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Morphological and Biochemical Abnormalities in Hearts of Cardiac Mutant Salamanders (Ambystoma Mexicanum)

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The effect of homozygosity for recessive gene *c* in Ambystoma mexicanum is the absence of a heartbeat even though initially heart development appears normal. Mutant embryos (c/c) are first distinguishable from their normal siblings (+/+; +/c) at stage 34 (7 days after fertilization) when the normals develop contracting hearts. The mutant hearts at this stage, upon gross examination, appear structurally normal but fail to beat. Nevertheless, the mutants survive through stage 41, which is about 20 days beyond the heartbeat stage, and they exhibit normal swimming movements, indicating that gene *c* does not affect skeletal muscle. Electron microscopic studies of normal hearts show some myofibrils to be present at stage 34; by stage 41, the normal myocardial cells have become highly differentiated muscle cells. Although some mutant heart cells contain a few thin 60 A and thick 150 A filaments, organized myofibrils are absent. Instead, amorphous proteinaceous collections are prominent. Heavy meromyosin (HMM) binding experiments were performed on mutant hearts to determine whether the myocardial cells contain actin. Mutant myocardial cells that are glycerinated but not treated with HMM contain intact amorphous bodies. After incubation in HMM, the amorphous collections are no longer present and large numbers of decorated actin filaments appear. The results suggest that the amorphous proteinaceous collections contain actin in a nonfilamentous form, and the addition of HMM induces this actin to polymerize into filaments. SDS-polyacrylamide gel electrophoresis of mutant heart tissue supports this conclusion by showing a prominent 43,000 dalton band suggestive of actin. The electrophoresis experiments also demonstrate a significant reduction of myosin heavy chain (200,000 daltons) in mutant hearts when compared to normal, and this latter observation is confirmed by radioimmunoassay experiments. Muscle tropomyosin (34,000 daltons), prominent in normal hearts, is virtually nonexistent in mutants. Thus, it appears that this single gene mutation affects the accumulation and organization of several different muscle proteins, including actin, myosin, and tropomyosin.

Key words: gene defect, muscle proteins, heavy meromyosin, radioimmunoassay, electrophoresis, ultrastructure

INTRODUCTION

Humphrey (1) reported the discovery of a naturally occurring recessive mutant gene, designated *c* for "cardiac lethal," in a dark strain of imported Mexican salamanders, Ambystoma mexicanum. In homozygous condition (c/c), there is a total absence of a heartbeat, even though the heart at first seems to develop in a normal way. The defective embryos (c/c) are obtained from spawnings of heterozygous (+/c) adults and are first recognizable from their normal siblings at stage 34 (about 7 days after fertilization of the egg), when the hearts in normal embryos begin to beat. The mutant heats at this time, upon gross examination, appear morphologically normal but never contract. Later the hearts become distended and thin-walled. Mutant embryos survive for about 20 days beyond the heartbeat stage and during this time swim normally, indicating that gene *c* does not affect skeletal muscle. When transplants of mutant (c/c) heart primordia into the cardiac regions of normal (+/+; +/c) siblings are performed, the cardiac defect is corrected (2). If reciprocal transplants are made, of +/+ or +/c donor heart primordia into c/c recipients, no heartbeat is observed. Furthermore, it has been shown that parabiosis of normal with mutant embryos does not correct the cardiac defect, nor are the normal twins adversely affected by this procedure. These conjoined normal-mutant pairs live indefinitely and the mutants, except for lacking functional hearts, appear normal. These studies 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 apparent that such effects are present in the immediate region of the developing heart and that the substances responsible are not circulating in the blood.

Electrophysiological experiments measuring transmembrane potentials from in vivo hearts of mutant axolotl embryos show the resting potentials of heart myoblasts to be identical to those in normal siblings; however, cardiac lethal heart cells do not display recordable action potentials nor do they respond to acetylcholine, norepinephrine or extrinsic electrical stimulation (3).

In the work reported here, I attempt to describe some of the morphological and biochemical abnormalities of mutant embryonic hearts at various developmental stages.

METHODS

Procurement of Tissues

Fertilized eggs were obtained from spawnings between adult salamanders heterozygous (+/c) for the cardiac lethal gene. The embryos were incubated at 18°C until ready for study; embryos were staged according to the Schreckenberg and Jacobson method (4). Hearts from normal and mutant siblings from stage 34 (7 days after fertilization) through stage 41 (30 days after fertilization) were investigated.

Histology and Ultrastructure

The methods used for preparing the embryonic hearts for routine light and electron microscopy have been described in earlier papers (5–8). For histological observation, the embryos were fixed in formaldehyde, infiltrated with 3% celloidin, and embedded in paraffin, and the sections were stained with hematoxylin and eosin stains (9–10). For electron microscopic study, the hearts were fixed in a glutaraldehyde-formaldehyde-picric-

acid-styphnic acid mixture (11), and secondarily fixed in 1% osmium tetroxide and embedded in Epon. Thin sections were mounted on copper grids and doubly stained with lead citrate and uranyl acetate.

Heavy Meromyosin (HMM) Binding

Myosin was extracted from chicken skeletal muscle (12), and the heavy meromyosin subunit was obtained by brief tryptic digestion (13). For the preparation of electron microscope sections, embryonic hearts were excised and processed through 50% glycerol-standard salt solution for 12 hr followed by 6 hr each in 25% glycerol and 5% glycerol (14). The tissues were incubated 18 hr in a 12% glycerol-standard salt solution containing 6 mg HMM/ml. Controls for the HMM binding experiments included normal and mutant hearts that were processed as above but without HMM, or rinsing 30–60 min in 5 mM $MgCl_2$ and adenosine triphosphate (ATP) after incubation in the HMM solution. The tissues were rinsed for 1 hr in 0.10 M KCl (14) and fixed for 4–8 hr in 4% glutaraldehyde buffered to pH 7.3 with 0.10 M phosphate buffer, followed by 1 hr in 1% osmium tetroxide buffered to pH 7.2 with 0.10 M phosphate buffer. The specimens were dehydrated, embedded, thin-sectioned, and stained using standard methods (6, 15).

