Immunofluorescence studies for myosin, α-actinin and tropomyosin in developing hearts of normal and cardiac lethal mutant Mexican axolotls, *Ambystoma mexicanum*

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SUMMARY

Recessive mutant gene *c* in axolotl embryos results in an absence of normal heart function. Immunofluorescence studies were done to determine the distributions of myosin, tropomyosin and α-actinin in the hearts of normal and mutant siblings. Anti-myosin specifically stains the A bands of myofibrils in normal hearts and reveals a progressive increase in myofibril organization with development. Mutant hearts display less staining for myosin than normal and localization is mainly in amorphous collections. Anti-α-actinin stains the Z lines of myofibrils in normal myocytes. Mutant cells also have significant staining for α-actinin but show no striations. Antitropomyosin intensely stains the I bands of myofibrils in normal cells; however, there is very little staining for tropomyosin in mutant hearts. Thus, mutant myocardial cells have reduced but significant amounts of actin (Lemanski, Mooseker, Peachey & lyengar, 1976) and myosin, even though non-filamentous, and substantial amounts of α-actinin. The cells appear to contain little tropomyosin.

INTRODUCTION

A recessive mutant gene, designated *c* for ‘cardiac lethal’, was discovered in the Mexican axolotl, *Ambystoma mexicanum* (Humphrey, 1972). The gene appears to exert its effect by abnormal inductive processes from the anterior endoderm (Lemanski, Paulson & Hill, 1979), a potent heart inductor tissue in salamander embryos (Jacobson & Duncan, 1968; Fullilove, 1970). Mutant (*c/c*) embryos are obtained by mating heterozygous (+/*c*) adults and can be first distinguished from their normal (+/+ or +/c) siblings at stage 35 (Schreckenberger & Jacobson, 1975) when the normals develop vigorously contracting hearts. The hearts of mutant embryos at this stage appear normal upon gross examination except they fail to beat properly in vivo and circulation is not

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established (Justus & Hollander, 1971; Humphrey, 1972; Justus, 1978; Kulikowski & Manasek, 1977, 1978; Lemanski, 1978). During later stages the mutant hearts distend and remain thin-walled. In spite of an absence of circulation the mutant embryos survive through stage 41 (about 20 days beyond the heart-beat stage), presumably by simple diffusion of oxygen directly into the tissues. Skeletal muscle is not affected by gene c (Lemanski et al. 1976).

Morphological studies on mutant and normal embryonic hearts from stage 34 (normal heart-beat stage) to stage 41 reveal that normal hearts become trabeculated and the myocytes contain numerous well-organized myofibrils (Lemanski, 1973a, b). The mutant ventricular myocardium remains a single cell layer thick and the cells contain relatively few thin (6 nm) and thick (15 nm) myofilaments; there are no distinct sarcomeric myofibrils. The mutant cells contain instead large amorphous proteinaceous collections in their peripheral sarcoplasm where myofibrils organize initially in normal cells (Lemanski, 1973c). These morphological data make it apparent that gene c results in abnormal heart cell differentiation. Most striking is the absence of well-organized muscle sarcomeres.

Biochemical experiments extend these basic morphological studies. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (Lemanski et al. 1976) and radioimmunoassay studies (Lemanski, Joseph & Iyengar, 1975; Lemanski, 1976) suggest that mutant hearts contain approximately 50–75% of the normal quantity of myosin. Heavy-meromyosin (HMM)-binding studies in combination with the gel electrophoresis data indicate that actin (43000 daltons) also is present in somewhat reduced but substantial quantity in mutant hearts; however, most of the actin is not in a filamentous form (Lemanski et al. 1976). Electrophoresis experiments indicate further that tropomyosin (34000) is very significantly reduced in quantity when compared to normal controls. Thus, the published morphological and biochemical data suggest that mutant hearts contain myosin and actin in somewhat reduced, but significant amounts, even though non-filamentous; however, tropomyosin appears to be substantially reduced.

