# STUDIES OF MUSCLE PROTEINS IN EMBRYONIC MYOCARDIAL CELLS OF CARDIAC LETHAL MUTANT MEXICAN AXOLOTLS (*AMBYSTOMA MEXICANUM*) BY USE OF HEAVY MEROMYOSIN BINDING AND SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

LARRY F. LEMANSKI, MARK S. MOOSEKER, LEE D. PEACHEY, and M. RAJA IYENGAR

From the Department of Anatomy, School of Medicine, University of California, San Francisco, California 94143, the Cell Biology and Cardiology Research Sections of the San Francisco Veterans Administration Hospital, San Francisco, California 94121, and the Departments of Biology and Animal Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19174

ABSTRACT

In the Mexican axolotl *Ambystoma mexicanum* recessive mutant gene *c*, by way of abnormal inductive processes from surrounding tissues, results in an absence of embryonic heart function. The lack of contractions in mutant heart cells apparently results from their inability to form normally organized myofibrils, even though a few actin-like (60-Å) and myosin-like (150-Å) filaments are present. Amorphous "proteinaceous" collections are often visible. In the present study, heavy meromyosin (HMM) treatment of mutant heart tissue greatly increases the number of thin filaments and decorates them in the usual fashion, confirming that they are actin. The amorphous collections disappear with the addition of HMM. In addition, an analysis of the constituent proteins of normal and mutant embryonic hearts and other tissues is made by sodium dodecyl sulfate (SDS) gel electrophoresis. These experiments are in full agreement with the morphological and HMM binding studies. The gels show distinct 42,000-dalton bands for both normal and mutant hearts, supporting the presence of normal actin. During early developmental stages (Harrison's stage 34) the cardiac tissues in normal and mutant siblings have indistinguishable banding patterns, but with increasing development several differences appear. Myosin heavy chain (200,000 daltons) increases substantially in normal hearts during development but very little in mutants. Even so, the quantity of 200,000-dalton protein in mutant hearts is significantly more than in any of the nonmuscle tissues studied (i.e. gut, liver, brain). Unlike normal hearts, the mutant hearts lack a prominent 34,000-dalton band, indicating that if mutants

contain muscle tropomyosin at all, it is present in drastically reduced amounts. Also, mutant hearts retain large amounts of yolk proteins at stages when the platelets have virtually disappeared from normal hearts. The morphologies and electrophoresis patterns of skeletal muscle from normal and mutant siblings are identical, confirming that gene *c* affects only heart muscle differentiation and not skeletal muscle.

The results of the study suggest that the precardiac mesoderm in cardiac lethal mutant axolotl embryos initiates but then fails to complete its differentiation into functional muscle tissue. It appears that this single gene mutation, by way of abnormal inductive processes, affects the accumulation and organization of several different muscle proteins, including actin, myosin, and tropomyosin.

Studies have recently been reported on a naturally occurring genetic mutation, designated *c* for "cardiac lethal," in an imported stock of dark Mexican salamanders *Ambystoma mexicanum* (16, 22, 23). The effect of homozygosity for recessive gene *c* is the absence of a heartbeat, even though heart development initially appears normal. Mutant embryos are obtained from matings between heterozygous parents and are first distinguishable from their normal siblings at Harrison's stage 34, when the normal embryos develop contracting hearts. The mutant hearts at this stage, upon gross examination, appear structurally normal but fail to beat. Subsequently, the heart becomes distended, remains thin walled, and the embryo acquires an ascites condition. Mutants survive for about 20 days beyond the heartbeat stage and exhibit normal swimming movements, indicating that gene *c* does not affect skeletal muscle. Humphrey (16) performed transplants of mutant (*c*/*c*) heart primordia into the heart regions of normal (+/+ or +/*c*) siblings at stages 29–30 and found the cardiac lethal defect to be corrected. When reciprocal transplants were made, of +/+ or +/*c* donor heart primordia into *c*/*c* recipients, no heartbeat was observed. Humphrey further demonstrated that parabiosis of normal embryos with mutant siblings at stage 25 did not correct the cardiac deficiency, nor were the normal parabiotic twins adversely affected by this procedure; such conjoined animals lived for up to several months and the mutant twins, except for lacking a functional heart, appeared normal. These experiments suggest that gene *c* specifically affects the heart and indicate that the failure of normal heart differentiation in cardiac lethal embryos results from abnormal inductive effects from surrounding tissues. It is evident that such effects are restricted to the area of the developing heart and that the substances responsible are not circulating in the blood.

Light and electron microscope studies of mutant embryonic hearts from Harrison's stage 34 (at which normal heartbeat initiates) through stage 41 (at which mutant embryos die) have been reported (22, 23). The hearts from normal and mutant embryos display increasing differences as development progresses, and by stage 41 mutant abnormalities are striking. The normal myocardium has well-organized sarcomeres at stage 34, and numerous intercalated disks subsequently appear. By stage 41, the normal myocardium is composed of highly differentiated muscle cells and shows extensive trabeculation (20, 21). The mutant myocardium throughout development remains only one cell layer in thickness with no indication of developing trabeculae. Mutant cells at stage 34 have a few 150-Å and 60-Å filaments along with what appear to be Z bodies (22). A partial organization of myofibrillar components is noted in some mutant myocardial cells at stages 38–41; however, distinct sarcomeres are not apparent and intercalated disks are very rarely seen. In general, the mutant cells appear less differentiated than controls at the same stage and in some respects are reminiscent of pre-heartbeat normal cells. Although many mutant heart cells exhibit some pathological characteristics (e.g., pleomorphic mitochondria, membranous whorls, and numerous autophagic vacuoles), selective myocardial cell death, a phenomenon associated with normal heart trabeculation in this species is not evident (21, 22). It is clear that gene *c* in homozygous condition interferes with the normal course of heart cell differentiation. The most striking feature of heart morphology resulting from this mutation is a lack of organized myofibrils.

We undertook the present study to investigate what, if any, differences exist in major muscle proteins of normal and mutant hearts relating to the observation that mutant hearts fail to form

organized myofibrils. Our results suggest the following differences: (*a*) actin accumulates at almost normal levels in the cardiac mutant hearts, but the bulk of it is not assembled into filaments; (*b*) myosin is present in very reduced quantities; and (*c*) muscle tropomyosin, prominent in normal hearts, is not detectable in mutants. On the basis of our findings, we conclude that the precardiac mesoderm in cardiac lethal embryos initiates but then fails to complete its differentiation into functioning muscle tissue. It is implied that this single gene mutation, by way of abnormal inductive activities from surrounding tissues, affects the synthesis and/or accumulation and organization of several muscle proteins.

