Protein Synthesis during Heart Development in Normal and *Cardiac* Mutant Axolotls

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Abstract

Recessive mutant gene *c* in axolotls causes a failure of the hearts of affected embryos to function. The mutant hearts (*c*/*c*) lack organized sarcomeric myofibrils. The present study was undertaken to determine the overall pattern of *in vivo* protein synthesis and subsequent accumulation of the newly synthesized proteins for a 24 hour period in normal (+/+ or +/*c*) and *cardiac* mutant (*c*/*c*) axolotl hearts at various stages of development. Additionally, selected cytoskeletal/myofibrillar proteins were analyzed in detail for their synthesis during heart development. For such analyses, the hearts were radiolabeled with [35S]-methionine for 24 hours and subjected to SDS-PAGE analyses and autoradiography. Quantitative densitometric analyses of the bands show that even though the overall protein pattern is similar in normal and mutant heart tissues, a general reduction in the synthesis of the proteins in mutant hearts is observed even at the earlier stages of development (stages 35 and 38). Synthesis and accumulation of most of the proteins is significantly inhibited in mutant hearts at later stages (stage 41-42). In the mutant hearts, tropomyosin, a 32 kd polypeptide in this species (Moore and Lemanski, 1982b) and troponin T, when grouped together, are synthesized at a level only 70% of that in normal embryonic hearts at stage 35. The synthesis and the accumulation of the tropomyosin/troponin T complex of proteins in mutant hearts decreases with increasing age until the proteins essentially stop being synthesized by stage 41. Most interestingly, actin is synthesized in normal amounts in mutant hearts at stage 35. Actin synthesis and accumulation over 24 hours in the mutant hearts is reduced as the hearts advance in age and drops to only 26% of that in normal hearts by stage 41, just before the mutant embryos die. Moreover, desmin and vimentin are reduced by 50% in the mutant hearts at stages 35 and 38 when compared to normal; vimentin synthesis is not detectable at stage 41 in mutant hearts although normal hearts show significant amounts of vimentin. Desmin in mutant hearts accumulates to levels that are 70% of normal.

Introduction

The *cardiac* lethal mutation in Mexican axolotls, *Ambystoma mexicanum*, results in lack of a heartbeat in affected embryos (Humphrey, 1972). The recessive gene that causes this mutation is designated as gene *c* for "*cardiac nonfunction*." According to the staging system of Bordzilovskaya et al. (1975), normal axolotl embryos develop functional hearts at stage 34-35, approximately 6 days post-fertilization, and hatch at stage 40, about three weeks post-fertilization. During the prehatching period, the individual embryonic cells contain numerous yolk platelets, from which they receive their nutrients. Oxygen is provided by simple diffusion into the embryonic tissues. The mutant embryos are first distinguishable from their normal siblings at stage 34-35, when the normals develop contracting hearts. The mutant hearts fail to beat and eventually die at stage 41-42 from lack of heart function.

Electron-microscopic studies reveal that mutant hearts lack well-organized myofibrils (Lemanski, 1973a). Subsequent immunohistochemical studies confirm that the mutant ventricular myocardium contains most of the myofibrillar proteins examined, but many of these proteins accumulate in the form of amorphous collections or randomly-arranged filaments instead of organized sarcomeric myofibrils as in normal myocardial cells (Lemanski et al., 1980; Starr et al., 1980; Shen and Lemanski, 1989; Fuldner et al., 1984).

SDS-polyacrylamide gel electrophoresis (Lemanski et al., 1976) and radioimmunoassay (Moore and Lemanski, 1982b) analyses of constituent contractile proteins in normal and mutant hearts show that myosin is slightly reduced from normal; however, actin is present in approximately normal amounts in the mutant hearts.

