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STUDIES OF HEART DEVELOPMENT IN NORMAL
AND CARDIAC LETHAL MUTANT AXOLOTLS: A REVIEW

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Abstract

The morphology of developing hearts in axolotls, *Ambystoma mexicanum*, has been studied by scanning electron microscopy in order to provide a chronology of morphogenesis that can be correlated with ongoing biochemical and immunocytochemical studies. In addition to normal embryos we have studied homozygous recessive cardiac lethal mutant axolotls. The mutant myocardium undergoes aberrant sarcomere development and lacks a normal heartbeat. Morphogenesis of mutant hearts appears to be nearly normal with respect to myocardial cell shape changes, epicardial formation, and the distribution of extracellular matrix fibrils in the cardiac jelly. This suggests that the deficient arrangement of contractile proteins in mutant myocardial cells does not prevent the normal organization or function of cytoskeletal isoforms of these proteins in the developing myocardium and epicardium. The implications of biochemical and morphological investigations of axolotl hearts are considered in the context of the entire developmental history of the cardiogenic mesoderm.

KEY WORDS: Axolotl, *Ambystoma mexicanum*, cardiogenesis, epicardium, myocardium, extracellular matrix, cell shape changes, cardiac lethal mutation.

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Introduction

The cardiac lethal mutant axolotl is the homozygous carrier of recessive gene *c* that causes the failure of the heart to beat throughout its length (Humphrey, 1972). The resultant lack of circulation to the kidneys causes an accumulation of ascites fluid that distends the heart and ultimately causes death of the early post-hatching larva. Lemanski (1973c) ascertained by transmission electron microscopy that mutant ventricular cardiomyocytes fail to contract because they lack normal sarcomeres. Numerous biochemical and immunocytochemical studies have since been undertaken to analyze the accumulation of contractile and cytoskeletal proteins in normal and cardiac lethal mutant axolotl hearts (Lemanski, 1979; Lemanski et al., 1976, 1980; Kulikowski and Manasek, 1978; Woodroffe and Lemanski, 1981; Moore and Lemanski, 1982; Fuldner et al., 1984; Shen et al., 1986; Shen and Lemanski, 1989; Starr et al., 1989). Although originally designed to investigate the sequence of events in cardiac myofibrillogenesis, these and future studies, if correlated with morphological data, could ultimately reveal the molecular basis of cardiac shape changes in the axolotl embryo. The cardiac lethal mutant has also been used to investigate the role of anterior endoderm in the regulation of the myogenic phenotype in cardiac mesoderm. It is thought that abnormal inductive processes by the anterior endoderm in cardiac mutant axolotls is ultimately responsible for the sarcomeric deficiencies in the mutant heart (Lemanski et al., 1977, 1979; Davis and Lemanski, 1987).

In this paper we review the results of our scanning electron microscopic investigations on the developing heart in normal and cardiac lethal mutant axolotls. The aim of these studies was to obtain more precise information on the substructure of the developing axolotl heart than was provided by earlier histological and ultrastructural descriptions which concentrated primarily on the myocardium (Lemanski, 1973a,b,c). We have consolidated this new morphological data with other published work from this laboratory in order to establish a chronology of heart development in the axolotl as it is currently understood.

Materials and Methods

Animals

The axolotl colonies at Indiana University and here at the SUNY Health Science Center at Syracuse provided fertilized eggs from matings of eleven pairs of adults. Fertilized eggs were removed from mating tanks and reared in 50% Holtfreter's solution. Younger pre-hatch embryos were removed from their jelly capsules, and along with post-hatch larvae, were transferred briefly into a calcium-free, magnesium-containing Holtfreter's solution to reduce muscular contraction during primary fixation for electron microscopy. Finquel (MS-222) or cerebral puncture was used to anesthetize embryos prior to fixation.

Cardiac lethal (c/c) mutant embryos were obtained by mating seven pairs of adult axolotls heterozygous (+/c) for the cardiac lethal gene. These were distinguished from phenotypically normal siblings (either +/+ or +/c, but referred to as +/- in figure legends) by the absence of a regular heartbeat at stage 34/35. Known +/- normal embryos were obtained from the mating of three pairs of normal (+/+) homozygotes. Additional normal (+/+ and +/c) embryos were obtained from one mating between a normal heterozygote (+/c) and normal (+/+) homozygote.

Staging Systems

We have tried to clarify the discrepancies between the various staging systems used to identify the developmental ages of axolotl embryos. Lemanski's (1973a,b,c) original papers were based on Harrison's schedule for *Ambystoma maculatum* as illustrated in Rugh (1962); however, the detailed descriptions of these illustrations were only published years later in a book not readily available (Harrison, 1969). Subsequently, papers from this laboratory used Schreckenbergs and Jacobson's (1975) staging system for the axolotl, *A. mexicanum*, which was based on Harrison's scheme. More recently the literature on the developmental biology of the axolotl has increasingly cited the system of Bordzilovskaya and Detlaff (1979). This has since been republished with a comparison of the three staging systems (Bordzilovskaya et al., 1989). In the following chronology the corresponding staging systems are referred to by initial: H for Harrison, SJ for Schreckenbergs and Jacobson, BD for Bordzilovskaya and Detlaff. Readers may refer to Bordzilovskaya et al. (1989) for a description of embryonic features diagnostic for each stage.

Processing for Scanning Electron Microscopy (SEM)

A total of 63 normal and 38 cardiac lethal mutant embryos as well as five pre-heartbeat embryos of unknown phenotype (??) were examined by scanning electron microscopy. Embryos were fixed in 2% glutaraldehyde (Ladd or Polysciences) in 0.1M cacodylate buffer, pH 7.3, plus 5 mm magnesium chloride at room temperature for the first hour and at 4°C subsequently. After a minimum of two hours in primary fixative, specimens were dissected to expose the heart, then returned to 4°C to complete the primary fixation.

Ventral views of the heart were obtained by removing the ventral body wall and pericardium with fine watchmaker's forceps. For a view of internal cardiac structures, some embryos were sliced sagittally during fixation by a scalpel blade cut through the anterior body. These split embryos were processed as one piece and separated just before mounting on SEM stubs.

After overnight washing in 0.1 M cacodylate buffer, pH 7.3, some specimens were secondarily fixed in 1% osmium tetroxide (EM Sciences or Pelco) in cacodylate buffer for two hours at 4°C. Other specimens (indicated in figure legends) were secondarily fixed for two hours in 0.2% tannic acid (Polysciences) in cacodylate buffer and washed four hours to overnight in buffer prior to tertiary fixation in osmium tetroxide as above. Osmicated samples were washed overnight in cacodylate buffer at 4°C, then dehydrated in a series of ethanols. Specimens in absolute ethanol were dried at the critical point of carbon dioxide in either a Sorvall or a Tousimis Autosamdri apparatus. Dried samples were mounted on coverslips using sticky-tabs (E. Fullam) and sputter-coated on stubs with gold-palladium (Technics: Hummer II apparatus). Embryos were examined in an Hitachi S-520 scanning electron microscope and images recorded with Polaroid 55 positive/negative film.

After observation by the SEM, selected specimens were dissected further to expose internal heart organization. Glass microneedles were coated with contact cement to produce sticky, flexible tools for removing outer tissue layers. Specimens dissected in this way were sputter-coated again prior to examination with the scanning electron microscope.

Results

Chronology of Heart Development

Stage H26-29; SJ25-28; BD26-29

The heart primordium arises from lateral plate mesoderm that migrates to a midventral position between the gills (Lemanski, 1973a). Each lateral plate consists of an outer pericardial epithelium joined at the leading edge to an inner, thicker myocardial layer (Fig. 1a). During the spreading of the lateral plate, pericardial cells are primarily elongated in the direction of migration. However, the cells of the leading edges are noticeably oriented with the long axis in the anterior-posterior (rostral-caudal) direction (Fig. 1a). Cells appear to detach from the leading edges of the migrating plates to form a midventral endocardial mass (not shown).

Earlier experiments indicate that anterior endoderm from normal embryos of this stage exerts a final inductive influence on cardiac mesoderm. Normal anterior endoderm "rescues", that is, restores normal myocardial structure and function to, mutant hearts at stage 34-35 in organ cultures (Lemanski et al., 1979). Subsequent investigations suggest that conditioned medium or crude RNA extracted from normal anterior endoderm (but not liver or neural tube) could duplicate the rescue of mutant hearts *in vitro* (Davis and Lemanski, 1987).