## MATERIALS AND METHODS

### *Procurement of Tissues*

Fertilized eggs were obtained from matings between adult axolotls heterozygous (+/*c*) for the cardiac lethal gene. The embryos were incubated at 16°–18°C until ready for study, at which time they were removed from their jelly coats and prepared for subsequent processing. The Harrison staging system for *Ambystoma* as described by Rugh (38) was used. Hearts from normal and mutant siblings from Harrison's stage 34 (7 days after fertilization) through stage 41 (30 days after fertilization) were investigated. Also included in the study as controls were a variety of different normal tissues ranging from fertilized eggs through adult muscle and nonmuscle tissues. In addition, the following purified muscle proteins were used as standards: myosin, actin, tropomyosin, and α-actinin (13) (the latter was a generous gift from Dr. D. Goll of Iowa State University).

### *Heavy Meromyosin Preparation*

Myosin was extracted from chicken skeletal muscle according to the procedure of Perry (31). Heavy meromyosin was obtained by brief tryptic digestion of the myosin and was then separated from the light meromyosin fraction by ammonium sulfate precipitation (43). The HMM was stored at −20°C in 25% glycerol dissolved in standard salt solution (0.01 M KCl, 0.005 M $MgCl_2$, and 0.006 M phosphate buffer, pH 7.0). The final concentration of the stored solution was 10–12 mg HMM/ml.

### *Electron Microscope Sections*

For the preparation of electron microscope sections, embryonic hearts were excised with glass dissecting needles and then placed in 50% glycerol-standard salt solution for 12–24 h followed by 6 h each in 25% glycerol and 5% glycerol (18). The tissues were incubated for 18 h in HMM solution diluted immediately before use with an equal volume of standard salt solution at pH 7.0. Thus, the final concentration of the incubation mixture was 5–6 mg HMM/ml and the concentration of glycerol was 12–13%. Controls for the HMM binding experiments included normal and mutant hearts which were processed as above but without HMM or with the addition of 5 mM $MgCl_2$ and adenosine triphosphate (ATP) to the incubation solution. The tissues were rinsed for 1 h in 0.10 M KCl (18) and fixed for 4–8 h in 4% glutaraldehyde buffered to pH 7.3 with 0.10 M phosphate buffer, followed by 1 h in 1% osmium tetroxide buffered to pH 7.2 with 0.10 M phosphate buffer. Some of the tissues were stained *en bloc* before dehydration with 0.5% uranyl acetate in acetate-Veronal buffer, pH 5.0 (7). All procedures before dehydration were carried out at 4°C. After dehydration in graded ethanol solutions and propylene oxide, the tissues were embedded in Epon. Thin sections were mounted on bare copper grids and stained with uranyl acetate and lead citrate (35). For routine ultrastructural observation the tissues were processed as previously described (22).

### *Negative Staining*

For each negative staining experiment, 10 stage 32 (pre-heartbeat) normal, 10 stage 41 normal, and 10 stage 41 mutant hearts were homogenized separately in vials, each containing 1 ml of standard salt solution with 0.001 M EDTA at 0°C. Homogenization was accomplished by rapidly drawing the hearts in and out of a 1-ml tuberculin syringe without a needle (18). One drop of the homogenate and one drop of HMM solution (diluted immediately before use with 0.10 M KCl to approximately 1 mg HMM/ml) were placed on a Formvar-coated, carbon-stabilized grid, and were allowed to react for 30–60 s (18). The grids were rinsed with several drops of 0.05 M KCl and were stained with 1% aqueous uranyl acetate (17). Controls included the above preparations containing 5 mM $MgCl_2$ and ATP or negative staining in the absence of HMM.

### *Electron Microscopy*

Specimens were viewed on a JEM 100B electron microscope at an acceleration voltage of 80 kV. Negatives taken at magnifications ranging between 1,000 and 120,000 were photographically enlarged. Measurements of filament diameters and HMM arrowhead periodicities were made on electron micrograph prints at × 120,000 or × 180,000 taken immediately after an accurate magnification calibration of the electron microscope had been performed with a carbon grating replica (Ernest F. Fullam, Inc., Schenectady, N. Y.).

### *SDS-Polyacrylamide Gel Electrophoresis*

A variety of tissues from normal and mutant embryos were dissected in quantity (30–50 animals were used per gel sample) and prepared for analysis by SDS gel electrophoresis. These included: (*a*) normal and mutant hearts at Harrison's stages 34, 39, and 41, and normal hearts at stage 46; (*b*) normal skeletal muscle at stages

30, 34, 39, 41, and 46; (*c*) mutant skeletal muscle at stages 34, 39, and 41; (*d*) normal gut at stages 30, 34, 39, and 41, and mutant gut at stages 34 and 41; (*e*) normal liver at stages 30, 34, 39, 41, and 46; (*f*) normal brain at stages 30, 34, 39, 41, 46, and juvenile; (*g*) homogenates of whole normal fertilized eggs (stage 2). The tissues were dissected from embryos which had been placed in a 0°C Steinberg's solution (38) to which had been added the proteolytic inhibitor phenyl methyl sulfonyl fluoride to a final concentration of 1.0 mM. In addition, myofibrils from juvenile and adult axolotl heart and skeletal muscle and chicken skeletal muscle were prepared for SDS gel electrophoresis. The myofibrils were made by homogenization of fresh muscle tissue in 50 mM KCl, 5 mM EGTA, 1 mM dithiothreitol, 1.0% Triton X-100, 10 mM Imidazole, pH 7.1. Myofibrils were collected by centrifugation of this homogenate at 7,000 *g* for 10 min. Molecular weight calibrations were made using the following muscle proteins as standards: chicken and axolotl myosin heavy-chain (200,000), porcine $\alpha$-actinin (100,000), chicken and axolotl actin (42,000), and chicken tropomyosin (34,000).

Protein solutions for gel electrophoresis were prepared from the above samples by standard methods (41, 48). The samples were suspended in 0.2–0.4 ml of 1.0% SDS, 1.5% $\beta$-mercaptoethanol, 1.0 mM phenyl methyl sulfonyl fluoride (proteolytic inhibitor), 10 mM phosphate, pH 6.8, heated at 100°C for 5 min, and then sonicated for an additional 5 min. At this point any undissolved material was removed from the gel samples by centrifugation in a clinical centrifuge. This step was not necessary for any of the embryonic cardiac tissues. Most gel samples were then concentrated by the addition of 9 vol cold acetone (−20°C) to precipitate the proteins from solution. The precipitates were collected by centrifugation and redissolved in small volumes of the above SDS solution (0.1–0.2 ml). The total protein concentrations of solutions containing the proteins were approximated by using the microbiuret method of Goa (12) as described by Bailey (1). Samples were run on both 5.0% and 7.5% polyacrylamide gels containing 0.1% SDS and then stained. The gels were examined visually to estimate roughly the amounts of proteins in the various stained protein bands (i.e. more than, less than, the same as), but in order to confirm these visual impressions densitometer tracings were performed. The gels were stained routinely with 0.25% Coomassie brilliant blue R250 and for the quantitative studies with 1% acid fast green, the latter being well known for its linearity of staining for a variety of proteins (14), including all of the muscle proteins compared in the present study (34). Graphic traces were made using a Gilford Spectrophotometer model 4200 (Gilford Instruments, Oberlin, Ohio) equipped with a gel scanner attachment at an absorbance wave length of 550 nm for Coomassie blue and 630 nm for fast green. To insure as nearly as possible linearity between optical density readings and protein quantity in the gels, the same samples were run at two or three different concentrations. Measurements were made on the resulting gel traces to determine molecular weights. The relative ratios of myosin heavy chain to actin at different stages were determined. In establishing these ratios, we assumed that the molecular weight of myosin heavy chain was 200,000 daltons and that actin was 42,000 daltons. The ratios were arrived at by comparing the weights of paper cutouts of the 200,000 dalton peak (myosin heavy chain) and the 42,000 peak (actin) as well as by determining their relative area ratios with a tracing device linked to a digital computer (29). Maximum and minimum values for each were determined by using the methods of Orkin et al. (28), and a mean value for each was used in establishing the relative ratios.