Both SDS-PAGE and immunohistochemical analyses suggest that the amount of tropomyosin is significantly reduced in mutant hearts when compared to normal (Lemanski,

1979; Lemanski et al., 1980). This observation has been subsequently confirmed immunologically by quantitation of absolute amounts of alpha-tropomyosin in normal and mutant axolotl hearts by employing a solid-phase radioimmunoassay method (Moore and Lemanski, 1982a). Although it is now well established that the accumulations of some selected myofibrillar/cytoskeletal proteins, such as myosin and, particularly tropomyosin, are quantitatively lower in mutant hearts, there has never been a systematic study of the overall protein synthetic/accumulation patterns in normal and mutant hearts. A major complication of using conventional comparative quantitation approaches in comparing the proteins in normal and mutant hearts results from the large amounts of unused yolk proteins in normal and mutant embryonic hearts at early stages and in mutant hearts at later stages. These yolk proteins mask the analyses of various proteins when using conventional electrophoresis methods. Moreover, the content of unused yolk in mutant axolotl is far higher than in normal embryos during the later developmental stages (41-42). Nothing has been reported to date on the patterns of protein synthesis in normal and mutant axolotl hearts during development.

In the present investigation, we compare overall protein synthesis patterns for several known cytoskeletal/contractile proteins in normal and mutant embryonic axolotl hearts at various stages of development using the ^{35}S-methionine labeling method. This approach permits the observation of specific proteins without interference from the masking yolk platelet proteins and provides insight into the differences between normal and mutant hearts with respect to protein synthesis and accumulation of the newly-synthesized proteins.