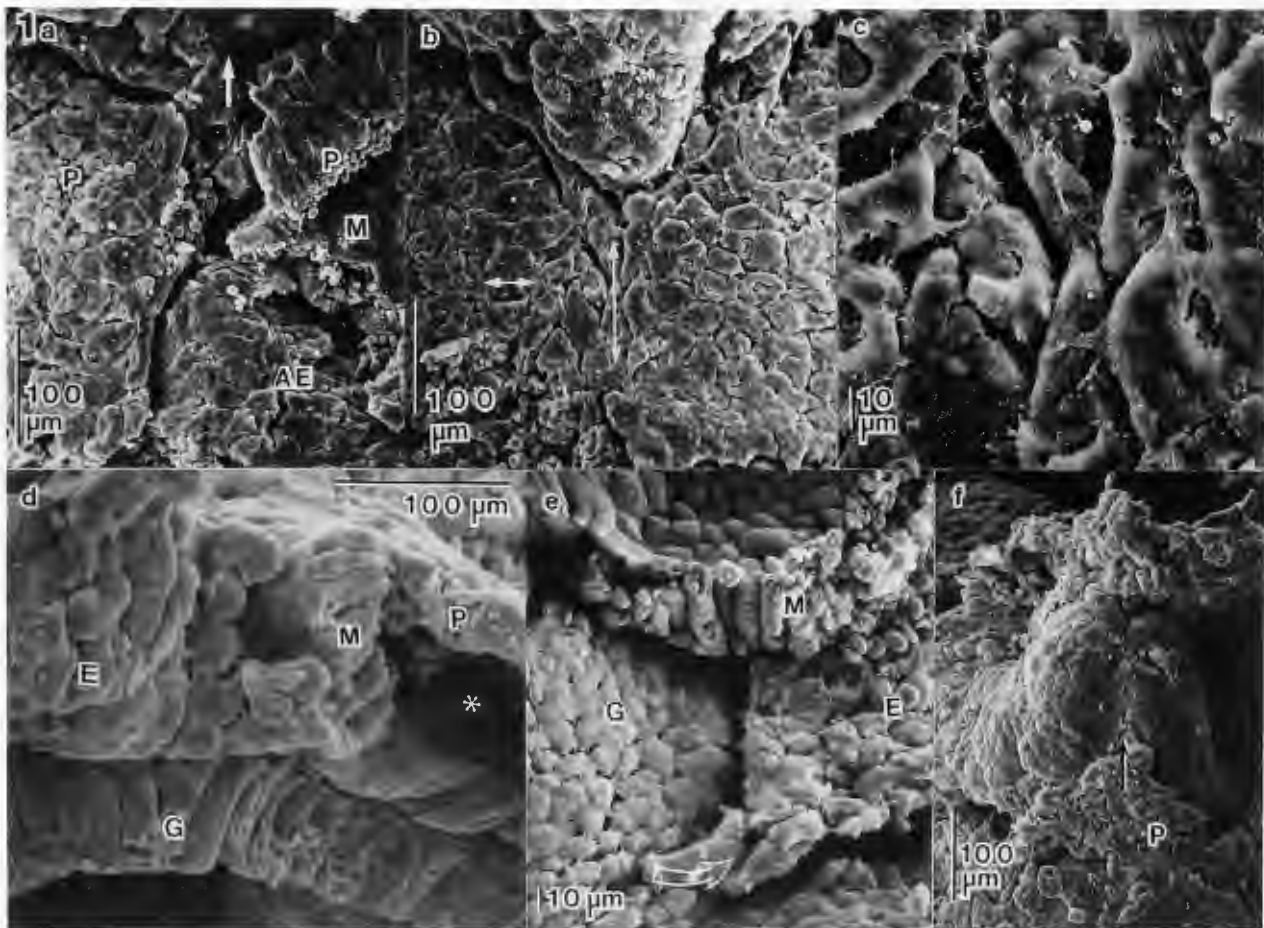


Fig. 1a-c. SEMs illustrating ventral views of lateral plate mesoderm during the period of its migration towards the midline in the cardiac region of axolotl embryos.

a. BD Stage 29 $+/+$ normal embryo. The lateral plates are not yet fused. The anterior endoderm (AE) is visible between the lateral plates. (Sections of whole embryos indicate that endocardial cells should be present here. However, as the ventral thoracic ectoderm had been peeled away from this embryo during fixation, it is likely that the loosely attached endothelial cells were washed away during processing.) The pericardial (P) portion of the left lateral plate was removed after critical point drying to reveal myocardial mesoderm (M). (Tannic acid post-fixation). Midline arrow denotes anterior.

b. BD Stage 30 $+/+$ normal embryo. The lateral plates are fused in the midline. Note the axial elongation of pericardial cells in the midline compared to their transverse orientations elsewhere. (Tannic acid post-fixation).

c. Enlargement of the mid-ventral pericardial cells in b.

d. BD Stage 32 embryo (unknown genotype). View from posterior of the anterior face of a transversely fractured embryo (ventral side up) showing close association of cardiac mesoderm to the columnar epithelium of the gut (G). The endocardial cell mass (E) is in the midline. The pericardial cavity (asterisk) is situated between the cuboidal epithelium of the pericardium (P) and the columnar epithelium of the myocardium (M). (Tannic acid post-fixation).

e. BD Stage 32 $+/?$ normal embryo. A view of the right side of heart after the pericardium and part of myocardium were removed after critical point drying. The endocardial cell mass (E) has hollowed out, as can be seen at the exposed inflow region (arrow). Both the endocardium and myocardium (M) are in contact with the gut epithelium (G). (The spherical objects to the right of the micrograph are yolk platelets released from myocardial cells during fracturing.)

f. BD Stage 33 $+/+$ normal embryo. A ventral view of a tubular heart exposed by removing the pericardium (P) after critical point drying. This operation caused tearing of the ventral mesocardium (arrow). (Tannic acid post-fixation).

Stage H30; SJ29-30; BD30

As the two lateral plates fuse in the midline, the pericardial leading edge cells are still recognizable by their elongation in the anterior-posterior (rostral-caudal) axis (Fig. 1b,c). These cells mark the position of the ventral mesocardium, the connection between the pericardium and the myocardial layer.

Normal anterior endoderm from this stage can still rescue the mutant phenotype (Lemanski et al., 1979; Davis and Lemanski, 1987).

Stage H31-32; SJ30-32; BD31-32

Dorsal to the cuboidal pericardial epithelium and attached to it at the ventral mesocardium is the columnar myocardial epithelium consisting of cells 30-40 μm high (Fig. 1d,e). The myocardial epithelium shifts ventrally to surround the mid-ventral endocardial mass, establishing the so-called myocardial trough. The right and left dorsal mesocardia are the top edges of the trough, where the myocardium is still attached to non-cardiac splanchnic mesoderm. Concomitant with the development of the myocardial trough the pericardial and myocardial lateral plate layers separate to form the pericardial cavity (Fig. 1d). The endocardial mass hollows out to create the lumen of the heart (Fig. 1e).

Normal anterior endoderm from this stage is less effective in "rescuing" the mutant phenotype (Davis and Lemanski, 1987).

Stage H33; SJ33; BD33

While the ventral mesocardium is still intact (Fig. 1f), both dorsal mesocardia shift medially and fuse. This closes the myocardial trough to form a myocardial tube surrounding the endocardium and separates the heart from the dorsal pericardial wall (Lemanski, 1973a). This process appears to involve a change in shape, or at least orientation, of columnar myocardial cells which become elongated circumferentially rather than radially with respect to the heart tube (Fransen and Lemanski, 1988a). Circumferential elongation involves a significant increase in the apical surface area of myocardial cells accompanied by a decrease in the height (apical to basal dimension) of the myocardium (Fransen and Lemanski, 1988a).

Indirect immunofluorescent studies of pre-contractile tubular hearts have revealed amorphous accumulations of myosin, α -actinin, tropomyosin (Lemanski et al., 1980), troponin-T (Fuldner et al., 1984) and desmin (Shen et al., 1986; Shen and Lemanski, 1989) within myocardial cells. Two-dimensional gel electrophoresis (IEF: SDS-PAGE) analysis indicates that homogenates of tubular hearts contain similar amounts of the α and β isoforms of actin (Woodroffe and Lemanski, 1981).

Medium conditioned by normal anterior endoderm explanted at this stage is no longer competent to rescue the mutant heart phenotype (Davis and Lemanski, 1987).

