negative staining, almost no 60-Å filaments can be found (Fig. 13); however, "bare" thin (60-Å) filaments can be seen all over the grid after tropomyosin treatment (Fig. 14) and decorated filaments are numerous with the addition of HMM (Fig. 15).

DISCUSSION

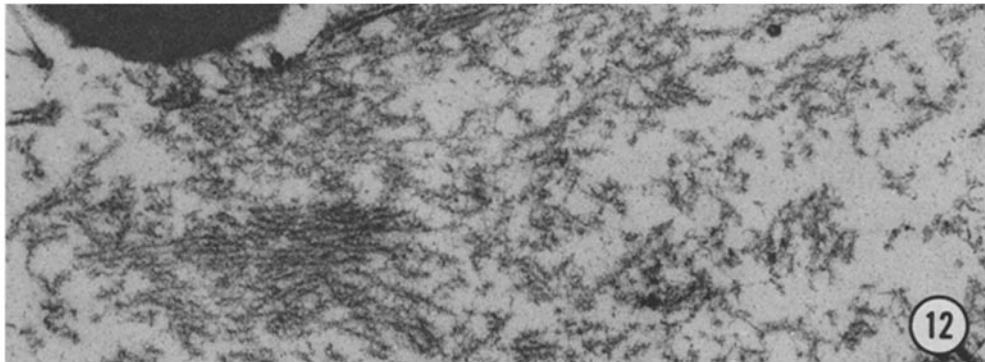
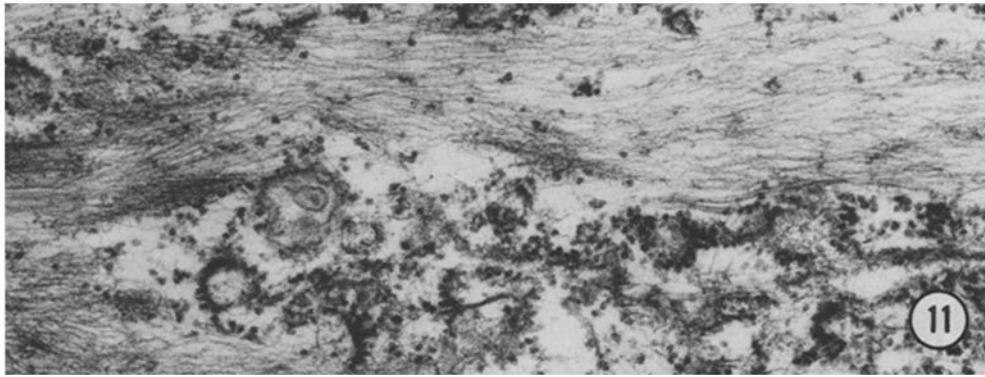
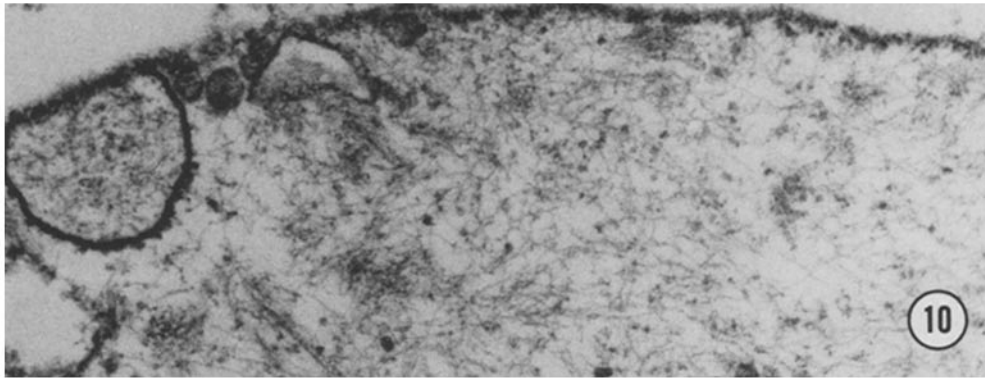
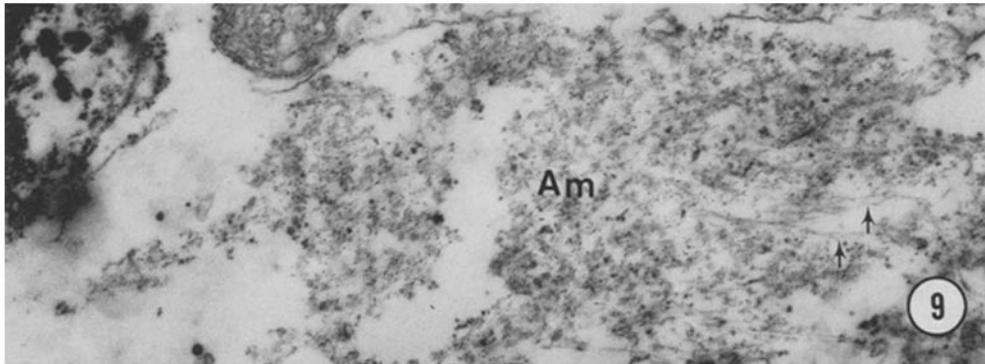
Homozygosity for recessive mutant gene *c* results in an absence of organized myofibrils in developing hearts of axolotl embryos. Even though the mutant hearts are nonfunctional, a few thin 60-Å filaments (actin-like) and thick 150-Å filaments