Negatively stained preparations were made following the methods developed by Ishikawa et al. (14). Embryonic hearts were homogenized in glass vials containing standard salt solution with 0.001 M EDTA. One drop of homogenate was mixed with one drop of HMM solution (1 mg HMM/ml) on a formvar-coated, carbon-stabilized grid, and were allowed to react for 60 sec. The grids were rinsed and then stained with 1% aqueous uranyl acetate. Controls included the above preparations containing 5 mM $MgCl_2$ and ATP or negative staining in the absence of HMM.

SDS-Polyacrylamide Gel Electrophoresis

Hearts and control tissues from normal and mutant embryos were dissected in quantity and prepared for analysis by SDS gel electrophoresis. The tissues were dissected from embryos and placed in a 0°C Steinberg's solution (4) to which had been added the proteolytic inhibitor phenylmethylsulfonyl 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. The myofibrils were collected by centrifugation of this homogenate. Molecular weight calibrations were made using the following muscle proteins as standards: chicken and axolotl myosin heavy chain (200,000 daltons), porcine α-actinin (100,000 daltons) (kindly provided by Dr. Darrel Goll and Dr. Judith Schollmeyer), chicken and axolotl actin (43,000 daltons), and chicken tropomyosin (34,000 daltons). Protein solutions for gel electrophoresis were prepared from the above samples by standard methods (16, 17). The samples were suspended in 1.0% SDS, 1.5% β-mercaptoethanol, 1.0 mM phenylmethylsulfonyl 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. This step was not necessary for any of the embryonic heart tissues. The total protein concentrations of solutions were approximated by using the microbiuret method of Goa (18) and as necessary were concentrated. Samples were run on both 5.0% and 7.5%

polyacrylamide gels containing 0.1% SDS and then stained routinely with 0.25% Coomassie brilliant blue R250 and for the semiquantitative studies with 1% acid fast green, the latter being well known for its linearity of staining for a variety of proteins (19–20). 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 three different concentrations. Relative ratios of myosin heavy chain (200,000 daltons) to actin (43,000 daltons) were determined by comparing the relative areas made in densitometer traces for 200,000 and 43,000 dalton peaks. A tracing device linked to a digital computer was employed for this purpose (25). Maximum and minimum values for a given peak were determined by using the methods of Orkin et al. (26), and a mean value for each was used in establishing the relative ratios.

Radioimmunoassay

Radioimmunoassays were performed to quantify absolute amounts of myosin in normal and mutant hearts. The methods developed by Iyengar et al. (21) were used. Briefly, purified cardiac myosin was injected into rabbits and the antimyosin was isolated from rabbit antiserum by ammonium sulfate precipitation and DEAE-cellulose chromatography. The antibody was coupled to cyanogen bromide-activated Sephadex (22, 23). Myosin was labeled with ^{14}C-iodoacetate in 0.5 M KCl, 0.01 M Tris-HCl, pH 8.0 (24). The immobilized antimyosin was saturated with labeled myosin and the ^{14}C-myosin-antibody complex was placed in a small counting vial. The embryonic heart tissues were homogenized and an aliquot of homogenate was added to the vial and allowed to react with gentle agitation for 18 hr at 0°C. Free radioactivity in the supernatant and the residual (bound) radioactivity in the insoluble state were measured in a liquid scintillation counter. The readings were compared with calibration standards obtained by adding known amounts of homologous purified myosin to the system.

RESULTS

Morphological Observations

Gross examination of stage 34 mutant embryos reveals that, except for lack of a heartbeat, the mutants appear identical to normal. In later development, due to an absence of circulation (2), mutant animals develop ascites and microcephaly, lack well-developed gills and are shorter in length than normals. Also by later stages, the mutant hearts appear thin-walled and abnormal in shape (7).

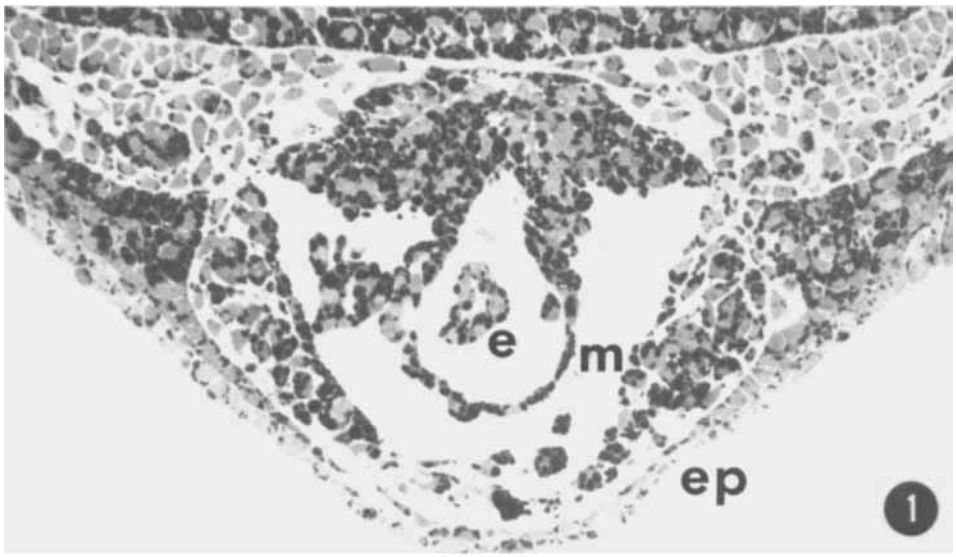
Despite the functional difference, light microscope studies of embryonic hearts at early stages reveal no striking histological differences between hearts of normal and mutant siblings (Fig. 1). The myocardium in each is one cell layer thick, forming a completed heart tube. By stage 41, however, the histological differences between normals and mutants have become obvious. Normal hearts at this stage have extensively trabeculated ventricles and myofibril tracts are numerous (Figs. 2–3). The mutant hearts show no sign of trabeculation or myofibrils (Figs. 4–5). Yolk platelets and lipid droplets, absent from normal cells at stage 41, are still abundant in mutants.