We wish to stress that the quantitative data we present represent relative amounts of protein on the gels and not absolute quantities. Furthermore, we compare mutant hearts with known muscle tissue (normal hearts). We additionally reiterate that the main purpose of presenting these quantitative measurement is to verify with numbers our visual judgments of the relative quantities of proteins represented by the different stained bands in the gels.

## RESULTS AND DISCUSSION

### *HMM Binding*

Numerous investigations have shown that the heavy meromyosin (HMM) fragment of myosin specifically interacts with thin 60-Å filaments in muscle and other cells to form characteristic arrowhead structures visible in the electron microscope (3–5, 11, 15, 18, 26, 32, 33, 42, 44, 47) and it is widely accepted that filaments capable of decorating with HMM are actin filaments.

In the present study, cells of normal hearts processed through decreasing concentrations of glycerol dissolved in standard salt solution without incubation in HMM show the appearances expected after glycerination. Distinct A bands, I bands, and Z lines are discernible in the myofibrils (Figs. 1, 3). After treatment with HMM, the thin filaments of the I band interact with the HMM molecules, increasing the overall density of the I bands (Fig. 2); these thin filaments display typical arrowhead structures with periodicities of 360 Å (Fig. 4). HMM binding is limited to 60-Å filaments, and the HMM molecules do not complex with 150-Å filaments (myosin), 100-Å filaments, collagen, membranes, or other cellular components.

In general, the morphology of mutant myocardial cells after glycerination is less well preserved than that of normal cells at the same developmental stages, presumably because mutant cells

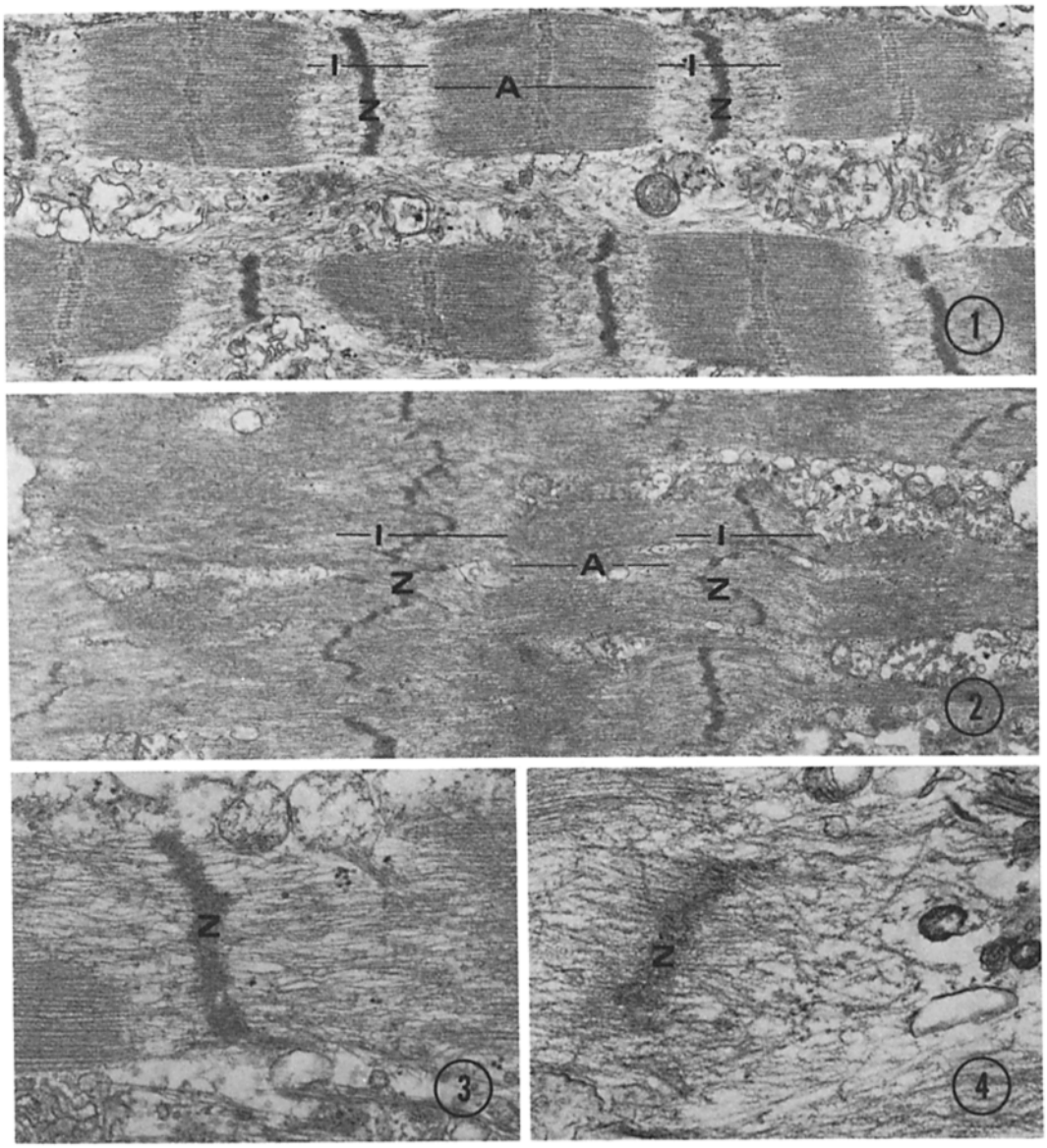
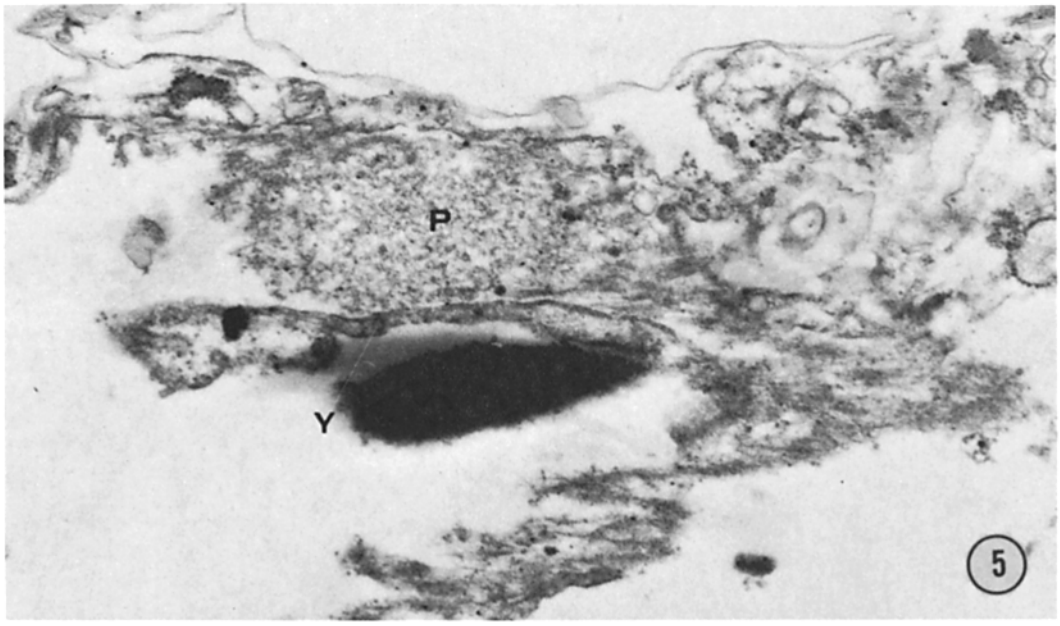