Stage H34; SJ33-34; BD34

The tubular heart, now more cylindrical rather than conical in shape, and the pericardial cavity elongate in the anterior-posterior axis. Shortly however, the pericardial cavity length becomes fixed and does not change substantially until after hatching (Fransen and Lemanski, 1988a). As the myocardial tube continues to grow by addition of cells to the ends (Fransen and Lemanski, 1988a), the tube bends to a bilaterally asymmetrical shape: dextral looping (Fig. 2a). Myocardial cells, still organized as a monolayer, overlap apical surfaces of adjacent cells as dextral looping proceeds. The ventral mesocardium begins to be resorbed during this time, initially at the center of the heart tube. The site of the ventral mesocardium, however, remains detectable in this and succeeding stages, by the orientation of mid-ventral myocardial (MVM) cells which elongate axially rather than around the circumference of the heart.

This stage marks the beginning of myocardial contractions in normal, but not c/c cardiac lethal mutant embryos. Mutant hearts explanted after the onset of dextral looping have been "rescued" by either medium conditioned by BD stage 29 anterior endoderm, or total RNA extracted from that endoderm (Davis and Lemanski, 1987). Rescued mutant hearts have normal propagated contractions and normal sarcomeric ultrastructure.

Stage H35; SJ34-35; BD35

The heart assumes a C-shape as dextral looping brings the ventricle to the right side of the pericardial cavity (Fig. 2b). Elongation of midventral myocardial cells is parallel to the dextral shift; neighboring circumferentially-oriented myocardial cells appear to undergo a slight oblique shift in orientation (Fransen and Lemanski, 1988a). The posterior end of the heart shifts to the left side of the pericardial cavity.

At this stage the regularity of the contractions in normal hearts is easily distinguishable from the quiescence of cardiac lethal mutant hearts. However, no morphological difference is detectable in gross structure of the hearts or in shape or orientation of myocardial cells (Fransen and Lemanski, 1988a). We infer from sections and from comparison with previous and later stages, that during this time all cells of the myocardial monolayer are elongating along the basal lamina to produce a typical bipolar cell morphology. Comparison with sectioned hearts suggests that the surface polygonal shapes visible by SEM represent the perinuclear portions of cardiomyocytes. These wider regions cover the narrow bipolar growths of adjacent cells. In both normal and mutant hearts, collagen fibrils are observable in the cardiac jelly between the basal lamina of myocardium and endocardium (Fig. 2d). The fibrils are mostly randomly oriented, but there is a tendency to orient parallel to the direction of elongation of the myocardial cells (Fransen and Lemanski, 1986 and in preparation).

That mutant hearts have lowered amounts of tropomyosin has been suggested from densitometric

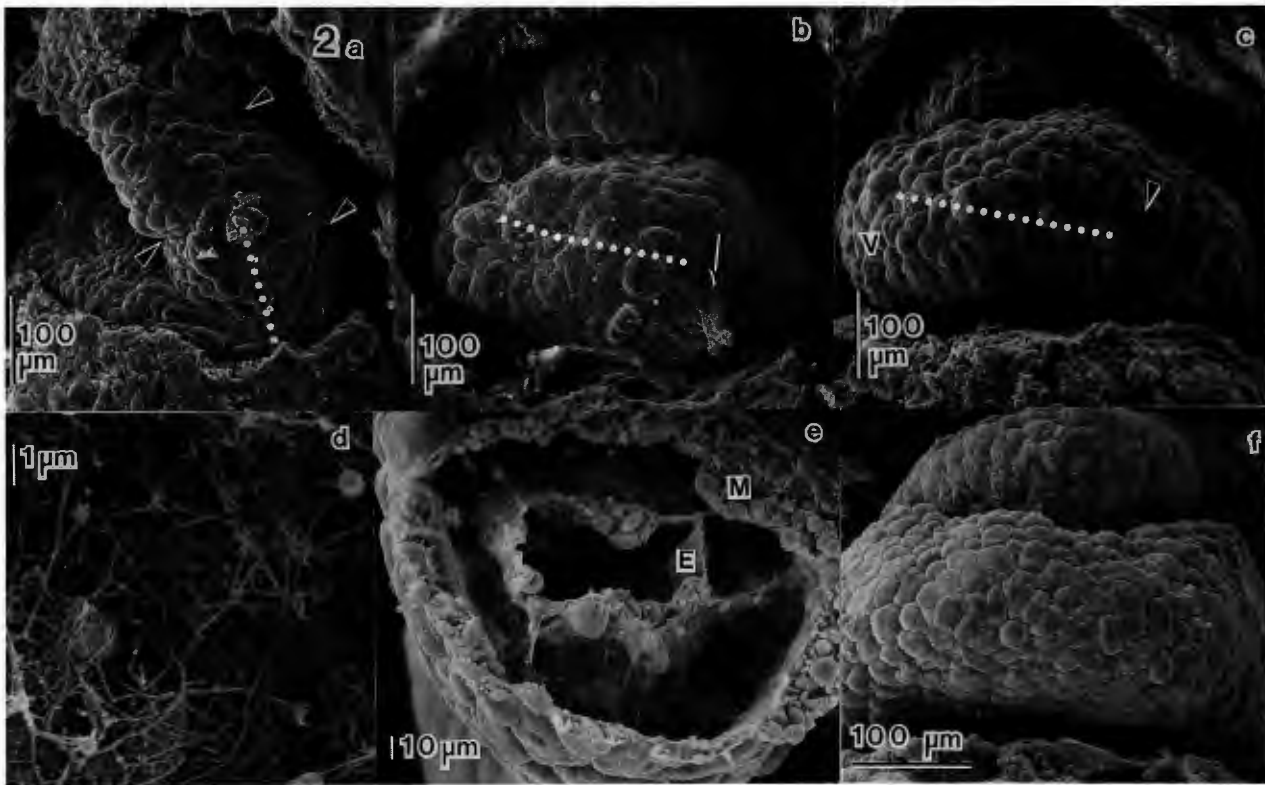


Fig. 2. a-f. SEM of embryonic axolotl hearts (all post-fixed in tannic acid).

a. BD Stage 34 embryo (genotype unknown). This ventral view illustrates the bilateral asymmetry of the bending tubular heart. Note creases in the surface of myocardium (arrowheads). Midventral myocardial cells (MVM) are indicated by dotted lines in this figure as well as in b and c.

b. BD Stage 35 c/c mutant embryo. This ventral view illustrates the C-shaped heart after dextral looping. The arrow indicates the posterior remnant of the ventral mesocardium.

c. BD Stage 36 c/c normal embryo. In ventral view the heart assumes an S-shape as the conus end of the ventral (V) is shifted dorsally and the atrial (Posterior) end shifts increasingly to the left. The posterior remnant of the ventral mesocardium (arrowhead) has completely detached. Note the varying orientations of myocardial cells with respect to this landmark.

d. BD Stage 35 +/- normal embryo. Collagen fibrils are randomly arranged at the basal surface of the atrial endocardium.

e. BD Stage 37 +/- normal embryo. The atrium as sliced during fixation. The endocardial tube (E) is surrounded by collagenous extracellular matrix within myocardial tube (M).

f. BD stage 39 +/- normal embryo. Note the compact surface profiles of myocardial cells in this ventral view. Sections reveal that these represent the perinuclear portions of cardiomyocytes. The basal bipolar extensions of myocardial cells are not visible from the surface. (Compare to mutant Fig. 3a).

analysis of heart homogenates fractionated on SDS-polyacrylamide gels (Lemanski et al., 1976), Western blotting and silver-stain analyses of IEF-SDS polyacrylamide gels (Starr et al., 1989) and corroborated by radioimmunoassay experiments (Moore and Lemanski, 1982). The latter experiments indicated that mutant hearts contain about one-quarter the normal amount of tropomyosin. Densitometric analysis of gels combined with heavy meromyosin labelling studies suggest that actin is present in apparently normal amounts in mutant myocardia, but not in filamentous form (Lemanski et al., 1976; Lemanski, 1979). In normal and mutant hearts, the α and β isoforms of actin are present in similar amounts as shown by IEF-SDS PAGE (Starr et al., 1989). In experiments with glycerinated mutant myocardia, Lemanski (1979) showed that addition of exogenous tropomyosin could vastly increase the number of 6 nm filaments that could be labeled with heavy meromyosin. Thus, it appears that the deficiency in tropomyosin, by impeding thin filament assembly, is at least partly responsible for the absence of sarcomeres in mutant hearts (Lemanski, 1973c; Lemanski et al., 1980; Starr et al., 1989).