In the present study, we have determined the distributions of myosin, α-actinin and tropomyosin in hearts of normal and mutant siblings at stages 35–41 by using immunohistochemical techniques. In addition, correlations were made with hearts of stage-33 (pre-heart-beat) normal embryos.

MATERIALS AND METHODS

Embryos. The axolotl embryos were reared in 25% Holtfreter's solution and staged according to the system of Schreckenberg & Jacobson (1975). Hearts from stage-33 known-wild-type normals (+/+ ) and from normal (+/+ or +/+ ) and mutant (c/c) siblings (obtained by +/+ x +/+ ) matings) at stages 35, 39 and 41 were investigated.

Morphology. The embryonic hearts were prepared for histological and ultrastructural study by methods detailed in earlier papers (Lemanski, 1973a, b, c).
Contractile proteins in developing axolotl hearts

Fig. 1. Double immuncdiffusion plate to test antimyosin. AM, antimyosin antibodies; MY, purified myosin from axolotl skeletal muscle.

Fig. 2. Double immuncdiffusion plate to test antitropomyosin. AT, antitropomyosin antibodies; TR, purified tropomyosin from chicken skeletal muscle.

Fig. 3. Double immuncdiffusion plate to test anti-α-actinin. AA, anti-α-actinin antibodies; αA, purified α-actinin.

Antigens and antibodies. Antibodies raised against highly purified muscle-protein antigens, prepared by ‘conventional’ as well as by SDS-gel electrophoresis methods, were used in the immunohistochemical studies. Myosin was extracted from fresh minced adult axolotl skeletal muscle and purified by DEAE Sephadex column chromatography (Richards, Chung, Menzel & Olcott, 1967). Tropomyosin was purified from ether-dried chicken skeletal muscle (Bailey, 1948). α-Actinin was prepared from porcine skeletal muscle (Goll, Suzuki, Campbell & Holmes, 1972). In addition to the above ‘conventional’ preparative methods, antibodies were prepared against chicken heart myosin (heavy chain), tropomyosin and α-actinin that had been purified by slab SDS-gel electrophoresis (Lazarides, 1975). Antibodies against the above proteins were prepared in young rabbits. Preimmune rabbits were screened carefully to eliminate non-specific staining on the tissue samples to be used in the experiments. Antibodies against the native (conventionally prepared) antigens were obtained by giving initial subcutaneous injections of 4 mg of protein in Freund’s complete adjuvant followed by three subcutaneous booster injections of 2 mg
of protein in Freund's incomplete adjuvant at 2-week intervals. The rabbits were bled from the lateral ear veins 8 days after the final injection and the γ-globulins obtained by ammonium sulfate fractionation. The antibodies were stored at −70 °C until ready for use. Antibodies to the antigens purified by SDS polyacrylamide gel electrophoresis were prepared as above except that the relevant bands were cut from the gels after slight Coomassie blue staining, homogenized in physiological saline and injected into the rabbits without Freund's adjuvant. The purity of antibody preparations prepared from both native and electrophoresed antigens was tested by double immunodiffusion and immunoelectrophoresis against purified antigen and against crude heart muscle homogenates from chicken and salamander. Each antibody gave a single precipitin line indicative of high degrees of purity (Figs. 1–3). For a given antigen the tissue-staining pattern for the two different antibody preparations was identical.

**Immunofluorescence staining.** Tissues to be examined immunohistochemically were fixed 4 h at 0 °C in a periodate-lysine-paraformaldehyde solution (McLean

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**Figures 6 and 7**

Fig. 6. Electron micrograph of portions of heart cells from a stage-41 normal embryo. Myofibrils are numerous and well organized. A, A band; I, I band; Z, Z line. X28400.