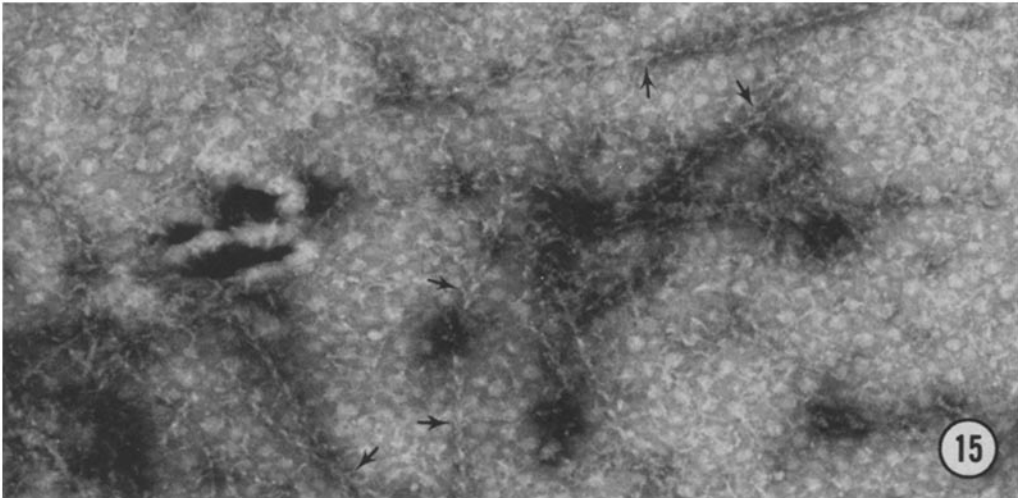
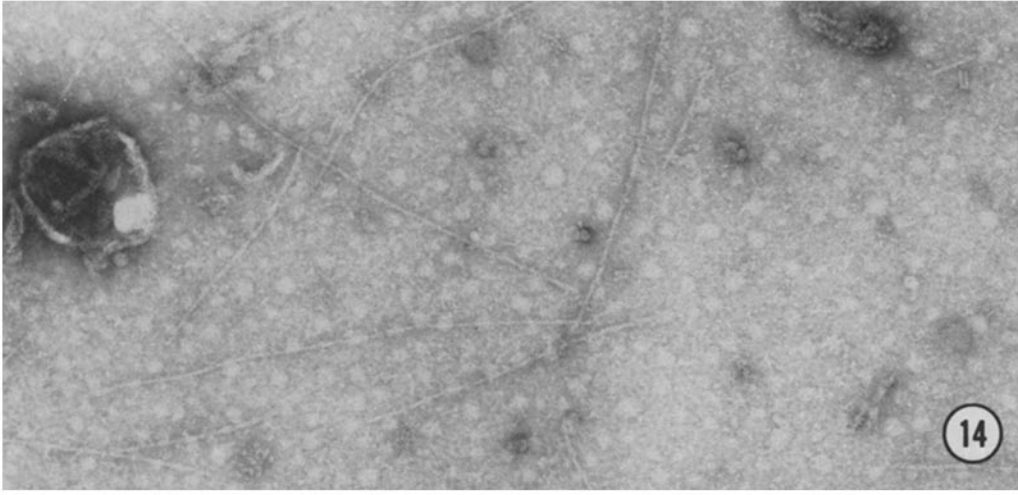
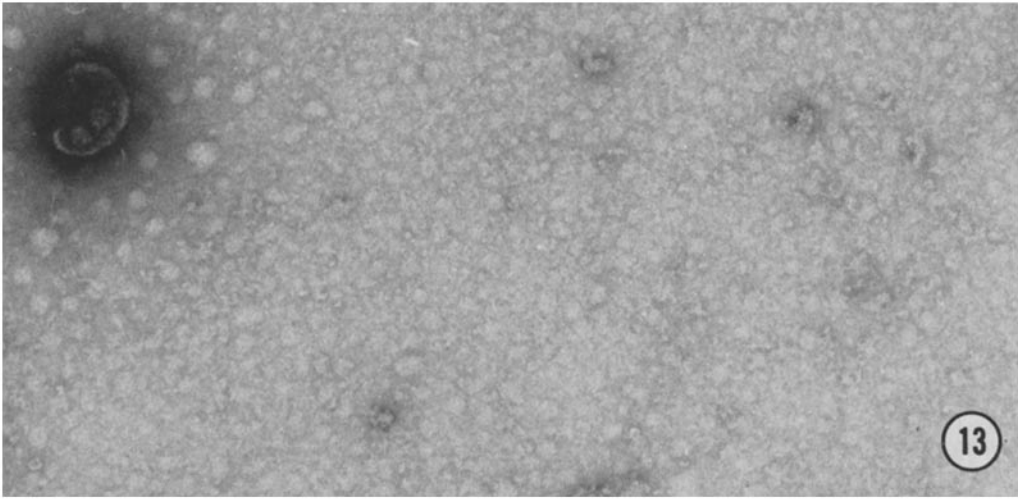
FIGURE 9 Electron micrograph of a portion of a heart cell from a stage 41 cardiac lethal mutant embryo which has been glycerinated for 18 h without incubation in tropomyosin. The amorphous collection (*Am*) has become somewhat diffuse but nonetheless remains in the periphery of the cell. A few thin filaments are seen in association with the amorphous material (arrows). $\times 34,500$.