Indirect immunofluorescent studies indicate that the intermediate filament protein desmin has a diffuse peripheral distribution in both normal and mutant myocardial cells (Shen et al., 1986; Shen and Lemanski, 1989). Mutant and normal hearts exhibit a diffuse distribution of contractile proteins, but only in normal hearts are these also detected at specific sarcomeric

positions: myosin in the A-band, α -actinin at the Z-line, tropomyosin at the I-band (Lemanski et al., 1980), troponin-T at the I-band (Fuldner et al., 1984), and actin at the I-band (Starr et al., 1989). Tropomyosin is barely detectable by indirect immunofluorescence, indicating a drastic reduction in the quantity of that protein, in mutant hearts (Lemanski et al., 1980; Starr et al., 1989).

When dissected and cultured *in vitro* in the presence of Holtfreter's solution, a simple salt solution, normal hearts will continue to beat with propagated contractions throughout their lengths. Mutant hearts, on the other hand, do not beat throughout their lengths when cultured *in vitro* (Hill and Lemanski, 1979). This suggests that the deficiency in mutant sarcomeric organization is not caused by a mere inhibitory effect of surrounding tissues. However, mutant hearts will start to propagate contractions over the whole heart when cultured in the presence of normal anterior endoderm from BD stage 29 (Lemanski et al., 1979).

Electrophysiological studies indicate that normal and mutant hearts exhibit action potentials of varying amplitude and duration appropriate to the heart region measured (Epstein and Lemanski, 1980). In addition, both normal and mutant myocardial cells have been shown to be electrically coupled.

Stage H36; SJ36-37; BD36

The ventral mesocardium is finally obliterated, but its posterior-most extent is marked on the myocardial surface by midventral myocardial (MVM) cells oriented at right angles to the circumferential rings of cells added later (Fig. 2c; Fransen and Lemanski, 1988a). The posterior end of the heart is shifted to the left side whereas the dextral loop begins to shift dorsally on the right side of the pericardial cavity (Lemanski, 1973a). The normal heart contracts vigorously throughout its length, whereas the c/c mutant heart is largely inactive, except for contractions of the conus regions in some specimens.

Two dimensional gel analyses indicate that normal and mutant hearts begin to accumulate relatively more α than β actin (Woodrooffe and Lemanski, 1981; Starr et al., 1989).

Mutant hearts at this stage are no longer capable of being rescued by co-culture with competent anterior endoderm (Davis and Lemanski, 1987). Kulikowski and Manasek (1977, 1978) reported that mutant hearts can undergo normal contractions if cultured in the presence of 0.1% glucose. However, Hill and Lemanski (1979) were unable to repeat these results, suggesting the discrepancy might be the result of the different genetic background of the mutant strains used in the two laboratories.

Stage H37-38; SJ38-39; BD37-38

The heart tube continues to lengthen, with no difference in the surface myocardial morphology between normal and mutant hearts detectable by scanning electron microscopy (Fransen and Lemanski, 1988a). The myocardium is still a

monolayer, separated from the endocardial tube by cardiac jelly (Fig. 2e). The cardiac jelly contains more fibrillar (probably collagen) elements than previously in both normal and mutant hearts (Fransen and Lemanski, 1986 and in preparation). Transmission electron microscopy indicates that mutant hearts have relatively fewer endocardial and mesenchymal cells and sparser collagen fibrils in the cardiac jelly of the bulbus region (Lemanski and Fitzharris, 1989). Alcian blue histochemical staining indicates that the cardiac jelly of normal and mutant bulbus regions contain the same amounts of sulfated and non-sulfated glycosaminoglycans (Lemanski and Fitzharris, 1989).

Electrophysiological measurements of normal and mutant hearts are similar to the results for stage 35 embryos (Epstein and Lemanski, 1980). The absence of a circulation in mutant embryos causes erythrocytes to remain at their site of origin in the "blood islands." As hemoglobin synthesis begins in these cells they are visible through the epidermis as red patches in the ventral endoderm. Such patches are useful for making a preliminary identification of mutant embryos because they are generally not observed in genetically normal embryos. (However, a final identification of mutants can only be made on the basis of the heartbeat, because red patches can appear in normal embryos under some conditions.)

Stage H39; SJ39; BD39

At this stage the first gross difference between normal and mutant hearts becomes apparent: the mutant heart appears to be longer than normal in that portion of the heart posterior to the cono-ventricular boundary (Fransen and Lemanski, 1988a). Viewed from the surface, normal myocardial cells continue to look like closely-packed polygons as described for stage 35 hearts (Fig. 2f). The normal ventricle undergoes trabeculation, in which myocardial cells grow inward to establish a complex three-dimensional structure (Lemanski, 1973a).

The mutant ventricular myocardium does not undergo trabeculation although it does contain mitotic figures (unpublished observations). In addition, mutant myocardial cells expose more surface area and more length than normal (Fig. 3a; Fransen and Lemanski, 1988a). Both of these phenomena could underlie the observation that the mutant heart tube is significantly longer than normal (Fransen and Lemanski, 1988a).

This stage represents the last in which the myocardial surface in mutant and normal embryos is exposed to the pericardial fluid. At this time mesothelial cells begin to round up and separate from the transverse septum. Only a few of these presumptive epicardial cells are detectable at this stage (Fransen and Lemanski, in press).

Myosin, actin, and α -actinin continue to be detected at normal levels in mutant heart homogenates fractionated by SDS-PAGE (Lemanski et al., 1976). Immune blotting analysis of IEF-SDS polyacrylamide gels demonstrates that mutant and normal hearts continue to undergo the isoform shift to α -actin reported for BD stage 36