Fig. 7. Electron micrograph of portions of heart cells from a stage-41 mutant embryo. There are no organized myofibrils. Amorphous proteinaceous collections are present in the peripheral areas of the cell. Sometimes dense bodies (d) and a few filaments are visible within or associated with the amorphous collection (arrow). X 28400.
Frozen sections, 2 µm thick, were air dried on glass slides and stained by an indirect method. The primary antibodies in 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. After several rinses in PBS-glycerol totalling at least 1 h, the sections were stained for 1 h at 37 °C with FITC-labeled IgG fraction of antirabbit IgG prepared in goat (Miles Laboratories, Kanakee, Ill.). The controls for the antibody staining included: (a) incubation with preimmune globulins from the same rabbit that produced the specific antibodies, (b) staining with antibodies that had been absorbed with excess purified antigen, and (c) staining with the second antibody only. There was very little background staining on the control slides. The FITC-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 sec exposure times. Light micrographs of the same section areas were taken using phase optics for comparison.

RESULTS

Morphology. In spite of the functional difference, studies using the light microscope show no discernible variance between normal and mutant hearts.
Contractile proteins in developing axolotl hearts
during early stages (Lemanski, 1973c). By stage 41, however, histological abnormalities in mutant hearts are obvious. Normal embryonic hearts at this stage have well-developed trabeculae composed of myocytes containing numerous organized myofibril tracts (Fig. 4). The mutant hearts fail to trabeculate and show no sign of sarcomeric myofibrils (Fig. 5). Yolk platelets, virtually absent from normal hearts by stage 41, still remain in the cytoplasm of some mutant cells.

Electron microscopy confirms the absence of sarcomeric myofibrils in mutant heart ventricles. Normal myocytes contain organized myofibrils at stage 35 directly beneath and parallel to the plasma membrane; by stage 41, the normal myocardium is composed of highly differentiated muscle cells containing numerous well-organized myofibrils (Fig. 6). The mutant ventricular cells at early stages contain amorphous proteinaceous collections along with a few 6 nm (actin-like) and 15 nm (myosin-like) filaments, and what appear to be Z bodies; however, even as late as stage 41, organized sarcomeres are absent (Fig. 7).

**Immunohistochemistry.** Immunofluorescence studies reveal that antimyosin very specifically stains the A bands of organized myofibrils (Fig. 8 a, b). The normal embryonic heart tissues at stage 34 show a few organized myofibrils

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**Figures 16-23**

Fig. 16. (a, b) Corresponding phase contrast (a) and immunofluorescence (b) light micrographs of adult heart tissue stained with anti-α-actinin. The antibody stains the Z lines of organized myofibrils. Arrows illustrate corresponding areas. ×800.

Fig. 17. Immunofluorescence micrograph of anti-α-actinin-stained stage-35 normal heart. The myofibrillar Z lines are stained and there is staining at the peripheries of the cells. ×800.

Fig. 18. Immunofluorescence micrograph of anti-α-actinin-stained stage-39 normal heart. There is staining of the myofibrillar Z lines along with some staining at the cell peripheries.

Fig. 19. Immunofluorescence micrograph of anti-α-actinin-stained stage-41 normal heart. Most of the α-actinin appears to be localized within the Z lines of organized myofibrils. ×800.

Fig. 20. Immunofluorescence micrograph of stage-33 normal heart (pre-heart-beat stage) stained with anti-α-actinin. The staining pattern is diffuse for the protein with no obvious Z line staining. ×800.

Fig. 21. Immunofluorescence micrograph of stage-35 cardiac mutant heart stained with anti-α-actinin. There seems to be localization of the protein at the peripheries in the cells. Some cells appear to contain more of the protein than others. No Z line staining can be seen. ×800.

Fig. 22. Immunofluorescence micrograph of anti-α-actinin-stained stage-39 cardiac mutant heart. There is an increase in staining from stage 35, but the general distribution and pattern of staining remains basically the same (see Fig. 21). ×800.