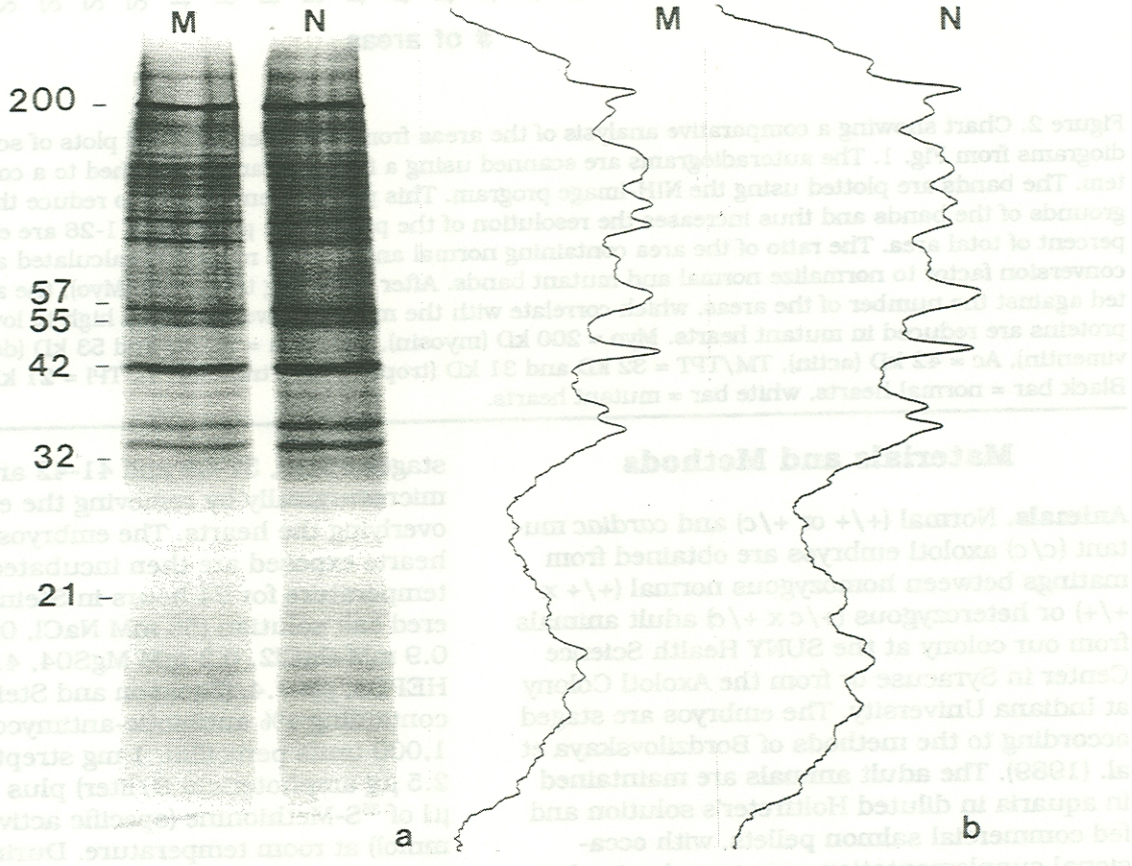

Figure 1. a) Autoradiogram of 35S-methionine labeled proteins synthesized by normal and mutant axolotl embryonic hearts at stage 35-36. Axolotl hearts are metabolically labeled with 35S-methionine and electrophoresed on a 10% SDS polyacrylamide gel. M: mutant, N: normal, MW: Molecular weight b) Densitometer traces of the mutant (M) and the normal (N) lanes. A significant reduction of a band with a MW of 21 kD in mutant hearts is observed on the autoradiographs (a) and the densitometric traces (b).

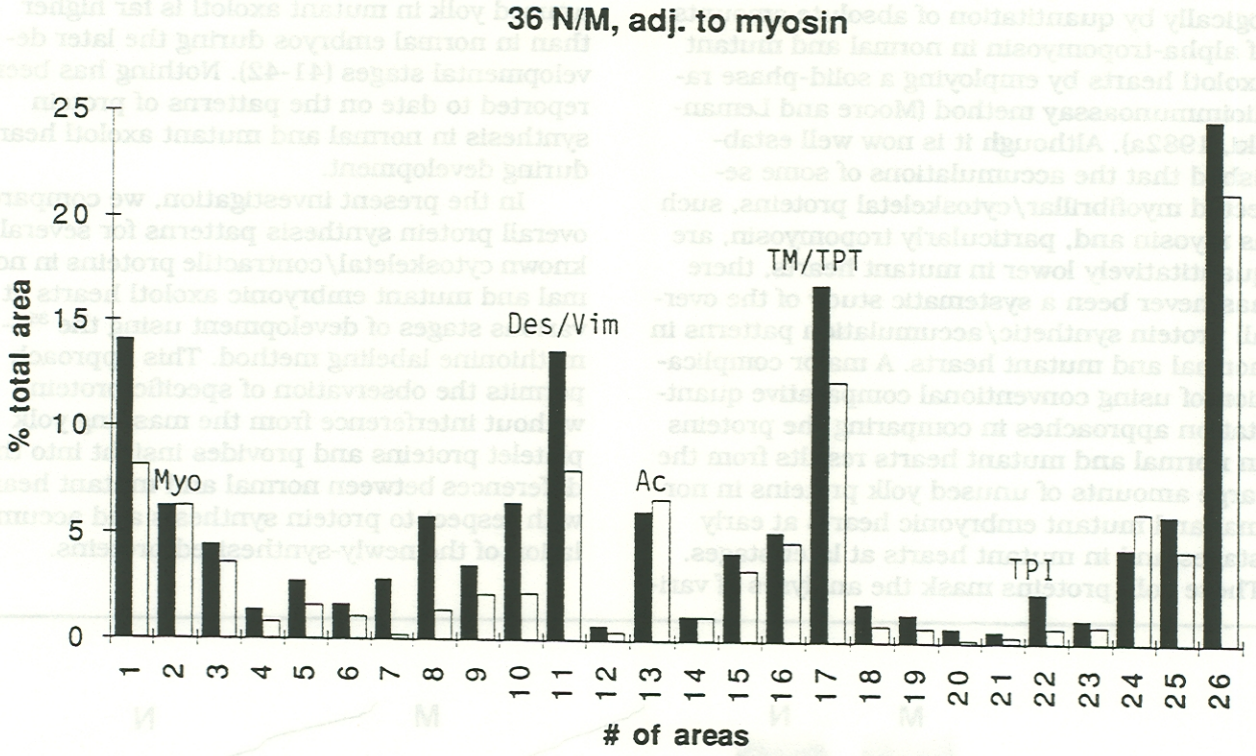

Figure 2. Chart showing a comparative analysis of the areas from computer analyzed plots of scanned autoradiograms from Fig. 1. The autoradiograms are scanned using a flatbed scanner attached to a computer system. The bands are plotted using the NIH-Image program. This program enables us to reduce the backgrounds of the bands and thus increases the resolution of the peaks. The peak areas 1-26 are expressed as percent of total area. The ratio of the area containing normal and mutant myosin is calculated and used as a conversion factor to normalize normal and mutant bands. After adjusting to myosin (Myo), the areas are plotted against the number of the areas, which correlate with the molecular weights from high to low. Most of the proteins are reduced in mutant hearts. Myo = 200 kD (myosin), Des/Vim = 55 kD and 53 kD (desmin/vimentin), Ac = 42 kD (actin), TM/TPT = 32 kD and 31 kD (tropomyosin/troponin T), TPI = 21 kD (troponin I). Black bar = normal hearts, white bar = mutant hearts.