FIGURE 1 Portion of a glycerinated myocardial cell from a stage 41 normal embryo processed without HMM. The myofibrils show distinct I bands (*I*), A bands (*A*), and Z lines (*Z*). The thin filaments of the I band region appear uniform in diameter and do not display periodic projections. × 23,000.

FIGURE 2 Section of a myocardial cell from a stage 41 normal embryo after treatment with HMM for 18 h. The I bands (*I*) are more dense than in control preparations due to binding of HMM molecules to actin filaments. *A*, A band; *Z*, Z line. × 17,000.

FIGURE 3 High-magnification micrograph showing an I band of a myofibril in a glycerinated myocardial cell from a stage 41 normal embryo processed without HMM. The thin 60-Å filaments are uniform in diameter and do not display periodic projections. *Z*, Z line. × 50,000.

FIGURE 4 High-magnification micrograph showing an I band of myofibril in a glycerinated myocardial cell from a stage 41 normal embryo after 18 h of incubation in HMM. The thin 60-Å filaments show characteristic decorated images with arrowhead structures at 360-Å intervals. *Z*, Z line. × 50,000.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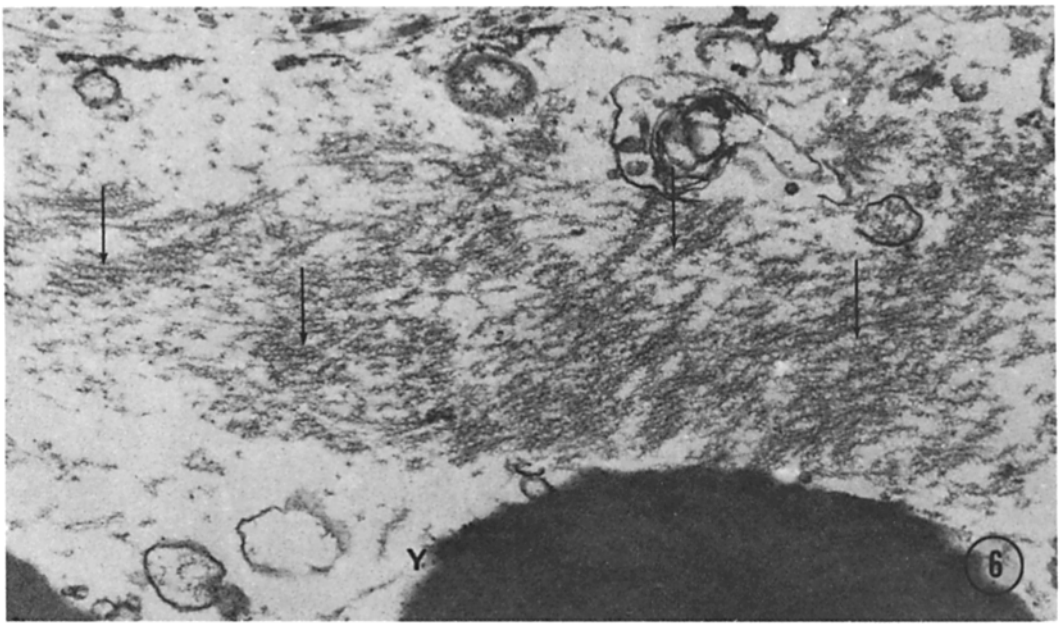
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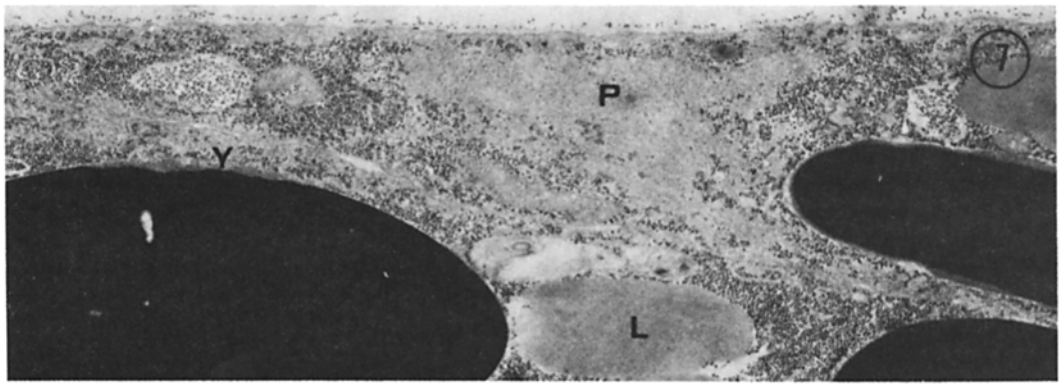
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lack the structural rigidity provided by organized myofibrils. Most mutant myocardial cells processed through glycerol solutions without HMM contain prominent collections of amorphous material but very few, if any, 60-Å filaments (Fig. 5); after incubation in HMM the amorphous collections disappear and large numbers of decorated filaments displaying arrowhead complexes with periodicities of 360 Å become apparent (Fig. 6, 8, 9). The HMM filament complexes are reasonably stable and they do not dissociate after repeated rinsing in 0.1 M KCl solution. They are dissociated, however, by rinsing in ATP (5 mM ATP, 5 mM $MgCl_2$, 6 mM phosphate buffer, pH 6.8) or in pyrophosphate (10 mM sodium pyrophosphate, 50 mM KCl, 1 mM $MgCl_2$, 10 mM sodium phosphate buffer, pH 6.8) for 30 min (18). Clearly, the number of visible filaments drastically increases in cells treated with HMM, and the increase seems to occur at the expense of the amorphous collections. One explanation for this result could be the induced polymerization of nonfilamentous actin, which may be present in the amorphous collections, into filaments in the presence of HMM molecules (32, 44). Further support for such a conclusion is obtained from routine ultrastructural observation which reveals that mutant heart cells contain amorphous proteinaceous collections in locations where myofibrils generally form in normal cells (22) (Fig. 7). Negatively stained mutant heart preparations corroborate the observations of sectioned specimens (Figs. 10–12). Without HMM treatment, virtually no 60-Å filaments can be found in mutants; however, the filaments are numerous after HMM treatment, and arrowhead structures at 360-Å intervals are clearly visible (Fig. 12). Thus, with respect to HMM binding the actin filaments in mutant myocardial cells appear to be identical to those in normal cells. Most of the actin in mutant hearts, however, is either present in a nonfilamentous form before HMM addition or is not preserved for electron microscopy after glycerination in the absence of HMM.