Fig. 23. Immunofluorescence micrograph of anti-α-actinin-stained stage-41 cardiac mutant heart. There is extensive staining of the tissue indicating rather substantial quantities of α-actinin. There are no obvious sarcomeric Z lines stained, although 'spots' are visible.
with distinct A band staining as well as myosin-containing amorphous areas in some regions of the cells (Fig. 9). With progressing development there is an increase in the organization of sarcomeres (Fig. 10) until by stage 41, most of the myosin in the cells is localized in the A bands of the now numerous organized myofibrils (Fig. 11). The mutant cells throughout development show no sarcomeric pattern of myosin localization. Most of the myosin-positive regions in the mutant hearts take the form of amorphous collections in the cell peripheral cytoplasm (Figs. 13–15), where myofibrils first organize in normal cells (Lemanski, 1973c). The pattern of staining in mutant cells is reminiscent of that seen in stage-33 (pre-heartbeat) normal cells (Fig. 12). While the accumulated myosin in mutant hearts increases with advancing development, there is clearly less in mutant hearts than in normal control tissues at the same stage. Nevertheless the mutant hearts do show substantial staining for the protein, certainly more than in any non-muscle tissues examined (i.e. gut, liver, brain).

Anti-α-actinin stains the Z lines of organized myofibrils (Fig. 16a, b). Sarcomeric Z line staining is present in stage-35 normal hearts (Fig. 17) and there is an increase in Z line staining with development (Figs. 18–19) corresponding to the increased numbers of organized myofibrils (Lemanski, 1973b). The α-actinin staining is often localized along the sarcolemma at the cell peripheries. The

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**Figures 24–31**

Fig. 24. (a, b) Corresponding phase contrast (a) and immunofluorescence (b) light micrographs of adult heart tissue stained with antitropomyosin. The antibody specifically stains the I bands of the organized myofibrils. Arrows illustrate corresponding areas. × 800.

Fig. 25. Immunofluorescence micrograph of antitropomyosin-stained stage-35 normal heart. Most of the protein is localized at the cell peripheries in amorphous collections. There is some sarcomeric I band staining. × 800.

Fig. 26. Immunofluorescence micrographs of antitropomyosin-stained stage-39 normal heart. Organized myofibrils show I band staining. A few stained amorphous areas are visible. × 800.

Fig. 27. Immunofluorescence micrograph of antitropomyosin-stained stage-41 normal heart. The myofibrillar I bands are stained as are some peripherally located amorphous areas. × 800.

Fig. 28. Immunofluorescence micrograph of antitropomyosin-stained stage-33 normal heart (pre-heart-beat stage). While there is no sarcomeric pattern of staining, substantial stain-positive areas are present as amorphous collections in the peripheral cytoplasm of the cells. × 800.

Fig. 29. Immunofluorescence micrograph of antitropomyosin-stained stage-35 mutant heart. There is very little stain-positive material present × 800.

Fig. 30. Immunofluorescence micrograph of antitropomyosin-stained stage-39 mutant heart. A few lightly stained amorphous areas can be distinguished in the cells. × 800.

Fig. 31. Immunofluorescence micrograph of antitropomyosin-stained stage-41 mutant heart. There is very little staining for the protein at this stage. It appears to be somewhat less than that seen in earlier mutant stages (see Figs. 29–30). × 800.
mutant heart cells also show substantial staining for α-actinin (Figs. 23–23); however, the pattern is more diffuse than in normal cells. While there is no obvious Z line staining in the mutant cells even as late as stage 41, there are little ‘spots’ of the protein which are similar in appearance to those seen in some normal cells at earlier stages (e.g. compare Figs. 17 and 18 and 20 with Fig. 23). We believe that these ‘spots’ in both normal and mutant cells represent collections of α-actinin which are closely associated with, or perhaps even plastered against, the plasma membrane. Thus, these immunohistochemical studies suggest that mutant cells have substantial staining for α-actinin but the protein does not become organized into sarcomeric Z bands as in normal cells. Advanced mutant hearts (stage 41) have certain staining characteristics of normal organs at earlier stages (stages 35 and 39).