FIGURE 10 Electron micrograph of a portion of a heart cell from a stage 41 cardiac lethal mutant embryo after glycerol extraction followed by incubation in a 6 mg/ml tropomyosin solution for 18 h. Amorphous collections are no longer visible and large numbers of 60-Å filaments appear in the cell periphery. The filaments are usually randomly arranged as is illustrated here. $\times 34,500$.

FIGURE 11 Electron micrograph of portions of heart cells from a stage 41 cardiac lethal mutant embryo after glycerol extraction followed by incubation in a 6 mg/ml tropomyosin solution for 18 h. Thin filaments are somewhat more "ordered" than in Fig. 10. $\times 34,500$.

FIGURE 12 Electron micrograph of a portion of a heart cell from a stage 41 cardiac lethal mutant embryo after glycerol extraction and incubation in a 6 mg/ml HMM solution for 18 h. Numerous decorated actin filaments are visible in the cell. $\times 34,500$.





(myosin-like) are visible in the cells after conventional electron microscope preparation. Most prominent are amorphous proteinaceous collections situated in the peripheral cytoplasm of the cells, where myofibrils initially organize in normal hearts (14). Immunofluorescence staining with antiactin antibodies suggests that most of the actin in mutant cells is located in these amorphous collections (L. Lemanski, R. Fuldner, and M. Nakanishi, unpublished observations). This and previously published studies in mutant hearts using HMM-binding and SDS-polyacrylamide gel electrophoresis methods agree that mutant hearts contain an almost normal amount of actin, enough it seems for organization into myofibrils provided that all of the other components necessary for organization are present. It was further clear from these studies that most of the actin in mutant hearts, although present, is in a nonfilamentous state (21).