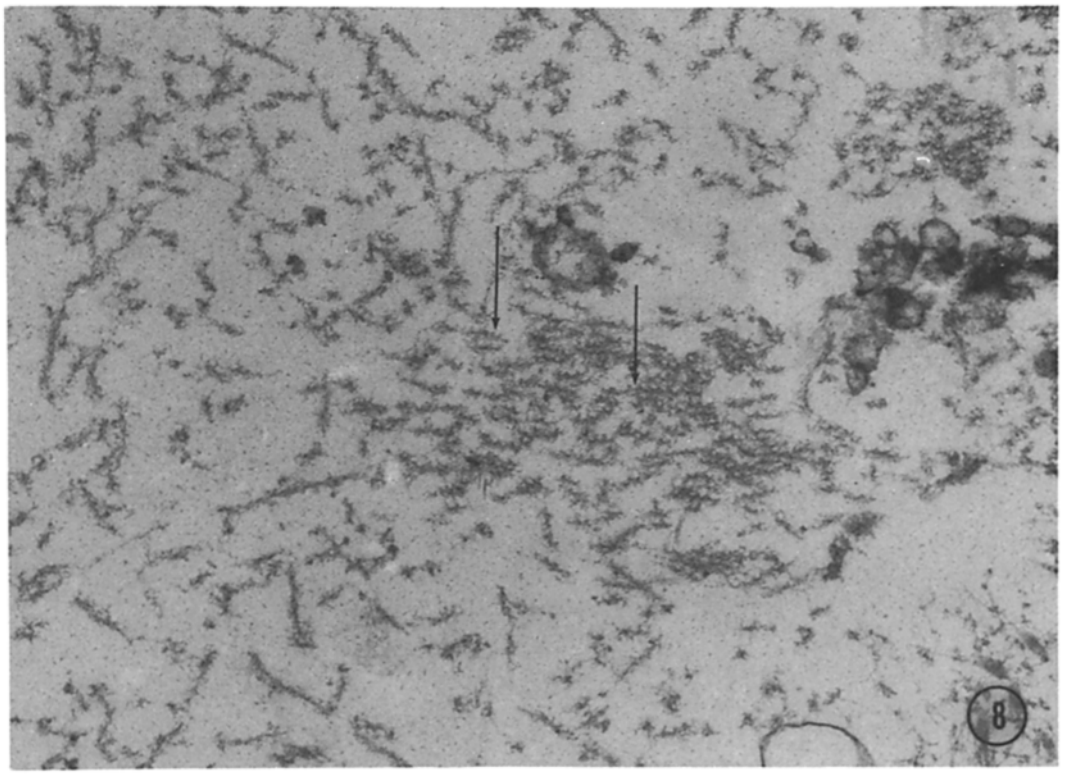
### *SDS-Polyacrylamide Gel Electrophoresis*

In using SDS-polyacrylamide gel electrophoresis to evaluate the various muscle proteins in embryonic tissues the following assumptions are made: (*a*) 200,000-dalton protein = myosin heavy chain, (*b*) 42,000-dalton protein = actin, and (*c*) 34,000-dalton protein = tropomyosin. The assumptions are based on the widely accepted premise that the molecular weights of these three myofibrillar proteins are well established and reproducible with electrophoretic methods (41, 48). Furthermore, it appears likely that actin (42,000 daltons) and probably myosin (200,000 daltons) are ubiquitous molecules in eucaryotic cells (33, 37). An additional assumption we make in analyzing the densitometer scans of the gels is: (*d*) the proteins (i.e. 200,000-dalton, 42,000-dalton, and 34,000-dalton) in mutant heart tissues bind the

FIGURE 5 Portion of myocardial cell from stage 41 cardiac lethal mutant embryo which has been glycerinated for 18 h without incubation in HMM. The cytoplasm lacks distinct filaments and contains instead a "proteinaceous" amorphous collection (P). We do not exclude the possibility that the filaments are simply not preserved for electron microscopy in the absence HMM. *Y*, yolk platelet. × 41,600.

FIGURE 6 Portion of myocardial cell from stage 41 cardiac lethal mutant embryo after incubation in HMM for 18 h. The HMM molecules decorate the thin filaments in a manner indistinguishable from binding in normal cells. In favorable planes of section, arrowhead complexes with 360-Å periodicities can be distinguished (arrows). Most significant is the large increase in number of filaments present in HMM-treated cells when compared to glycerinated controls (see Fig. 5). Note also the obvious absence of amorphous proteinaceous collections. Such evidence may indicate that a nonfilamentous form of actin (G-actin or "profilametnous" actin) is present in the mutant cells which is stimulated to form filaments in the presence of HMM. *Y*, yolk platelet. × 27,800.

FIGURE 7 Portion of mutant myocardial cells from a stage 41 mutant embryo prepared for conventional morphological study. Unlike normal cells in which organized myofibrils are abundant (see Fig. 1), these cells lack myofibrils. Instead, an amorphous "proteinaceous" collection (*P*) is present in the peripheral cytoplasm of the cell where myofibrils first organize in normal cells. This micrograph also illustrates an area of the mutant heart which still contains large amounts of yolk (*Y*); virtually all of the yolk platelets have disappeared from normal hearts by stage 41. *L*, lipid. × 13,300.