Electron microscopy of normal and mutant hearts confirms the absence of myofibrils in mutants. In normal hearts, organized myofibrils are first present directly beneath and parallel to the plasma membrane (Fig. 6). Large numbers of yolk platelets and lipid droplets characterize these early cells, but both gradually dissappear as contractile elements increase in amount. At stage 39, the normal myocardial cells contain abundant myofibrils, although they are still randomly arranged with respect to each other. Intercalated disks are present but not yet numerous or well developed. Yolk and lipid materials are significantly reduced from earlier stages. By stage 41 the myocardium in normal hearts is composed of highly differentiated muscle cells with numerous well-organized myofibrils and intercalated disks (Fig. 7). Mutant myocardial cells at stage 34 have a few 150 Å and 60 Å filaments along with what appear to be occasional Z bodies; however, even as late as stage 41, distinct sarcomeres are not apparent (Figs. 8–9). In examining those areas in the cell where myofibrils might be expected to form (i.e., the peripheral cytoplasm), one sees instead collections of amorphous proteinaceous material (Fig. 9).

Heavy Meromyosin (HMM) Binding

Cells of normal stage 41 hearts processed through glycerol-standard salt solutions in the absence of heavy meromyosin (HMM) show the appearance expected after glycerination. Sharply defined A bands, I bands, and Z lines are visible in the myofibrils and the 60 Å filaments are "bare" (Figs. 10 and 12). When the tissues are incubated in a solution containing HMM, the thin filaments of the I band interact with the HMM molecules, increasing the overall density of the I bands; these thin filaments decorate in a characteristic fashion for actin displaying periodicities of 360 Å (Figs. 11 and 13). The HMM complexes only with 60 Å actin filaments, and not with collagen, membranes, or other cellular components.

The morphology of mutant myocardial cells is less well preserved after glycerination than that of normal cells. Apparently this results from a lack of structural rigidity provided by organized myofibrils. Most of the myocardial cells in mutant hearts after processing through glycerol solutions in the absence of HMM contain collections of amorphous material but very few 60 Å filaments (Fig. 14); after incubation in HMM the amorphous collections disappear and numerous decorated filaments with 360 Å periodicities become apparent (Figs. 15–16). The filament-HMM complexes are quite stable and do not dissociate after repeated rinsing in standard salt solution or 0.1 M KCl. 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 (14). 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. Negatively stained mutant heart preparations corroborate the observations of sectioned specimens. In mutant heart homogenates that have been negatively stained, very few filaments can be found (Fig. 17); however, the filaments are numerous after HMM addition and arrowhead structures with 360 Å periodicities are clearly visible (Fig. 18). Thus, with respect to HMM binding, the actin filaments in mutant myocardial cells appear to be identical to those in normal cells. The bulk of actin in mutant hearts, however, appears to be present in a nonfilamentous state prior to the addition of HMM.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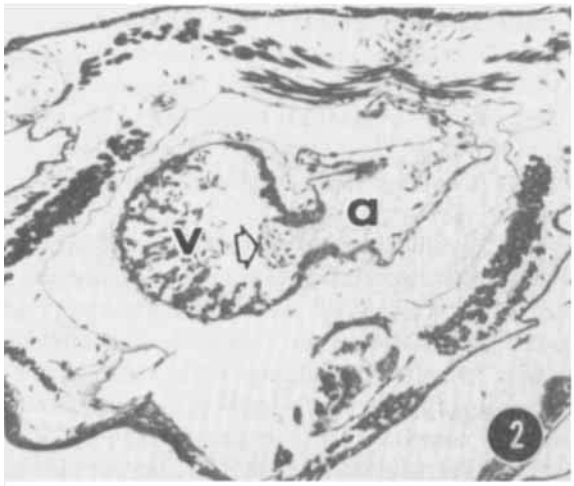
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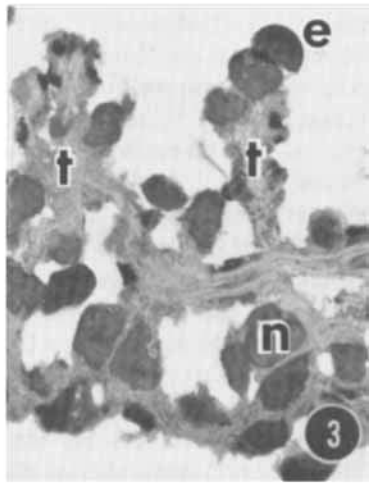
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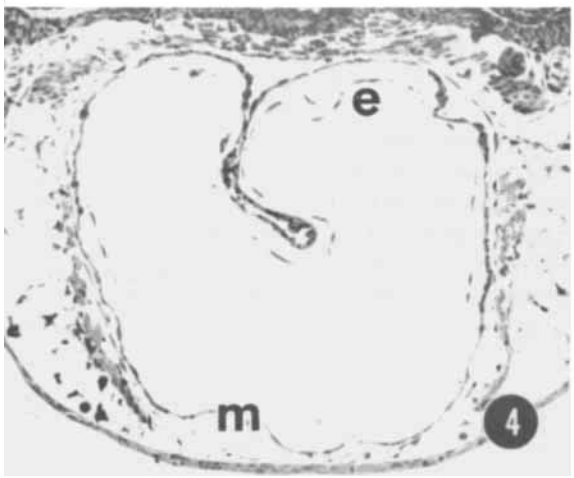
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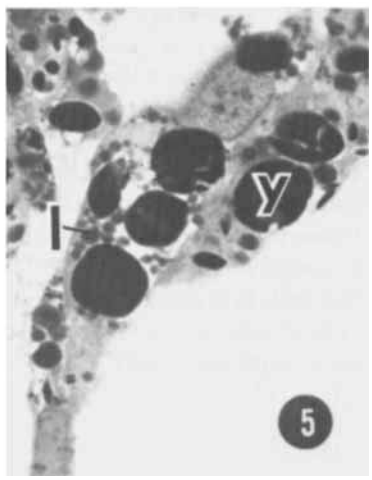
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SDS-Polyacrylamide Gel Electrophoresis

In evaluating the electrophoresis patterns of the various muscle proteins in the developing embryonic tissues of the present study, the following assumptions are necessary: a) 200,000 dalton protein = myosin heavy chain; b) 100,000 dalton protein = α-actinin; c) 43,000 dalton protein = actin; d) 34,000 dalton protein = tropomyosin. These assumptions are based on the widely accepted premise that the molecular weights of these four proteins are well established and are reproducible with electrophoretic methods (16, 17). An additional assumption made in analyzing the densitometer readings is that the proteins in mutant heart tissues bind the acid fast green stain with stoichiometries identical to those of the proteins in normal hearts and control tissues.