Materials and Methods

Animals. Normal (+/+ or +/*c*) and *cardiac* mutant (*c*/*c*) axolotl embryos are obtained from matings between homozygous normal (+/+ x +/+) or heterozygous (+/*c* x +/*c*) adult animals from our colony at the SUNY Health Science Center in Syracuse or from the Axolotl Colony at Indiana University. The embryos are staged according to the methods of Bordzilovskaya et al. (1989). The adult animals are maintained in aquaria in diluted Holtfreter's solution and fed commercial salmon pellets, with occasional supplementation using raw bovine liver and live Georgia red wiggler earth worms.

***In vivo* labeling of proteins in embryonic hearts with ^{35}S-Methionine.** The chest cavities of normal and mutant axolotl embryos at stages 35-36, 37-38 and 41-42 are opened microsurgically by removing the epidermis overlying the hearts. The embryos with their hearts exposed are then incubated at room temperature for 24 hours in Steinberg's buffered salt solution (58 mM NaCl, 0.67 mM KCl, 0.9 mM CaCl2, 0.2 mM MgS04, 4.6 mM HEPES, pH 7.4, (Zackson and Steinberg, 1986) containing 1% antibiotic-antimycotic (Gibco: 1,000 units penicillin, 1 mg streptomycin, and 2.5 µg amphotericin B/liter) plus 10 µCi/100 µl of ^{35}S-Methionine (specific activity 6000 Ci/mmol) at room temperature. During the incubation period, the normal hearts continue to beat vigorously. The hearts are rinsed with fresh Steinberg's solution and homogenized in gel electrophoresis buffer composed of 0.80 M Tris HCl, pH 8.0, 2% SDS, 100 mM DTT, 15% glycerol, 1 mM PMSF, and 0.001% Brom-