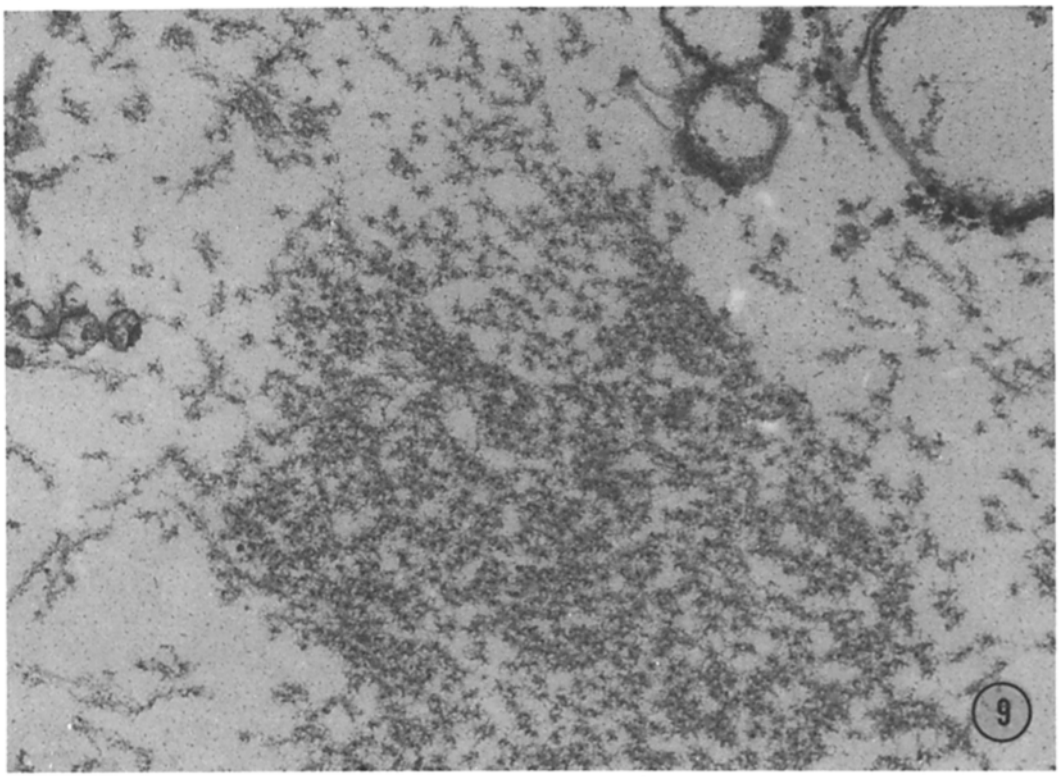




FIGURE 8 Section showing a portion of a myocardial cell from stage 41 cardiac lethal mutant embryo after incubation in HMM for 18 h. Numerous filaments are visible. Several can be seen in longitudinal profile illustrating the periodic arrowhead projections (arrows). × 41,600.

FIGURE 9 Section showing a portion of a myocardial cell from a stage 41 cardiac lethal mutant embryo after incubation in HMM for 18 h. Numerous decorated filaments can be seen in cross section. × 41,600.

acid-fast green stain with stoichiometries identical to those of the proteins in normal hearts and other control tissues.

Banding patterns for the hearts of normal and mutant embryos at stage 34 are indistinguishable from each other; however, as development progresses through stage 41 (when mutants begin to die), differences become striking (Figs. 13–14). The densitometer traces (Fig. 15) and the data in Table I show that as heart cells in normal embryos continue to differentiate, the 200,000-dalton protein (myosin heavy chain) progressively increases. The mutant hearts, on the other hand, show much smaller increases in the 200,000-dalton band and remain much like normal cells at earlier stages. Although mutant hearts fail to develop the amount of 200,000-dalton protein associated with normal heart muscle differentiation, the mutants during later stages do display substantially higher amounts of 200,000-dalton protein than the nonmuscle tissues studied (liver, brain, gut). The 42,000-dalton band (actin) is about the same in normal and mutant hearts.

A 34,000-dalton protein band becomes prominent in normal hearts by stage 41 (Fig. 14). The band is not obvious in the 5% gels of Fig. 13 because the gels illustrated are somewhat underloaded. Presumably, this band is muscle tropomyosin since the protein(s) coelectrophoreses with purified chick breast muscle tropomyosin (2). The mutant hearts have a very faint 34,000-dalton band, suggesting that muscle tropomyosin is drastically reduced in mutant heart cells and is, in fact, almost nonexistent (Fig. 14). In view of recent reports in the literature (6, 8), we cannot rule out the possibility that mutant hearts contain tropomyosin of the nonmuscle type (30,000 daltons). Mutant gels show a diffuse but prominent 30,000-dalton band which could contain this protein. The majority of the 30,000-dalton protein in mutant

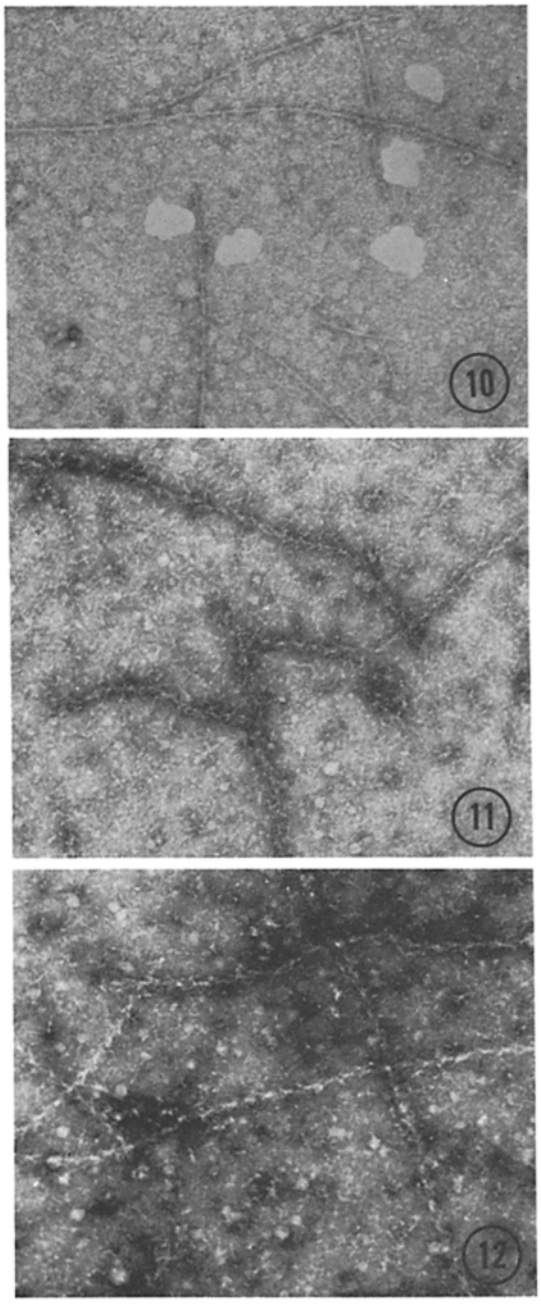



FIGURE 10 Negatively stained preparation of filaments from homogenized hearts of stage 41 normal embryos without HMM treatment. The filaments are uniformly smooth. × 75,000.

FIGURE 11 Negatively stained preparation of myofilaments from homogenized hearts of stage 41 normal embryos after HMM binding. Arrowhead structures on the 60-Å actin filaments exhibit 360-Å periodicities. × 69,000.

FIGURE 12 Negatively stained preparation of filaments from homogenized hearts of stage 41 cardiac lethal mutant embryos after HMM binding. Thin filaments are numerous and show HMM projections with 360-Å periodicities. The HMM binding of thin filaments in mutant heart preparations is indistinguishable from that of normal hearts. Without the addition of HMM, virtually no filaments can be found. × 78,000.