At stage 34, the banding patterns in normal and mutant hearts are identical; however, as development progresses to stage 41 the differences become obvious (Figs. 19–20). The data in Table I show that as the normal hearts continue to differentiate, the myosin heavy chain (200,000 daltons) relative to actin (43,000 daltons) progressively increases. The mutant hearts, however, show much smaller increases in myosin heavy chain and retain the myosin/actin ratios reminiscent of normal heart cells at earlier stages. Nonetheless the mutants develop higher myosin heavy chain/actin ratios than any of the nonmuscle tissues investigated (brain, gut, liver). Actin (43,000 daltons) appears to be present in similar quantities in normal and mutant hearts.

A 34,000 dalton protein band becomes very prominent in the normal heart gels by stage 41. Apparently the band represents muscle tropomyosin since it coelectrophoreses with purified chicken breast muscle tropomyosin. The mutant hearts at stage 41 have only a very faint 34,000 dalton band, suggesting that muscle tropomyosin in mutant hearts is almost nonexistent (Fig. 19). In view of recent studies of tropomyosin in nonmuscle systems (27, 28), the possibility that mutant hearts contain a nonmuscle type of tropomyosin (30,000 daltons) is not ruled out. The mutant gels show a dense 30,000 dalton band that could contain this protein. Most of the 30,000 dalton protein in mutants is likely a yolk component, however, since it is distinct only in those gels of embryonic tissues that contain numerous yolk platelets.

Fig. 1. Light micrograph of a cross section through the anterior region of the pericardial cavity and heart of a stage 35 mutant embryo. At this developmental stage, the mutant is similar to normal. e, endocardium; ep, epidermis; m, myocardium. × 195. From Lemanski, L. F., Develop. Biol. 33:312 (1973).

Fig. 2. Light micrograph of a cross section through the heart region of a stage 41 normal embryo. The ventricle (v) of the heart is trabeculated. A heart valve (arrow) is beginning to form between the atrium (a) and ventricle (v). ep, epidermis. × 64.

Fig. 3. High magnification light micrograph of a stage 41 normal heart wall illustrating trabeculation. The endocardial layer (e) is closely apposed to the myocardial cells that make up the developing trabeculae (t). n, myocardial cell nucleus. × 600.

Fig. 4. Light micrograph of a cross section through the heart region of a stage 41 mutant embryo. The myocardium (m) remains only one cell-layer thick and shows no sign of trabeculation. e, endocardium. × 70.

Fig. 5. High magnification light micrograph of a portion of a mutant myocardial wall at stage 41. There are no obvious myofibrils. Yolk platelets (y) and lipid droplets (l) are still prominent features in some regions of the mutant heart. × 700.