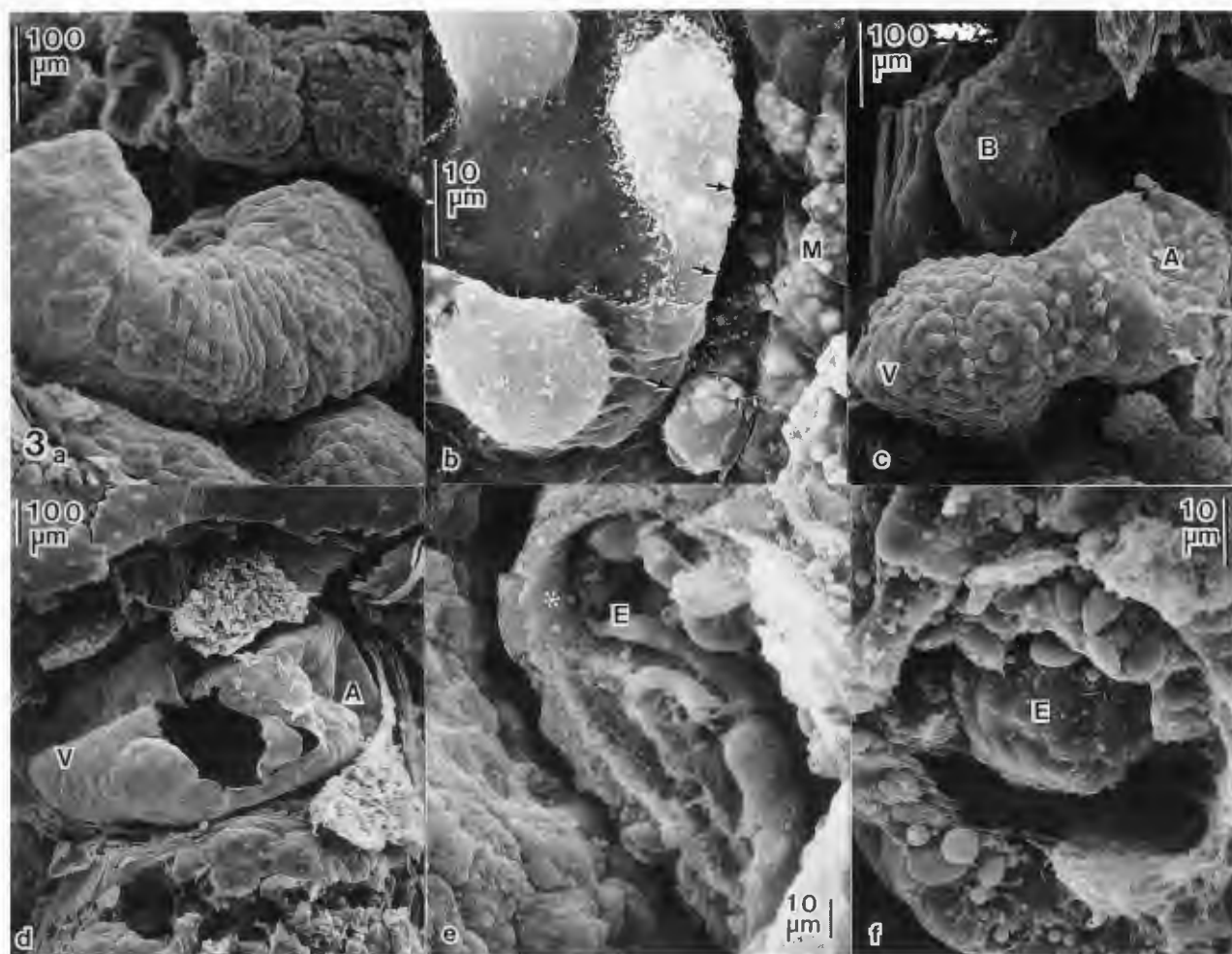


Fig. 3. a-f. SEMs of embryonic axolotl hearts. a. BD Stage 39 c/c mutant embryo. Note the elongated profiles of ventricular myocardial cells in this ventral view of a heart fractured during preparation. (Compare to normal Fig. 2f). b. BD Stage 41 +/- normal embryo. Relatively yolk-free epicardial cells spread over the atrial myocardium (M). (Arrows point to leading edge.). c. BD Stage 42 +/- normal embryo. By this stage the heart is complete covered by epicardium. (V=ventricle, A=atrium, B=bulbus arteriosus). d. BD Stage 42 c/c mutant embryo. By this stage the ventricle (V) and atrium (A) are distended by ascites fluid. Note the thin heart wall where it was damaged during preparation. e. BD Stage 42 +/- normal embryo. The ventricle was sectioned during fixation. Note the dense basement membrane beneath myocardium (asterisk). (E=endocardium). (Tannic acid post-fixation). f. BD Stage 42 c/c mutant embryo (sibling to Fig 3e). The myocardium of the truncus arteriosus was sliced during fixation. (E=base of endocardial layer). The fibrillar extracellular matrix is less well developed than normal. (Tannic acid post-fixation).

(Woodroffe and Lemanski, 1981; Starr et al., 1989). Tropomyosin continues to be detected at abnormally low levels in mutant hearts by either electrophoretic (Lemanski et al., 1976; Starr et al., 1989) or radioimmunoassay analyses (Moore and Lemanski, 1982).

Immunocytochemical analysis of contractile proteins are qualitatively as reported for BD stage 35. Myosin, α -actinin, tropomyosin (Lemanski et al., 1980), troponin-T (Fuldner et al., 1984) and actin (Starr et al., 1989) are detected in extensive sarcomeric arrays in normal myocardial cells. In mutant myocardial cells tropomyosin is barely detectable, whereas the other proteins accumulate only as peripheral amorphous deposits.

Stage H40; SJ40; BD40-41

The heart becomes increasingly S-shaped, which brings the posterior part of the ventricle in close contact with the transverse septum. Here the ventricle is surrounded by a mesothelial growth (the ventricular pericardial stalk) that is confluent with the squamous pericardium of the transverse septum. The flattening of mesothelial cells on the myocardium represents the initial site of epicardial formation (Fransen and

Lemanski, in press). Squamous epicardial cells contain fewer yolk inclusions than myocardial cells and are, therefore, easily recognized by SEM (Fig. 3b).

Post-hatching Stages H41-46; SJ40; BD42-43

During this period, mesothelial cell migration and spreading complete the epicardial coverage of the vigorously beating normal heart (Fig. 3c). As this proceeds, collagenous fibrils appear in the subepicardial space (Fransen and Lemanski, in press). In the mutant embryo, the lack of a normal circulation apparently causes an accumulation of ascites fluid within the heart and other tissues (Lemanski, 1973c). This probably contributes to the extreme distention of the ventricular and atrial regions (Fig. 3d). The thinning of the ventricular and atrial walls does not prevent these regions from becoming covered by epicardium. However, mutant embryos die before the bulbus region is completely invested.

Internally, the myocardium of the normal ventricle contains extensive trabeculae, whereas the mutant myocardium remains a monolayer. Despite this difference and in contradiction to one earlier report (Justus and Hollander, 1971), normal and mutant hearts have similar action potentials (Epstein and Lemanski, 1980; Moore and Satterlie, 1988). Furthermore, gap junctions, revealed in ultrathin sections and freeze-fracture preparations, have been identified in both normal and mutant myocardial cells (Epstein and Lemanski, 1980; Moore and Satterlie, 1988). These membrane specializations are thought to provide the molecular basis for the observed electrical coupling properties of the myocardium.

The collagenous matrix in normal cardiac jelly is very dense (Fig. 3e). Immunofluorescent labelling reveals that fibronectin and laminin are localized in the cardiac jelly of normal hearts (Fransen and Lemanski, 1988b). The collagenous fibrils of mutant hearts are generally sparser and of a thinner diameter (Fig. 3f). The bulbus region of mutant hearts contains more hyaluronate (Alcian blue positive material) but fewer mesenchymal cells than normal hearts (Lemanski and Fitzharris, 1989).

Electrophoretic analysis indicates that mutant hearts at this stage accumulate apparently normal amounts of myosin, actin and α -actinin but much less tropomyosin than normal (Lemanski et al., 1976; Starr et al., 1989). Radioimmunoassays have shown mutant tropomyosin levels to be only 5% of normal values at this stage (Moore and Lemanski, 1982). Two-dimensional gel analysis shows that the α isoform of actin is present in much larger quantities than the β isoform in both normal and mutant hearts (Woodroffe and Lemanski, 1981; Starr et al., 1989). On the other hand, mutant hearts retain a proportionally larger amount of yolk protein than normal hearts; as suggested by Lemanski et al. (1976), this could reflect a slowed metabolic rate because of a lack of oxygen.

Immunofluorescent labelling of contractile proteins yields the patterns mentioned in earlier stages. Normal myocardial cells contain more sarcomeric labelling than at earlier stages

(Lemanski et al., 1980; Fuldner et al., 1984; Starr et al., 1989). Vimentin and vinculin are diffusely distributed in both normal and mutant myocardial cells (Shen et al., 1986). Desmin is diffusely distributed in mutant hearts, but is associated with the central I-band near the Z-line between individual myofibrils of normal myocardial cells (Shen and Lemanski, 1989). Kulikowski and Manasek (1978) have detected longitudinal non-striated filaments in normal and mutant myocardial cells by polarization microscopy. However, the protein composition of these filaments has not been determined.

Discussion

Investigations by scanning electron microscopy and other morphological approaches, by defining the sequence of changes in tissue associations and cell ultrastructure, can provide a basis for analyzing the significant molecular events that regulate organogenesis. The Results section has summarized morphological studies on axolotl cardiogenesis as well as studies on induction and myofibrillogenesis. It is the purpose of this discussion to relate this information to other well-studied systems in order to consider the entire developmental history of the axolotl heart from its earliest specification as mesoderm to the later events of differentiation. Molecular cloning studies on mesodermal differentiation in the anuran *Xenopus laevis* can provide a framework for considering early events in the axolotl. However, any comparison of the two species must take into consideration the known developmental differences between them: *Xenopus* embryos are smaller, develop at an accelerated rate and in a more mosaic pattern than axolotl embryos, suggesting that the coordination of events may not be the same (Mohun et al., 1980; Slack et al., 1987; Slack, 1989). For later cardiogenic events the avian literature, more extensive than the recent amphibian literature (compare the review of Copenhaver (1955) with that of Icardo (1984) or Ferrans et al. (1985)), will serve to highlight the processes as yet incompletely understood in the axolotl.

Origin of Cardiac Tissue

Mesoderm origin. As the heart is a mesodermal organ, it is possible that current investigations on the mechanism of mesoderm formation in *Xenopus* may reveal how cardiac tissue is specified. To briefly sketch the findings of this rapidly progressing field of research, most recently reviewed by Smith (1989), the mesoderm is not the first portion of the embryo to differentiate. Rather, experimental evidence suggests that mesoderm is the product of an induction of marginal animal hemisphere blastomeres by cells of the vegetal hemisphere (Gurdon et al., 1985a,b). In amphibian embryos, the blastocoel limits the area of interaction between the two hemispheres to a marginal equatorial zone and therefore contributes to the overall patterning of mesodermal tissues in the embryo (Gurdon, 1989). In the axolotl the entire marginal zone participates in mesoderm formation (Smith and

Malacinski, 1983). This is in contrast to *Xenopus* in which the most superficial cell layer of the marginal zone differentiates as endoderm and only the interior cell layers differentiate as mesoderm (Smith and Malacinski, 1983).