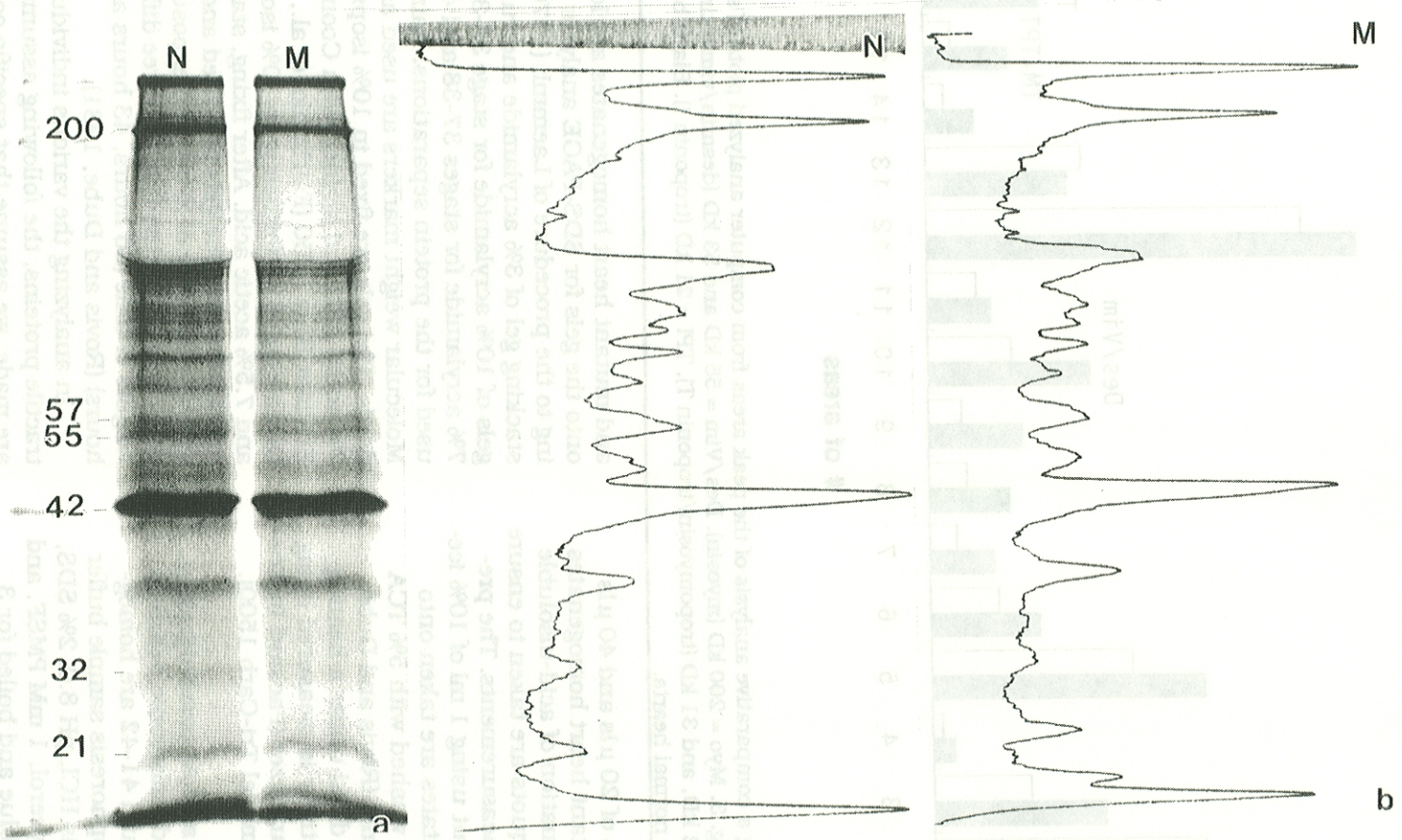

Figure 3. Autoradiogram of ^{35}S-methionine labeled proteins synthesized by the normal and mutant axolotl embryonic hearts at stage 37-38. Axolotl hearts are metabolically labeled with ^{35}S-methionine and electrophoresed on a 7% SDS polyacrylamide gel. N: normal and M: mutant, MW: molecular weight b) Densitometer traces of the normal (N) and the mutant (M) lanes. A reduction of the band with a MW of 32 kD (Tropomyosin) is observable on both the autoradiographs and the densitometric traces (arrow).