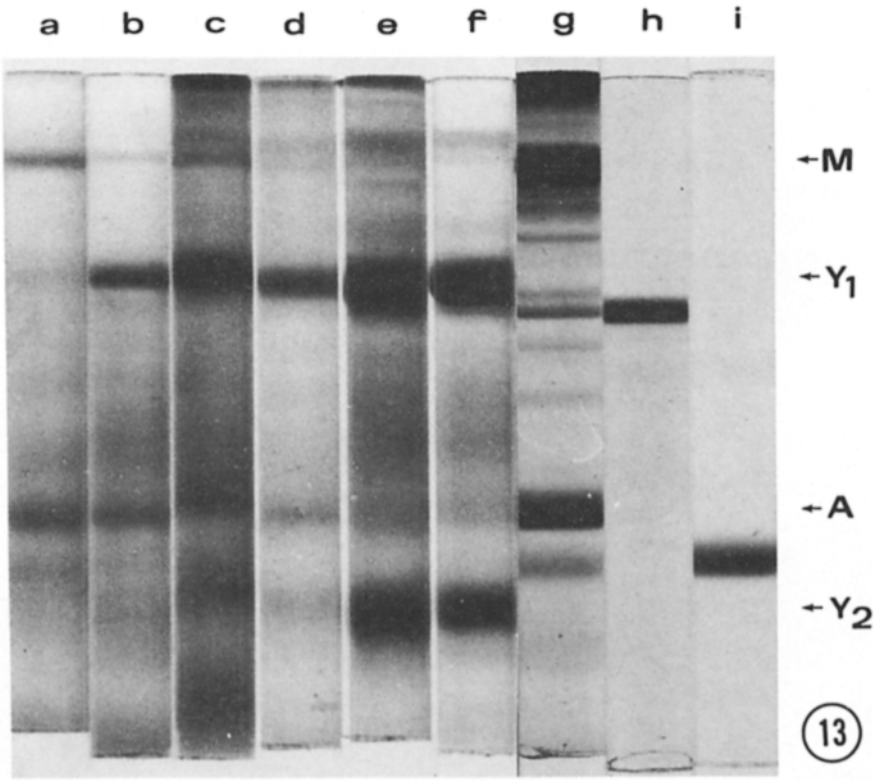



FIGURE 13 SDS-polyacrylamide (5% acrylamide) gel electrophoresis patterns of normal and mutant heart tissues at several developmental stages: (*a*) stage 41 normal heart; (*b*) stage 41 mutant heart; (*c*) stage 39 normal heart; (*d*) stage 39 mutant heart; (*e*) stage 35 normal heart; (*f*) stage 35 mutant heart; (*g*) chicken skeletal muscle myofibrils; (*h*) porcine skeletal $\alpha$-actinin; (*i*) chicken skeletal tropomyosin. *M*, myosin heavy chain (200,000); $Y_1$ yolk platelet protein (130,000); *A*, actin (42,000); $Y_2$, yolk platelet protein (30,000).

hearts is probably a yolk component, however, since it appears distinct only in gels of embryonic tissues that contain numerous yolk platelets (see Discussion, below).

The most obvious band in gels of mutant hearts at stage 41 ranges from 90,000 to 150,000 daltons (center measurement = 130,000 daltons) and also probably is a yolk component. This band is not prominent in normal heart cells at stage 41, and virtually all yolk platelets have disappeared (Figs. 13-15). Platelets remain a characteristic feature of myocardial cells in some areas of the mutant hearts, however (Fig. 7). Furthermore, electrophoresis studies combined with light and electron microscopy of a variety of early normal tissues (heart, gut, brain, liver, skeletal muscle, fertilized eggs, and others) reveal that whenever yolk platelets are present the 130,000-dalton band is present, and that whenever the cells lack platelets the large band is absent. The normal hearts at stage 41 and later have several electrophoretic bands that fall within the 90,000-150,000-dalton range, and some of these bands may represent different structural or modulatory muscle proteins (e.g., $\alpha$-actinin [13], C protein [27], etc.). Further studies will be required to determine if comparable bands are present in mutants hearts but masked by yolk. What remains fact is that yolk platelets are still abundant in mutant cells long after they have disappeared from normal cells. This, in itself, suggests that the mutant cells have failed to differentiate as normal.

Both normal and mutant hearts when electrophoresed on 7.5% acrylamide gels show distinct low molecular weight components including 18,000- and 27,000-dalton bands (Fig. 14).

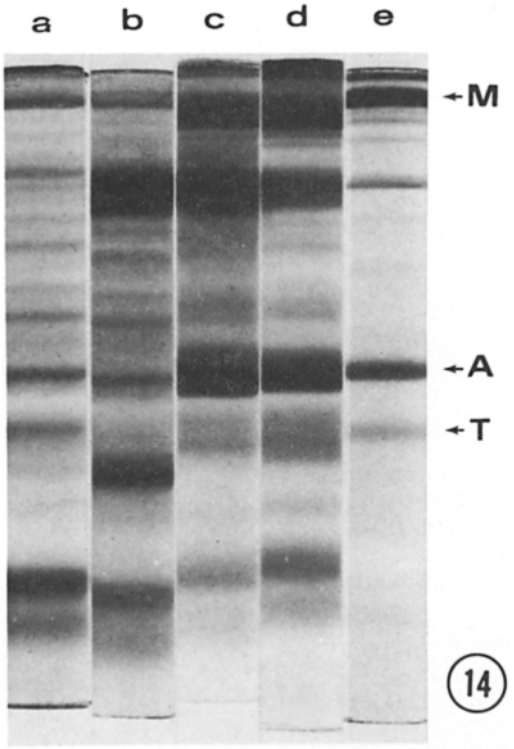



FIGURE 14 SDS-polyacrylamide (7.5% acrylamide) gel electrophoresis patterns of normal and mutant muscle tissues at stage 41: (*a*) stage 41 normal heart; (*b*) stage 41 mutant heart; (*c*) stage 41 normal skeletal; (*d*) stage 41 mutant skeletal; (*e*) chicken skeletal muscle myofibrils. *M*, myosin heavy chain (200,000); *A*, actin (42,000); *T*, tropomyosin (34,000).

Whether these proteins represent cardiac myosin light-chains (25, 39, 49), troponins, or some other unidentified proteins requires further investigation.