Anti-tropomyosin antibodies specifically stain the I bands of organized myofibrils (Fig. 24a, b). The normal heart cells show significant staining for tropomyosin at stage 35 and, like myosin and α-actinin, there is an increase in sarcomere staining with advancing development (Figs. 25–27). At stage 41, the bulk of the staining for tropomyosin in normal heart cells is in the I bands of organized myofibrils. The mutant hearts throughout development show little staining for tropomyosin (Figs. 29–31). While there does appear to be a slight increase of tropomyosin-staining in mutant cells between stages 35 and 39, this increase does not continue to stage 41. If anything, the individual cells seem to show progressively less staining for the protein with advancing development beyond stage 39. Stage-33 (pre-heartbeat) normal hearts show as much staining for tropomyosin as any of the three stages of mutant hearts we have examined (Fig. 28).

DISCUSSION

The present immunohistochemical studies were undertaken to determine the distributions of myosin, α-actinin and tropomyosin in the normal and mutant heart cells. Great care was taken to screen the rabbits and produce antigens of the highest possible purity for our studies. Myosin in the normal hearts is localized primarily in the A bands of organized myofibrils during the advanced developmental stages (39–41). At early stages the normal hearts have a number of amorphous myosin collections, which presumably represent areas in the cells where myosin has accumulated but not yet been incorporated into myofibrils. The pre-heartbeat (stage 33) normal myocardial cells show no sarcomere staining even though intensely stained amorphous collections are visible. The mutant hearts throughout development do not display sarcomere staining, although they do have rather extensive accumulations of amorphous myosin-positive areas which are reminiscent in distribution and appearance to the myosin of pre-heart-beat normal cells. It appears that myosin has accumulated in substantial quantity in mutant hearts, but fails to become incorporated into sarcomeric myofibrils.
The immunohistochemical data suggests that α-actinin is also abundant in mutant hearts, and like myosin and actin, is not incorporated into myofibrils as is the case in normal cells. Rather the α-actinin appears to be generally diffuse with a few concentrated collections of the protein at the cell peripheries.

Our immunohistochemical experiments corroborate the previously published electrophoresis data (Lemanski et al. 1976) suggesting that muscle-type tropomyosin (34000 daltons) is quantitatively reduced in mutant hearts when compared to normal. In fact, pre-heart-beat normal cells (stage 33) show as much staining for the protein as mutant hearts at stages 35-41. Furthermore, the mutant hearts do not show an increase in this protein with advancing development beyond stage 39. If anything, there appears to be a decrease in tropomyosin staining within individual mutant heart cells. Whether this decline represents a dilution effect from continued cell division, a breakdown of the protein, or some other factor, remains moot. What is clear is that mutant hearts have substantially lower than normal amounts of antigenically detectable tropomyosin at all of the stages studied. These studies do not exclude the possibility that mutant heart cells contain antigenically altered or abnormal tropomyosin, such that the protein escapes recognition by the antibodies used. Further, we cannot rule out the possibility that mutant heart cells contain significant quantities of non-muscle-type tropomyosin (30000 daltons) (Cohen & Cohen, 1972; Fine, Blitz, Hitchcock & Kamier, 1973). These questions require further study.

Thus, although the mutant hearts studied here contain almost normal complements of the myofibrillar proteins, myosin, actin and α-actinin, tropomyosin appears to be quantitatively reduced or, at least, biochemically altered. In any event, normal myofibrillogenesis fails. In view of the failure of mutant heart actin to form into filaments (Lemanski et al. 1976) and in view of the close molecular association between actin and tropomyosin in organized myofibrils of muscle (Gergeley, 1976), studies were undertaken recently in our laboratory to determine whether there might be a casual relationship between the deficiency of normal muscle tropomyosin in mutant hearts and their failure to form filamentous actin. The results of that study suggest that there is indeed a correlation (Lemanski, 1979). When glycerated mutant hearts are incubated in a solution containing purified tropomyosin from chicken or rabbit skeletal muscle, large numbers of 6 nm actin filaments form from the amorphous collections in the cells; negative staining experiments confirm this result. Therefore, all available evidence suggests that insufficient or abnormal tropomyosin in mutant heart cells may be a key in their failure to form normally organized myofibrils. It is inviting to speculate that the presence of normal muscle-type tropomyosin in developing embryonic hearts may be a necessary prerequisite for actin to become filamentous and for myofibrillogenesis to succeed.
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REFERENCES


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