Mesodermal induction is based upon ooplasmic segregation, which has been thought to create the regional differences in blastomere behavior. Recently molecular differences between the animal (reacting) and vegetal (inducing) regions have been described. By a mechanism not yet understood, developing *Xenopus* oocytes concentrate a specific mRNA (Vgl) in the cortex of the vegetal pole (Weeks and Melton, 1987). After fertilization, the Vgl mRNA is translocated throughout the cytoplasm of the vegetal hemisphere, the future endoderm, but is not present in animal blastomeres. Vgl codes for a protein that is homologous to transforming growth factor- β (TGF- β) of higher vertebrates. The vegetal region of *Xenopus* eggs also activates the gene for basic fibroblastic growth factor (bFGF) during the midblastula transition so that this product is present during the same time that mesodermal induction is thought to occur (Kimelman and Kirschner, 1987; Slack et al., 1987). Thus, both of these heparin-binding growth factors would appear to be excellent candidates for native mesodermal inducing factors (see Slack and Isaacs (1989) for criteria determining morphogenetic substances). Both bFGF and TGF- β or their analogues have been shown to induce blastomere shape changes equivalent to those that occur in mesoderm during gastrulation and, in addition, to induce different mesodermal structures in a dose-dependent manner in both *Xenopus* and the axolotl (Slack et al., 1987; Godsave et al., 1988; Grunz et al., 1988; Rosa et al., 1988). From the standpoint of the reacting tissue the induction appears to involve at least two consequences for gene expression. Induced animal blastomeres down-regulate the synthesis of cytokeratins that are characteristic of uninduced animal blastomeres (i.e. potential epidermal cells) (Gurdon et al., 1985a,b; Kimelman and Kirschner, 1987; Weeks and Melton, 1987). During this same period, mesoderm-specific genes, including the gene for cardiac α -actin, are activated in induced animal cells (Jamrich et al., 1987; Symes et al., 1989). Although the down-regulation of cytokeratin synthesis can occur in individual animal blastomeres cultured in the presence of soluble factors, the activation of the mesodermal program appears to require cell proximity (Symes et al., 1989; Gurdon, 1989). However, Warner and Gurdon (1987) have demonstrated that the coupling of cells by gap junctions is not required for the transmission of the mesodermal inducing signal.

Mesodermal patterning. Treatment of fertilized *Xenopus* eggs with heavy water (Scharf et al., 1989) or early blastulae with lithium ion (Kao and Elinson, 1988; Cooke et al., 1989) results in embryos which produce excessive amounts of dorsal/anterior structures at the expense of ventral/posterior structures. As the heart is present in dorsalized embryos, it is

likely that the mechanism by which "antero-dorsality" is specified occurs in pre-cardiac mesoderm. Recent studies implicate peptide growth factors, perhaps in gradients, in the establishment of embryonic axes; the TGF- β analogues apparently induce more dorsal structures than bFGF (Slack et al., 1987; Cooke et al., 1989; Ruiz i Altaba and Melton, 1989; Slack and Isaacs, 1989). In an important new development Ruiz i Altaba and Melton (1989) have shown that inducing animal pole caps with varying amounts of these growth factors alters the expression of a homeobox gene *xhox3*. In the normal embryos *xhox3* mRNA is present at low levels anteriorly and at higher levels posteriorly. Similarly, treatment with the TGF- β analogues results in less *xhox3* expression than with bFGF. Although this particular homeobox gene is probably not important for heart development, it is possible that another, as yet untested, homeobox gene might be. Because homeobox proteins seem to act as specific transcription factors (Levine and Hoey, 1988) they could be part of the process by which heart tissue becomes specified.

Cardiac Origin. In *Xenopus*, embryonic skeletal and cardiac muscle are both mononucleate (Hamilton, 1969; Muntz, 1975). In addition, both types of embryonic striated muscle express both sarcomeric (cardiac and skeletal) isoforms of α -actin (Mohun et al., 1984). Because the growth factor experiments described above did not distinguish between cardiac and skeletal muscle (but did use cardiac actin probes) it is difficult to be certain of their relevance to heart differentiation (Slack et al., 1987; Godsave et al., 1988; Grunz et al., 1988). However, on the basis of experiments involving *in vitro* explantation of pre-cardiac mesoderm, Sater and Jacobson (1989) have concluded that heart mesoderm in *Xenopus* begins to be specified as early as the gastrula stage and is completely determined by midneurula stages. This chronology suggests that the regulatory events described in the Mesoderm Origin section above could play a major role in specifying cardiac mesoderm in *Xenopus*. However, it remains to be shown how these regulatory events are modulated, perhaps by variations in tissue associations, to give rise to the early components of the cardiac region: the endocardium, myocardium and pericardium.

It is clear that the processes of mesodermal differentiation and heart specification in urodeles occurs over a longer developmental period than in *Xenopus* (Mohun et al., 1980; Sater and Jacobson, 1989). At the end of gastrulation the heart forming regions are at the anterior and dorsal portions of each lateral plate. Experiments on the urodele *Taricha torosa* (Fullilove, 1970, and other work reviewed in Jacobson and Sater, 1988) suggest that the underlying endoderm of the early to mid neurula (stage 14-16) is a specific heart inducer. Prospective heart mesoderm explanted at this stage differentiates at low levels (10-14% of explants), increasing by stage 26, presumably because of a longer association with the endoderm, to 75% of explants. Jacobson and Duncan (1968) have partially puri-

fied from anterior endoderm a heat-insensitive molecule with apparent heart-inducing activity but further biochemical characterization has not yet been reported.

Hirakow et al. (1987) have described the process of lateral plate migration in the mid-neurula of the Japanese newt *Cynops pyrrhogaster*. During the initial stages of anterior and ventral migration, the lateral plate mesoderm is in close contact with the basal surface of the ectoderm. The heart mesoderm migrates along strands of extracellular matrix that have been shown, by immunofluorescence labelling, to contain fibronectin. Such fibrils are absent at the base of the endoderm at this time. Interestingly, Jacobson (1960) demonstrated in *I. torosa* that the ectoderm appears to be necessary for lateral plate migration at this stage. In neurulae whose overlying ectoderm had been removed, the lateral plates failed to migrate ventrally, sometimes differentiating into a heart on the side of the body. Hirakow et al. (1987) found that as the lateral plate nears the ventral side it becomes more adherent to the basal surface of the anterior endoderm which has similar extracellular matrix fibrils for the first time. These fibrils disappear from the endoderm as the mesoderm passes over it, suggesting that they may function in contact guidance during migration.

This earliest precardiogenic period has not been investigated by SEM in the axolotl embryo. However, from our observations of embryos just before plate fusion it appears that the concerted elongation of individual cells within the sheet might play a role in the ventral extension of the plates. From morphological evidence in this paper as well as Lemanski (1973a,b,c), it is clear that cell differentiation has begun to specify the components of the cardiac region: columnar myocardial cells, cuboidal pericardial cells and nearly mesenchymal endocardial cells. It is not known what cell surface changes occur in pre-endocardial cells to cause them to leave the lateral plate and later collect in the mid-ventral region. It is likely, given the electrophoretic detection of α -cardiac actin in whole axolotl embryos at SJ stage 23 (Mohun, et al., 1980), that specific gene expression may already be underway; however, this requires further investigation of specific pre-cardiac layers. Slack (1984, 1989) in his search for regional specific markers in embryos of *Xenopus* and the axolotl identified a glycoprotein "epimucin" specific to the epidermis. It is not known whether this might modulate the response of the outer lateral plate mesoderm (the pericardium) to the endodermal inducer molecules. In this regard, Jacobson's (1960) experiments with ectodermless *I. torosa* neurulae would repay a histological re-investigation as his original report did not specify whether the lateral-forming hearts were also surrounded by pericardium.