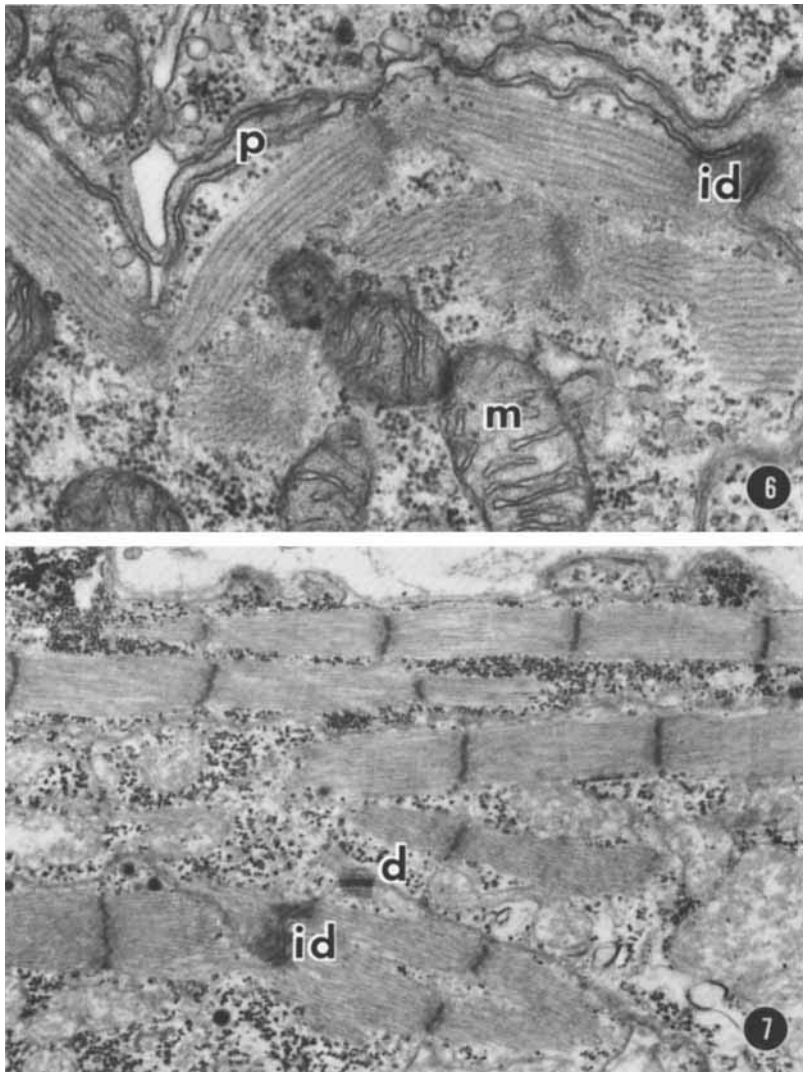
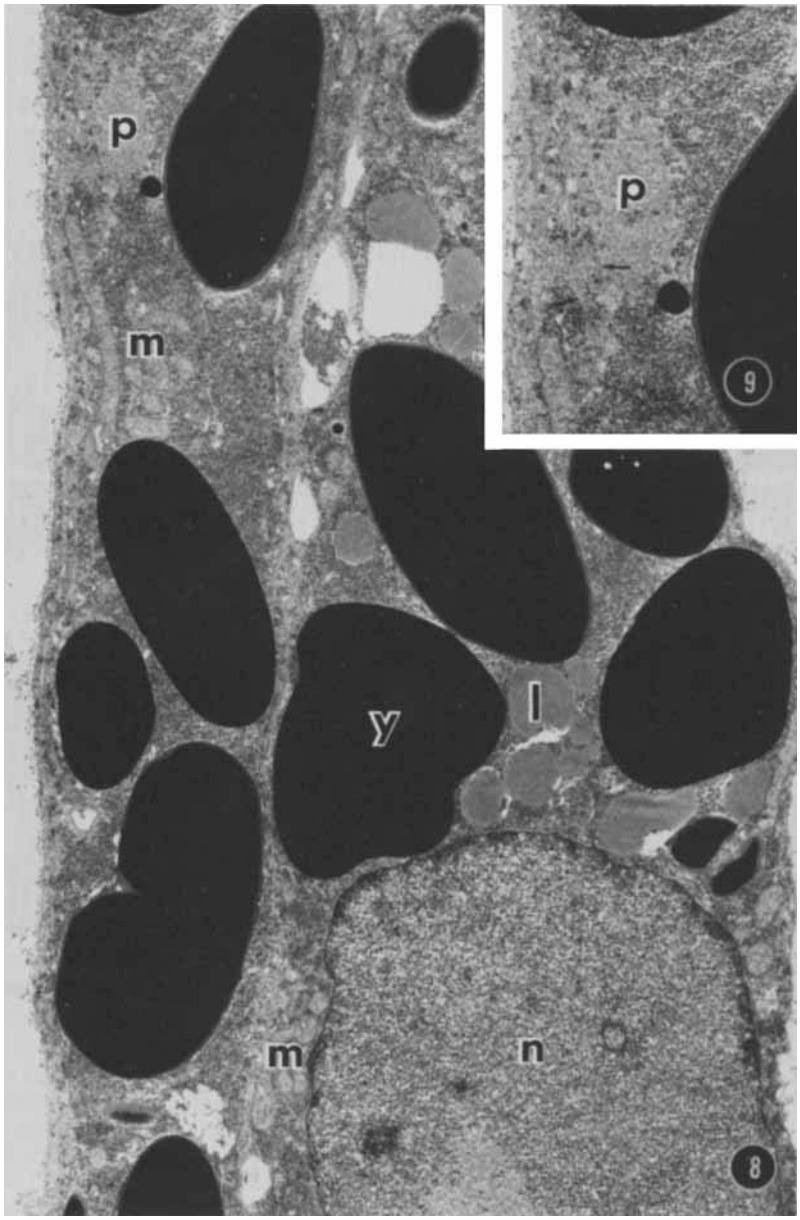


Fig. 6. Electron micrograph of portions of myocardial cells from a stage 35 normal embryo. Myofibrils are organized in the peripheral portion of one cell. An intercalated disk (id) appears to be at an early stage of formation. m, mitochondrion; p, plasma membrane. × 24,240.

Fig. 7. Electron micrograph of portions of myocardial cells from a stage 41 normal embryo. Myofibrils are numerous and well organized. Intercalated disks (id) and desmosomes (d) are prominent structures in normal hearts by this stage. × 20,000.

The most conspicuous band in gels of mutant hearts at stage 41 ranges from 90,000 to 150,000 daltons, with a center measurement of 130,000 daltons. This band also appears to be a yolk component, since it is observed only in tissues containing yolk platelets. The band is not visible in normal heart cells at stage 41, and all of the yolk platelets have disappeared (7, 15). Yolk is still present in certain mutant heart regions at this stage, however. Furthermore, electrophoresis studies combined with light and electron microscopy

of a variety of early normal tissues (heart, gut, liver, brain, fertilized eggs, etc.) make clear that whenever yolk platelets are present the 130,000 band is present, and whenever the tissues lack platelets the 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.,



Figs. 8–9. Electron micrograph of portions of myocardial cells from a stage 36 mutant embryo. Myofibrils fail to form. Amorphous proteinaceous collections (p) are present in peripheral areas of the cell. Sometimes a few filaments are visible within or associated with the amorphous collections (arrows). l, lipid; m, mitochondria; n, myocardial cell nucleus; y, yolk. × 6,240; inset × 8,000.