Skeletal muscles of normal and mutant siblings are indistinguishable with respect to ultrastructure and constituent proteins as determined by SDS polyacrylamide gel electrophoresis (Fig. 14). That the mutant skeletal muscle is unaffected by gene *c* confirms previous postulates (22, 23) that gene *c* affects only muscle of the heart. It also supports the likelihood that the mutation exerts its effect by way of abnormal inductive influence(s) from surrounding tissues in the heart region (e.g., anterior endoderm). Gene deletions and gene dosage effects within the myocardial cells themselves for myosin and tropomyosin can be ruled out since mutant precardiac mesoderm in the right "environment" (i.e. in the heart region of a normal embryo) differentiates in a totally normal fashion (16). In fact, since mutant myocardial cells are indeed capable of differentiating normally, the possibility of a primary genetic flaw of any kind (e.g., abnormal yolk utilization, abnormal mitochondria, an altered membrane phenomenon, etc.) can be eliminated from consideration. The most likely explanation we are left with is that gene *c* alters the inductive patterns (19) required for normal heart differentiation. In further support of this conclusion, a preliminary electron microscope study of anterior endoderm in normal and mutant siblings at the heartbeat initiation stage (Harrison's stage 34) suggests that the mutant anterior endoderm has reached a more advanced level of differentiation than the normal endoderm (24). Among other

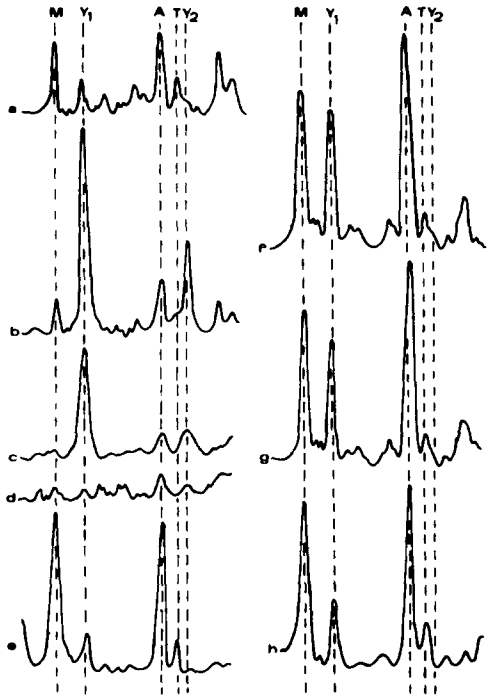



FIGURE 15 Densitometer tracings of SDS-polyacrylamide gels after staining with acid-fast green (14, 34). (*a*) stage 41 normal heart; (*b*) stage 41 mutant heart; (*c*) stage 34 brain; (*d*) stage 41 brain; (*e*) chicken skeletal muscle myofibrils; (*f*) stage 41 normal skeletal muscle; (*g*) stage 41 mutant skeletal muscle; (*h*) juvenile axolotl skeletal muscle. *M*, 200,000-dalton protein (myosin heavy chain); $Y_1$, 135,000-dalton protein (probably mostly yolk protein in *a*,*b*,*c*,*f*, and *g*, but probably α-actinin in *e* and *h*); *A*, 42,000-dalton protein (actin); *T*, 34,000-dalton protein (tropomyosin); $Y_2$, 30,000-dalton protein (probably mostly yolk but possibly some non-muscle type of tropomyosin).

TABLE I

*Relative Ratios of 200,000-Dalton (Myosin Heavy Chain) to 43,000-Dalton (Actin) Proteins*

| | Chicken skeletal myofibrils | Azolotl skeletal muscle | Mutant heart | Normal heart | Mutant skeletal | Normal skeletal | Normal brain |
|---|---|---|---|---|---|---|---|
| 200,000/43,000 | 1.15 | 0.85 | | | | | |
| stage 34 | | | 0.14 | 0.14 | 0.11 | 0.13 | 0.16 |
| stage 39 | | | 0.25 | 0.56 | 0.58 | 0.54 | 0.14 |
| stage 41 | | | 0.33 | 0.71 | 0.68 | 0.63 | 0.14 |

The numerical values are based on densitometer tracings of acid-fast green-stained SDS-polyacrylamide gels. The results show that mutant hearts compared to normal have lower 200,000 to 43,000 ratios during stages 39 and 41. The ratios in mutant hearts are nonetheless significantly higher than in the nonmuscle tissues studied. Skeletal muscle appears the same in normals and mutants.

possibilities, this might indicate that the inducing capability of the mutant anterior endoderm is "spent" before the mesoderm arrives on the scene. Whatever the details, this work proves that gene *c* affects the primary heart inductor tissue—the anterior endoderm. Thus, all of the available evidence supports an abnormal induction process as the cause of the cardiac lethal defect in axolotls.

## CONCLUSION

Gene *c* is the only known vertebrate mutation that drastically alters myofibril formation. Although thin 60-Å actin filaments and a few thick 150-Å myosin filaments are present in the myocardial cells of affected embryos, organized myofibrils do not form and, as a consequence, the mutant hearts fail to contract.

Heavy meromyosin binding, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and previous morphological (22, 23) studies agree that mutant hearts contain an almost normal amount of actin, enough, it seems, for organization into myofibrils provided the other components essential for organization are present. Nonetheless, the distribution and nature of actin in mutant cells remain unclear. Even though numerous filaments are visible in mutant tissue after HMM treatment, few are visible in either intact or glycerinated tissue before addition of HMM. This is a familiar result to investigators using HMM as a probe for actin in nonmuscle motile systems (9–11, 18, 33, 40). Although this may be an artifact of fixation, we believe that such a result suggests that much of the actin in mutant cardiac cells is in a nonfilamentous state and the addition of HMM causes this actin to polymerize (50) into a filamentous form. One problem with this interpretation is that one would expect G actin to be washed out of the tissue during the glycerination period before HMM addition. However, it has recently been found that actin in sea cucumber sperm and red blood cells exists *in situ* in a stable, nonfilamentous insoluble form (45, 46). This newly discovered state of actin is morphologically similar to the amorphous proteinaceous collections present in the mutant myocardial cells described here.

The constituent proteins of mutant and normal hearts as revealed by SDS polyacrylamide gel electrophoresis are virtually identical at early stages, but as development advances the mutants contain much less myosin than normal. Nevertheless, the 200,000-dalton band does reach higher levels in mutant hearts than in any of the nonmuscle tissues studied (see Table I). A band of 34,000 daltons, indicating muscle tropomyosin, is prominent in normal hearts by stage 41 but almost indiscernible in mutant gels. It is apparent that muscle tropomyosin, if present at all, is present in much reduced quantities in mutant heart cells.

Thus, in the mutant myocardial cells there is an accumulation of actin in almost normal amounts, albeit in a nonfilamentous form, myosin in reduced quantities, and very little, if any, tropomyosin. It is apparent that this simple recessive single gene mutation, by way of abnormal inductive processes, has affected the accumulation, assembly, and organization of several major muscle proteins. The end result is a failure of mutant precardiac mesoderm to complete its differentiation into functional muscle tissue.

*Note Added in Proof:* Quantitation by radioimmunoassay of absolute amounts of myosin in hearts of cardiac lethal mutant embryos confirms the SDS polyacrylamide

gel electrophoresis experiments reported in the present paper suggesting that the myosin content of mutant hearts is quantitatively lower than normal (Lemanski, L. F., X. Joseph, and M. R. Iyengar. 1975. *J. Cell Biol.* **67** [2, Pt. 2]:239 a. [Abstr.]).

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