As reviewed above, Lemanski and co-workers have determined that RNA from normal BD stage 26-29 anterior endoderm, but not other tissues, is capable of imparting an inductive effect on cardiac lethal mutant hearts. Until anterior endoderm from earlier embryonic stages has been

tested, it will not be certain whether the effects noted in experiments on neurulae of *I. torosa* (see Jacobson and Sater, 1988) are the result of the same molecule. The inducer identified by Davis and Lemanski (1987) appears to function in later stages of cytodifferentiation (see below) rather than during early organogenesis of the heart. The mechanisms by which the inducer molecule interacts with cardiac mesoderm and the immediate biochemical responses of that mesoderm have not been characterized. The normal cell shapes of early mutant heart tubes suggest that organogenesis is controlled by other factors not related to the mutant gene (Fransen and Lemanski, 1988a).

Heart Tube Formation

The factors controlling the formation of the endocardial tube are not known. At first its constituent cells have a shape reminiscent of their mesenchymal origin; a typical squamous shape is not attained until well after the myocardial tube has formed. This flattening of endocardial cells would appear to be an active process regulated by the cytoskeleton and membrane adhesions to the extracellular matrix of the basal lamina. The fact that this process also occurs in explanted heart rudiments where diffusion is less of a problem suggests that the flattening is not a passive accommodation to osmotic swelling of the lumen caused by a net accumulation of metabolic wastes. It is likely for similar reasons that the flattening of the pericardial cells is also an active process.

The formation of the myocardial tube from the trough of columnar myocardial cells also appears to involve active changes in cell shape. Although, as reviewed above, the myocardium expresses intermediate filament and contractile proteins at this time, the localizations are diffuse and not in a sarcomeric pattern. It is likely, especially considering the normal morphogenesis of cardiac lethal mutant embryos, that this myocardial shape change does not involve the sarcomeric but rather the cytoskeletal isoforms of these proteins. However localization at the ultrastructural level using isoform-specific antibodies may be required to monitor the relevant cytoskeletal changes. (See Fransen and Lemanski (1988a) for a comparison of heart tube formation in the axolotl and chick).

Heart Tube Differentiation

Looping. Although the phenomenon of dextral looping has been known for many decades, the mechanisms controlling this process are not completely understood. From the work of Manasek and co-workers it appears that the intrinsic looping of the chick heart is accompanied by regional variations in cytoskeletal organization of the myocardium in coordination with hydration of the cardiac jelly (Manasek et al., 1972, 1984). Their working hypothesis is that hydration of the cardiac jelly creates a force that works against the surrounding myocardial tube. Regional cytoskeletal variations may result in regional variations in the compliance of the myocardium to the force, thereby controlling the

directionality of looping. It is not clear how the regional variations in cytoskeletal organization arise.

On the basis of morphological data it appears as though the looping process in the axolotl differs from that of the chick (see Fransen and Lemanski (1988a) for a detailed comparison). Axolotl and chick hearts have different gross morphologies and behave differently when cultured *in vitro*. Like the chick heart (Butler, 1952 cited in Manasek et al., 1972) looping seems to be intrinsic to the axolotl heart (our unpublished observations). However, the straighter contours of explanted axolotl hearts, some L-shaped rather than S-shaped, suggests that the axolotl myocardium plays a more passive role in the looping process. It is possible therefore, that unlike the situation in the chick embryo, space limitations of the pericardial cavity may modulate looping of the axolotl heart. The precise cytoskeletal organization of the axolotl myocardium during the period of looping has not yet been described. However, the fact that cardiac lethal mutant axolotl hearts undergo the looping process in a normal manner suggests that sarcomeric isoforms of contractile proteins are not required for looping to occur. The forces that may interact with the axolotl myocardium during looping and subsequent shape changes have not been analyzed experimentally.

Myofibrillogenesis. The process of myofibrillogenesis within the developing myocardium of the axolotl is, as yet, only partially understood. The time of the first appearance of sarcomeric isoforms of contractile proteins in the axolotl heart has not been determined. However, as Woodroffe and Lemanski (1981) detected some α -actin by stage 33, some sarcomere protein synthesis clearly precedes the initiation of contractions. How the expression of the various muscle-specific genes may be coordinated is not known. However, deficiencies in tropomyosin accumulation within cardiac lethal mutant hearts suggest that this protein may be regulated independently of other sarcomeric proteins, perhaps via specific influences from anterior endoderm. Studies are underway to determine at what level of protein expression the tropomyosin deficiency occurs.

As has been described for other cardiac muscle systems (Tokuyasu and Maher, 1987; Greaser et al., 1989; Choi et al., 1989), sarcomeric proteins accumulate in axolotl myocardial cells prior to the appearance of organized myofibrils. Nascent myofibrils appear adjacent to the basal plasma membrane, but how specific proteins interact to create them is not known. Investigations at the ultrastructural level will be necessary to monitor these earliest events. Comparisons with the cardiac lethal mutant embryos should reveal how much of the myofibrillar assembly program can occur without the presence of thin sarcomeric filaments. Also not yet examined in the axolotl is the specific orientation of myofibrils within cardiomyocytes and the factors that might control that orientation.

Trabeculation. In order to ensure survival it is necessary for the growth of the ventricular pump to keep pace with the circulatory needs of the growing embryo. This increase in ventricular mass is not simply attained by myocardial cell growth, but also involves cell division. In the case of the axolotl, where coronary vessels are not well-developed, the physiological needs of the ventricular myocardium are supported by diffusion to and from the heart lumen across the intimately-associated endocardial layer. Therefore, the number of cardiomyocyte layers within the ventricular wall is limited, probably by rates of diffusion, to only two or three.

The normal axolotl embryo is able to achieve an increase in ventricular cell mass without sacrificing metabolic needs by means of trabeculation. Around stage 38 certain ventricular myocardial cells apparently change their axis of cell division so that, instead of both daughter cells remaining in the monolayer, one is positioned nearer the heart lumen (Lemanski, 1973a). It is not known what regulates this behavior so that it occurs in the ventricle but not other heart regions. Furthermore, the manner in which these cell divisions are regulated in particular groups of cells to give rise to organized ridges within the ventricle is not known. However, recent studies on cytokinesis in *Caenorhabditis elegans* embryos suggest some of the processes that may be occurring in trabeculating cells. Hyman and White (1987) have demonstrated that cell division axes in *C. elegans* are determined by the relative positions of pairs of centrosomes together with the nucleus. They have concluded that microtubules linking centrosomes to the cortical cytoskeleton are responsible for the alignment. As far as the axolotl is concerned, microtubules or their associated proteins in the heart have not yet been investigated. If this mechanism is relevant to trabeculation it may be possible that some related cytoskeletal deficiency could be the cause of the lack of trabeculation in cardiac lethal mutant embryos.

Extracellular Matrix. The cardiac jelly of the axolotl heart has not been studied to the same extent as that of the chick heart by Markwald et al. (1985). Although various matrix components have been detected in axolotl hearts by various means (SEM, immunofluorescence labeling, histochemistry), the organization of the cardiac jelly is not completely understood. The relative contributions of the endocardium and myocardium to the cardiac jelly have not been determined. As French-Constant and Hynes (1988) have demonstrated for the chick embryo, *in situ* hybridization analysis of mRNA would be necessary to answer this question. The role of the cardiac jelly in establishing the hemodynamic properties of the axolotl heart or in contributing to heart valve morphogenesis is not known. (see below for a consideration of sub-epicardial extracellular matrix).

Colonization of the Heart Tube

The initial embryonic heart consists of the inner endocardium separated by cardiac jelly from

the outer myocardium. After the heart has begun to contract it is "colonized" by the invasion of other cell types that ultimately contribute to definitive cardiac morphology and probably cardiac function.

Epicardium. It is clear that, as in chick and mouse embryos (Ho and Shimada, 1978; Komiyama et al., 1987; Viragh and Challice, 1973, 1981; Hiruma and Hirakow, 1989), the axolotl epicardium arises from the spreading of mesothelial cells over the surface of the myocardium (Fransen and Lemanski, in press). The proliferation of mesothelial cells posterior to the developing heart appears to be a common feature of vertebrate epicardial development, although the stimulus for this proliferation is unknown. In mouse and axolotl embryos, where the pericardial cavities are isolated compartments, mesothelial cells may be released singly or as aggregates to circulate within the pericardial fluid. In chick embryos, whose pericardial cavities are open during this period, mesothelial proliferation takes the form of cohesive villi that grow towards and subsequently spread over the myocardium. (See Fransen and Lemanski, in press, for a detailed comparison of epicardial development in these three species).