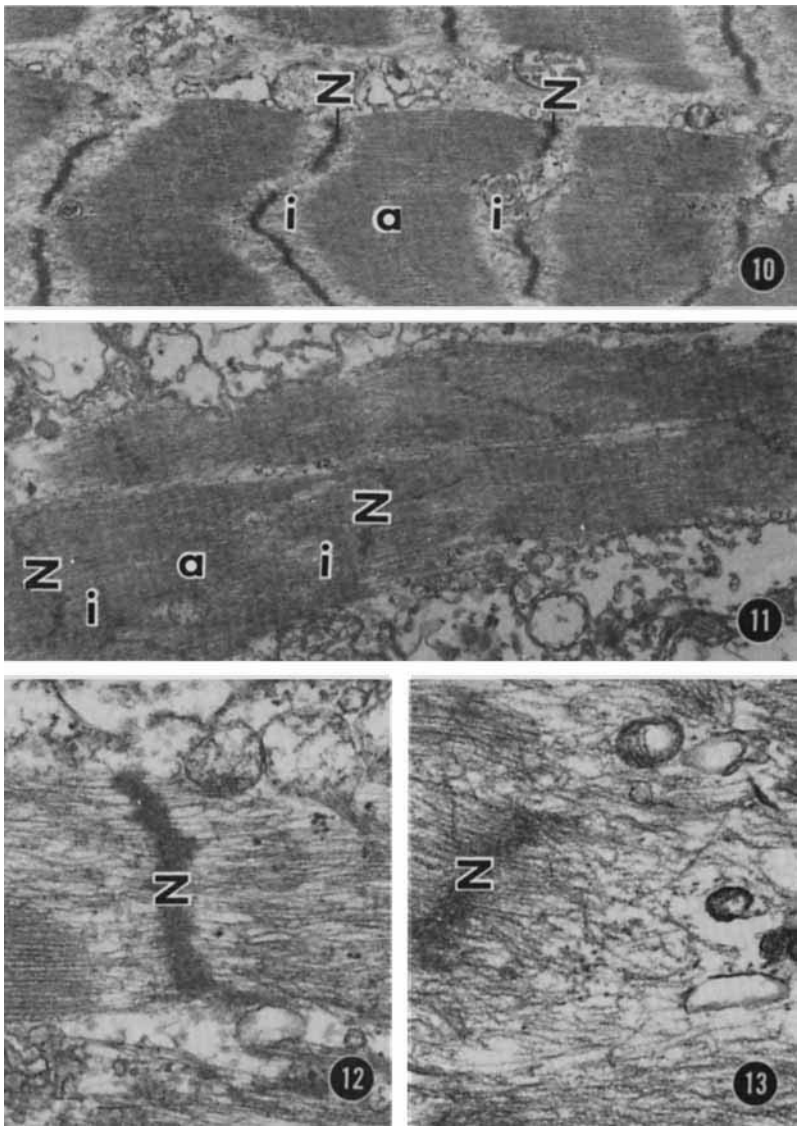


Fig. 10. Electron micrograph of cardiac myofibrils from stage 41 normal embryonic hearts after glycerol extractions in the absence of heavy meromyosin (HMM). A bands (a), I bands (i), and Z lines (z) are distinct. Note that the I bands are electron lucent compared to the A bands, which are more electron opaque. × 18,400.

Fig. 11. Electron micrograph of cardiac myofibrils from stage 41 normal embryonic hearts after glycerol extraction followed by incubation in heavy meromyosin (HMM) for 18 hr. The I bands have increased in density due to the HMM molecules forming complexes with the actin filaments. The electron opaqueness is now almost the same in I bands and A bands. a, A band; i, I band; z, Z line. × 22,400.

Fig. 12. High magnification electron micrograph of an I band region of a glycerol-extracted normal 41 myofibril that has not been incubated in heavy meromyosin (HMM). The thin actin filaments are smooth and bare in appearance. z, Z line. × 34,400.

Fig. 13. High magnification electron micrograph of an I band region after glycerol extraction followed by incubation in heavy meromyosin (HMM) for 18 hr. The thin filaments are "decorated" by the HMM molecules in a fashion characteristic for muscle actin. The HMM periodicity is 360 Å. z, Z line. × 33,200.

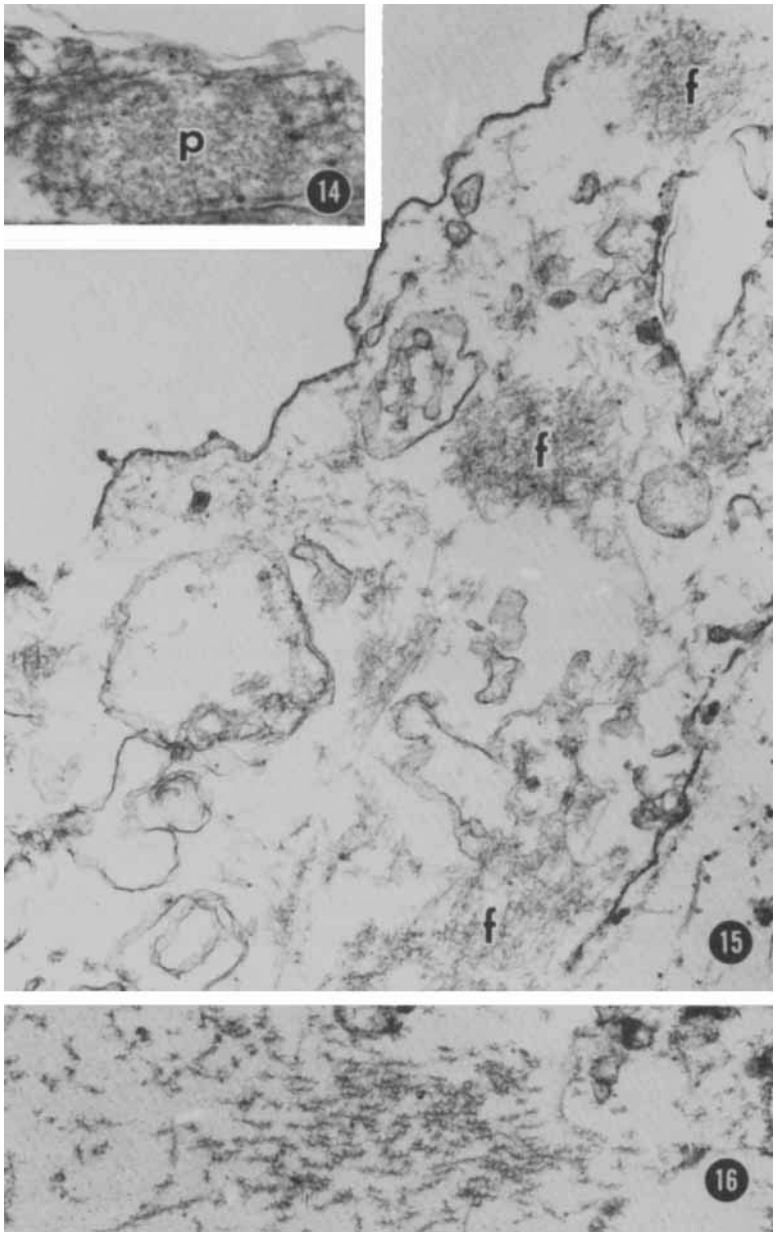


Fig. 14. Electron micrograph showing an amorphous proteinaceous collection (p) in a myocardial cell from a stage 41 mutant embryo after glycerination only. × 31,840.