While actin is abundant in mutant heart cells, muscle tropomyosin appears to be very much reduced. SDS-polyacrylamide gel electrophoresis demonstrates that normal hearts at stage 41 have a prominent 34,000-dalton protein indicative of muscle tropomyosin. Gels of mutant heart homogenates show only a slight 34,000-dalton band. Immunofluorescence studies using antibodies against tropomyosin correlate well with the electrophoresis studies. Sections of normal hearts stain intensely with antitropomyosin, and most of this staining is in the I bands of organized myofibrils. The mutant hearts show only slight staining for tropomyosin and there are no visible sarcomere striations. Recent immunofluorescence studies for myosin and α -actinin have been completed, and these proteins are present in significant quantities in mutant cells (18). Thus, although the mutant cells contain substantial amounts of actin, myosin, and α -actinin, all available evidence suggests that tropomyosin is reduced very significantly.

In view of the close structure-function relationship between actin and tropomyosin in organized myofibrils of muscle (5), the present study was undertaken to determine whether there might be some correlation between the deficiency of tropomyosin in mutant heart cells and their failure to form filamentous actin. The results suggest that there is indeed a correlation. Although 60-Å filaments are visible in mutant heart cells after incubation in tropomyosin, almost none are apparent in either intact or glycerinated tissue without the addition of tropomyosin. Negative staining experiments corroborate these observations. The actin filaments seem to form at the expense of the amorphous collections. One difficulty with this interpretation is that G-actin might be expected to wash out of the tissue during glycerination. However, recently it has been found that actin in sea cucumber sperm and erythrocytes exists in a non-filamentous, yet stable, form (33-36). This newly discovered state of actin is morphologically similar to the amorphous proteinaceous collections observed in the mutant heart cells. Recently, strong evidence has been presented suggesting that tropomyosin added to glycerol-extracted nonmuscle cells (*Acanthamoeba*) or to purified muscle actin serves to stabilize actin filaments that are already formed but that break down during fixation in osmium tetroxide (24, 27). In the present study on cardiac mutant hearts, results of the negative staining experiments with tissues that are neither glycerol-treated nor osmium-fixed do not allow a similar conclusion to be made for this system. Instead, I believe the overall results overwhelmingly support the conclusion that addition of tropomyosin induces the nonfilamentous actin in mutant heart cells to form into filaments.

Thus, gene *c*, by way of abnormal inductive processes (8, 12, 20, 22) from surrounding tissues, results in mutant heart cells having reduced but significant amounts of myosin and actin, even

FIGURE 13 Electron micrograph of a negatively stained preparation of stage 41 mutant heart homogenate without incubation in either tropomyosin or HMM. Very few filaments can be found on the grids. $\times 86,500$.

FIGURE 14 Electron micrograph of a negatively stained preparation of stage 41 mutant heart homogenate after a 30-min incubation with an equal volume of 4 mg/ml tropomyosin solution. Thin 60-Å filaments are numerous. $\times 86,500$.

FIGURE 15 Electron micrograph of a negatively stained preparation of stage 41 mutant heart homogenate after a 30-min incubation with an equal volume of 4 mg/ml HMM solution. Decorated actin filaments are numerous; several in this field show characteristic arrowhead structures (arrows). $\times 86,500$.

though nonfilamentous, and substantial amounts of α -actinin. There appears to be little tropomyosin in mutant cells and they fail to form organized myofibrils. While I do not rule out the possibility of a molecular abnormality of some kind in the mutant heart actin, the studies to date imply that the failure in actin filament formation in mutant hearts is related to the reduction of tropomyosin. Therefore, it is inviting to speculate that tropomyosin must be present in sufficient quantities in normal developing heart cells for actin to become filamentous and for myofibrillogenesis to take place.

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