The mechanism of mesothelial cell spreading over the myocardium undoubtedly involves cell surface adhesion molecules. In this regard it is interesting that a differential expression of the neural cell adhesion molecule (N-CAM) has been reported in developing hearts of chick and *Xenopus* (Thiery et al., 1982; Levi et al., 1987). NCAM is present in the developing myocardia of these species, but apparently not in their epicardia. The developmental history of NCAM expression in the axolotl heart should be investigated to determine whether proliferating mesothelial cells might initially express NCAM, but lose the molecule from their membranes as they release from neighboring cells during proliferation. Whatever such an investigation may reveal, it is certain that axolotl mesothelial cells that are forming the epicardium concomitantly secrete fibronectin and laminin over the apical myocardial surface (Fransen and Lemanski, 1988b, and in preparation). Presumably these matrix components assist in the anchoring, perhaps via integrin in the cell membrane, of the cytoskeletal apparatus. The changes in the organization of axolotl mesothelial cell cytoskeletons as they undergo spreading have not been investigated. Nor have the basal projections of epicardial cells been examined for the distribution of proteins that are known to contribute to focal contacts in stationary cells (Geiger, 1989).

Neural Crest. There is a growing body of evidence that neural crest cells play a significant morphogenetic role in normal heart development. Kirby and co-workers have established that the neural crest in the region of the anterior somites in the chick embryo contributes directly to septation of the outflow tract (Kirby and Bockman, 1984). More posteriorly, neural crest contributes to the autonomic innervation of

the heart (Kirby and Stewart, 1984). Despite several morphological investigations on neural crest migration in the axolotl, these have not focused upon the heart region (Loefberg et al., 1989). However, at least one recent paper on *Xenopus* species indicates that branchial neural crest does contribute to the truncus arteriosus in amphibians (Sadaghiani and Thieband, 1987) and therefore may contribute to outflow tract septation. With regard to the cardiac lethal mutant axolotl, it is not known whether the observed abnormalities in the truncal region (Fransen and Lemanski, 1988a; Lemanski and Fitzharris, 1989) are accompanied by an abnormal distribution of neural crest cells in that region. One detailed study of the development of the sympathetic system in the axolotl also does not focus on innervation of the heart (Vogel and Model, 1977). However, by extrapolation from data presented by these authors, it seems as though neural crest cells may migrate into the heart region by stage 35. It is clear that the role of neural crest cells in the development of the axolotl heart, needs to be examined in detail with regard to septation, innervation and, in addition, possible trophic effects on the myocardium, as has been suggested by Vogel and Model (1977).

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Discussion with Reviewers

A.W. Neff: What is the evidence that Xenopus laevis develops at an accelerated rate in reference to the axolotl? Could it not be possible that the axolotl develops at a decelerated rate? However, it is clear that Xenopus laevis develops faster than the axolotl.

Authors: Many authors (Jacobson and Sater, 1988; Mohun et al., 1980; Slack, 1989) have remarked on the relatively early occurrence of determinative events in Xenopus compared to the axolotl. A comparison of staging systems for amphibian embryos raised at 18°C (see Rugh, 1962 and Schreckenberg and Jacobson, 1975), indicates that X. laevis reaches the heartbeat stage in 2.4 days, compared to 7 days for both Ambystoma maculatum and Ambystoma mexicanum and 15 days for Taricha torosa. However X. laevis is also faster than Rana sylvatica (3.1 days) and R. pipiens (4.9 days). It is possible that both X. laevis and the axolotl have undergone shifts in developmental rate compared to ancestral forms, but there is no direct evidence on this point.

A.W. Neff: Evidence presented in this paper indicates that a morphological approach to studying the cardiac lethal will be fruitless. (1) What is the best future approach? (2) Does a molecular approach show any promise? (3) Could the cardiac mutation affect a regulatory step in the differentiation of the myocardiocytes, that has multiple inputs? (4) How can the interesting observation that tropomyosin may be separately regulated, be utilized to study the regulation of coordinated synthesis of muscle specific proteins be capitalized on in this system?

Authors: (1,2) A first step will be to use molecular methods to characterize the sarcomeric tropomyosin transcript in the mutant myocardium. If it is not found, the gene activation level would be a likely target. If a transcript is found it will be compared to the normal to determine whether it is translatable. (3) Given the ability of the defect to be corrected, it is likely that a regulatory event is the source of the tropomyosin deficiency. (4) We will be able to address your last question realistically when we have some solid information about the nature of this defect.

A.W. Neff: Please provide evidence that mitosis is not blocked in the mutant ventricular myocardium at stage 39.

Authors: Our original observation, confirmed by morphometric analysis (Fransen and Lemanski, 1988a), was that the mutant myocardial tube is longer than normal at stage 39. Furthermore, the width of myocytes that are arrayed concentrically in the heart tube is statistically the same in normals and mutants. We hypothesized that a lack of trabeculation (myocardial ingrowth) in mutant hearts could account for the increased tube length, if mitosis were normal. SEM examination of normal and mutant hearts at earlier stages seemed to support the idea that mitotic rates in mutant hearts are normal. We have subsequently examined histological sections of three pairs of normal and mutant stage 39 hearts. Our unpublished observation of mitotic figures in mutant hearts suggests that mitosis is not blocked.

T. Pexieder: Why is "ascites fluid" accumulated specifically in the mutant heart? Usually ascites fluid accumulates in all coelomic cavities.

Authors: Our mention of ascites fluid accumulation in mutant hearts was not intended to be exclusive. The phenomenon occurs in other mutant tissues as well.

T. Pexieder: Please provide references for Holtfreter's solution and Finquel (MS-222) for those readers unfamiliar with amphibian experimental embryology.

Authors: Both are discussed in Rugh (1962). Finquel and MS-222 are brand names for the anaesthetic tricaine methanesulfate.

T. Pexieder: Why were the embryonic hearts not fixed by perfusion (cf. T. Pexieder (1981) Prenatal development of the endocardium: a review. Scanning Electr. Microsc. 1981/II, 223-253)? Why was sagittal sectioning performed? Why wasn't the standardized method for cardiac microdissection applied (cf. T. Pexieder (1986) Standardized method for study of normal and abnormal cardiac development in chick, rat, mouse, dog and human embryos. Teratology 33, 91c-92c)?

Authors: In adults or embryos, amphibian hearts have much thinner walls than those of higher vertebrates. We know that glutaraldehyde fixative penetrates embryonic axolotl hearts very rapidly. We processed our embryos with hearts along with embryos of earlier stages (prior to heart formation) at the same time. We found that by including magnesium chloride in the fixative, we could maintain heart morphology and avoid distorting contraction artefacts even when fixing by immersion and agitation. We sectioned embryos sagittally in order to maintain the relationship of the heart to surrounding tissues. The microdissection method to which you refer would not have been appropriate for the axolotl hearts at the stages we examined: the hearts are still in the shape of coiled tubes.

T. Pexieder: What is meant by "both dorsal mesocardia" at stage 33?

Authors: The myocardial trough is U-shaped in cross-section. The two top points of the U, still connected to the parietal mesoderm, are the dorsal mesocardia.

T. Pexieder: What is the role of microtubules relative to dextral looping (cf. Manasek et al., 1972)?

Authors: We stated that the cytoskeleton of looping axolotl hearts had not yet been examined. Therefore, we do not know whether microtubules may function in this process, although it is a possibility.

T. Pexieder: Please explain what is meant by "the role of cardiac jelly in establishing the hemodynamic properties" of axolotl hearts.

Authors: It is known that the manner in which collagen fibrils are arranged in specific helical winding patterns can determine the limits of strength and flexibility of biological structures (Trelstad RL and Silver FH (1981) Matrix assembly. In: Cell Biology of Extracellular Matrix, E.D. Hay, ed. Plenum Press, New York, pp.

179-215). For example, Nakamura and Manasek (1981) have shown how enzymatic digestion of specific components of the cardiac jelly can cause the embryonic chick heart to become flaccid (Nakamura A. and Manasek F.J. (1981) An experimental study of the relation of cardiac jelly to the shape of the early chick embryonic heart. J. Embryol. exp. Morph. 65, 235-256). Manasek et al. (1984) have analyzed the cardiac jelly of the chick heart with respect to the principles of biomechanics. Such an analysis, which has direct bearing on the ability of the heart to act as a pump, has not been performed in axolotls.

T. Pexieder: What is meant by the "branchial neural crest"?

Authors: This refers to neural crest cells arising at the level of the branchial (gill) region of the neural tube.