Fig. 15. Electron micrograph of a portion of a myocardial cell from a stage 41 mutant embryo after incubation in heavy meromysin (HMM) for 18 hr. The amorphous collections are no longer visible and seem to be replaced by groups of decorated filaments (f). × 11,040.

Fig. 16. High magnification electron micrograph of one group of decorated filaments from a stage 41 mutant myocardial cell after incubation in heavy meromyosin (HMM) for 18 hr. The periodicity shown by the HMM on the actin filaments is 360 Å, which is identical with that in normal heart cells. × 22,720.

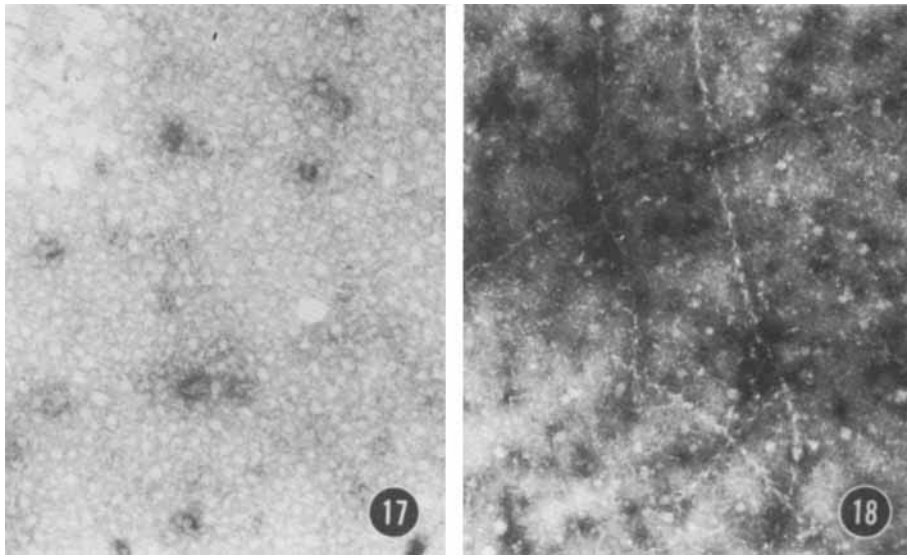


Fig. 17. Negatively stained preparation of an homogenate of stage 41 mutant hearts without heavy meromyosin (HMM) present. There are no filaments. × 64,000.

Fig. 18. Negatively stained preparation of an homogenate of stage 41 mutant hearts after the addition of heavy meromyosin for 1 min. Numerous decorated filament have now formed. The HMM periodicity on the actin is 360 Å. × 64,000.

α-actinin (29), C protein (30), etc.]. Further studies will be required to determine if comparable bands are present in mutants' hearts but masked by yolk.

Low-molecular-weight proteins of 18,000 and 27,000 daltons are present in both normal and mutant hearts. Whether these proteins represent myosin light chains, troponins, or some other unidentified proteins also requires further study.

Skeletal muscle in mutant embryos is identical to normal in fine structure and constituent proteins as determined by SDS-gel electrophoresis. Thus, gene *c* does not appear to affect skeletal muscle.

Radioimmunoassay

Quantitation by radioimmunoassay of absolute amounts of myosin in normal and mutant embryonic hearts confirms the results obtained by SDS-gel electrophoresis concerning this protein. At stage 34 the mutant hearts have only slightly less myosin per heart than normals; however, as development proceeds the differences become greater. By stage 41, normal hearts have twice as much myosin per organ as mutant hearts. The numerical data relating absolute amounts of myosin per heart are summarized in Table II. The total protein content as determined by the Lowrey method (31) shows that the quantities of total protein are comparable in normal and mutant hearts. In addition, the number of cells per heart is the same in normals and mutants as analyzed by dissociation with trypsin and counting in a hemocytometer. Thus, it appears that mutant cells at stage 41 contain only about half as much myosin as normal. The details of the radioimmunoassay procedure (21) and its application to the mutant heart system (32) are both being prepared for detailed publication elsewhere.

TABLE I. Relative Ratios of 200,000 Dalton (Myosin Heavy Chain) to 43,000 Dalton (Actin) Proteins

	Chicken skeletal myofibrils	Axolotl 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. From Lemanski, L. F., Mooseker, M. S., Peachey, L. D., and Iyengar, M. R., J. Cell Biol. 68:375 (1976).

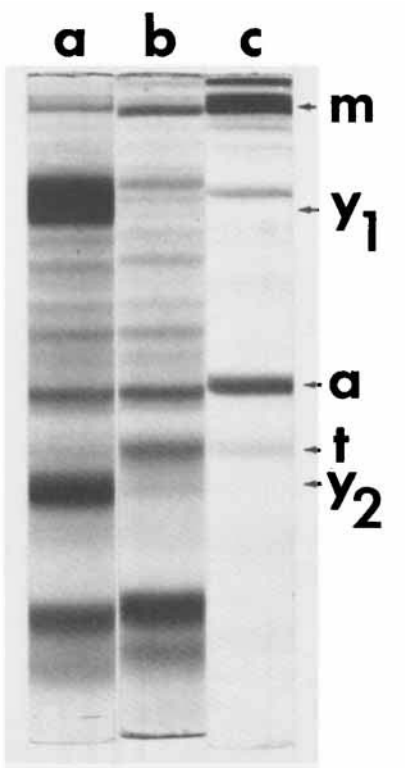


Fig. 19. SDS-polyacrylamide (7.5% acrylamide) gel electrophoresis patterns of stage 41 normal and mutant hearts: (a) stage 41 mutant heart; (b) stage 41 normal heart; (c) chicken skeletal muscle myofibrils. m, myosin heavy chain (200,000); a, actin (43,000); t, tropomyosin (34,000); y_1, yolk platelet protein (135,000); y_2, yolk platelet protein (30,000).

TABLE II. Absolute Quantities of Myosin (μ G Myosin/Heart)

Stage	Normal	Mutant
34	0.8	0.6
39	1.6	1.2
41	3.2	1.6

These absolute amounts of myosin have been determined by radioimmunoassay. Myosin heavy chain (200,000 daltons) represents about 95% of the above weights listed in μ G myosin/heart. Light chains of myosin represent the other 5% of the total myosin measured in our immunoassay system. The results of these assays clearly show that mutant hearts contain less myosin than normal.

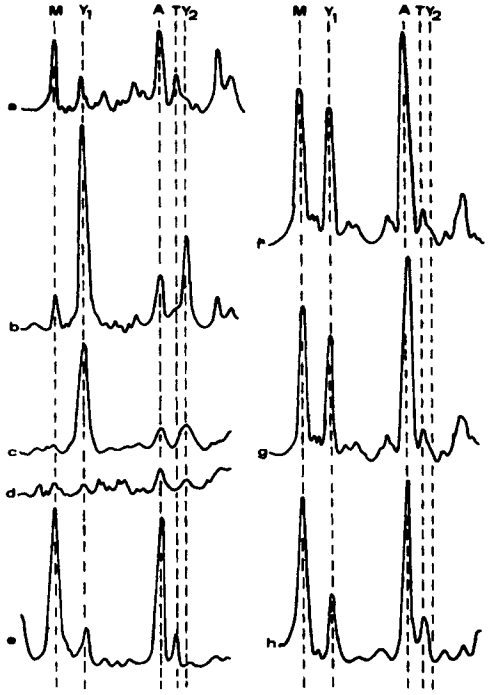


Fig. 20. Densitometer tracings of SDS-polyacrylamide gels after staining with acid fast green (a) stage 41 normal heart; (b) stage 41 mutant heart; (c) stage 34 brain; (d) stage 41 brain; (e) chicken skeletal muscle myofibrils; (f) stage 41 normal skeletal muscle; (g) stage 41 mutant skeletal muscle; (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, d, f, and g, but probably α-actinin in e and h); A, 43,000 dalton protein (actin); T, 34,000 dalton protein (tropomyosin); Y_2, 30,000 dalton protein (probably mostly yolk but possibly some nonmuscle type of tropomyosin). From Lemanski, L. F., Mooseker, M. S., Peachey, L. D., and Iyengar, M. R., J. Cell Biol. 68:375 (1976).

DISCUSSION

Recessive mutant gene *c*, in homozygous condition, results in an absence of organized myofibrils in the developing hearts of axolotl embryos. Morphological and biochemical studies show that skeletal muscle is not altered by gene *c*. Although the hearts fail to contract, there are a few thin 60 Å filaments (actin-like) and thick 150 Å filaments (myosin-like) visible in the cells after conventional electron microscopic preparations. Most prominent in these cells, however, are amorphous proteinaceous collections. These collections are located in peripheral regions of the cells where myofibrils would normally form (15).

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 for the nonmuscle tissues studied (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.

Heavy meromyosin binding and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis studies agree that hearts of mutant embryos contain an almost normal amount of actin -- enough, it seems, for myofibril organization, provided that all of the other components essential for organization are present. Nevertheless, it is not totally clear what the nature and distribution of actin is in the mutant cells. Although numerous actin filaments are visible in mutant cells after HMM incubation, very few are visible in either intact or glycerol-extracted tissues before the addition of HMM. This is a result that has been experienced by a number of investigators using HMM as a probe for actin in nonmuscle motile systems (33–37). Although this may be a fixation artifact, it is thought that this result suggests that the actin of mutant myocardial cells is present in a nonfilamentous state and the addition of HMM causes the actin to polymerize into filaments. There is a problem with this interpretation in that one might expect G actin to be lost from the tissue during the glycerination period prior to HMM addition. However, a newly discovered state of actin has been found in sea cucumber sperm that seems to exist in situ in a nonfilamentous yet stable and insoluble form (38). This new state of actin is similar morphologically to the amorphous proteinaceous collections present in the mutant myocardial cells described here. The strongest evidence that nonfilamentous actin is being transformed into filaments by the addition of HMM to mutant hearts is obtained from the negative staining experiments carried out in the present study. Homogenates of mutant hearts without HMM have very few actin filaments visible after negative staining. If HMM is added to the homogenate, however, large numbers of decorated filaments can be found. Thus, the evidence appears to overwhelmingly favor actin being "stored" in a nonfilamentous, yet stable, state in the mutant heart cells.

Recent preliminary studies in this laboratory show that the addition of purified chicken tropomyosin (39) to glycerinated mutant heart will essentially mimic the effects of HMM by "inducing" the actin to polymerize into filaments (40). This observation becomes extremely germane to the mutant heart system in view of the SDS-gel electrophoresis data that suggest an absence of tropomyosin in mutant hearts. On the basis of these new data, it is inviting to speculate that the absence of tropomyosin in mutant heart cells is the key to their failure to form organized myofibrils. If that turns out to be true, then the appearance of tropomyosin in normal developing heart cells may be the necessary prerequisite for the formation of filamentous actin, which in turn may be essential to the organization of normal myofibrils.

Whatever the details of future experiments along these lines yield, the following conclusions may be made at this time: 1) There is an accumulation of actin in mutant myocardial cells in almost normal amounts, although in a nonfilament form; 2) myosin is reduced in quantity; and 3) tropomyosin is virtually absent. It is apparent that this simple recessive single gene mutation, by way of abnormal induction (2, 41), 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.

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