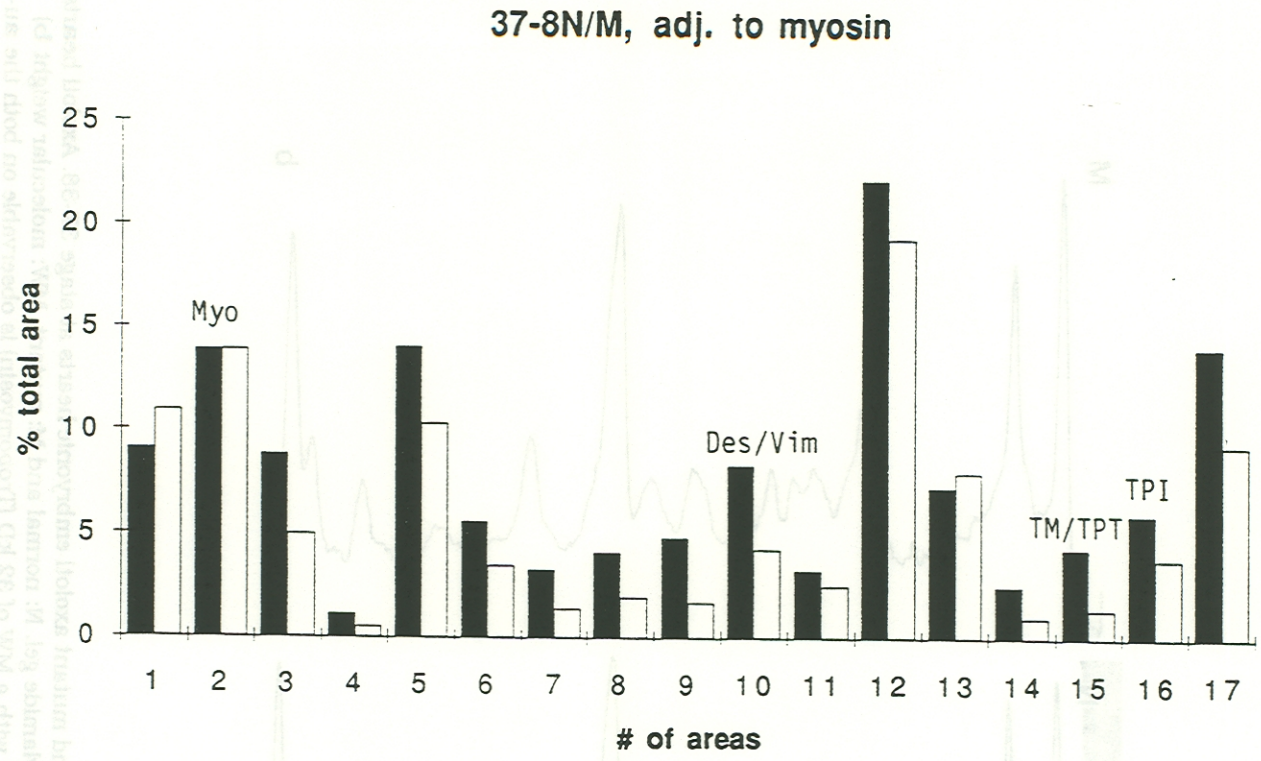

Figure 4. Chart showing a comparative analysis of the peak areas from computer analyzed plots of scanned autoradiograms from Fig. 3. Myo = 200 kD (myosin), Des/Vim = 55 kD and 53 kD (desmin/vimentin), Ac = 42 kD (actin), TM/TPT = 32 kD, and 31 kD (tropomyosin/troponin T), TPI = 21 kD (troponin I). Black bar = mutant hearts, white bar = normal hearts.

phenol blue. Aliquots of 20 µls and 40 µls from normal and mutant heart homogenates are taken for determination of acid-insoluble radioactivity. Two aliquots are taken to ensure the accuracy of the measurements. The precipitation is carried out using 1 ml of 10% ice-cold TCA. The precipitates are taken onto glass fiber filters and washed with 5% TCA followed by 95% ethanol (Rovis and Dube, 1981). The filters are dried under a heating lamp and the radioactivity is measured using a liquid scintillation analyzer (Packard Instrument Company, US, model Tri-Carb 1500).

Gel Electrophoresis and Autoradiography. ^{35}S-Methionine labeled embryonic hearts at stages 35-36, 37-38 and 41-42 are homogenized in a gel electrophoresis sample buffer containing 0.80 M Tris HCl, pH 8.0, 2% SDS, 100 mM DTT, 15% glycerol, 1 mM PMSF, and 0.001% Bromphenol blue and boiled for 3 minutes. The mixture is then centrifuged at 12,000 g for 5 minutes and the insoluble precipitates discarded. Aliquots of the supernatant containing equal amounts of radioactivity (acid insoluble counts per minute) for normal and mutant heart homogenates are loaded onto the gels for SDS-PAGE analysis according to the procedure of Laemmli (1970). A stacking gel of 3% acrylamide and running gels of 10% acrylamide for stage 35-36, and 7% acrylamide for stages 37-38 and 41-42 are used for the protein separations. RainbowR Molecular weight markers are used as standards. The gels are fixed in 10% isopropanol, 7% acetic acid and stained with Coomassie Brilliant Blue R250 (Fairbanks et al., 1971). Destaining is performed with 10% isopropanol and 7.5% acetic acid. After fixing, staining and destaining, the gels are dried and to ensure the linearity of X-ray film exposure, each gel is exposed to the film for three different lengths of time (26 hours, 43 hours and 102 hours) (Rovis and Dube, 1981).

In analyzing the various individual contractile proteins, the following assumptions are made: we assume that specific contractile proteins separate during the electrophoresis procedure used such that myosin heavy chain = 200 kd; vimentin = 55 kd; desmin = 53 kd; actin = 43 kd; tropomyosin = 32 kd; troponin T = 31 kd; troponin I = 21 kd. These assump-

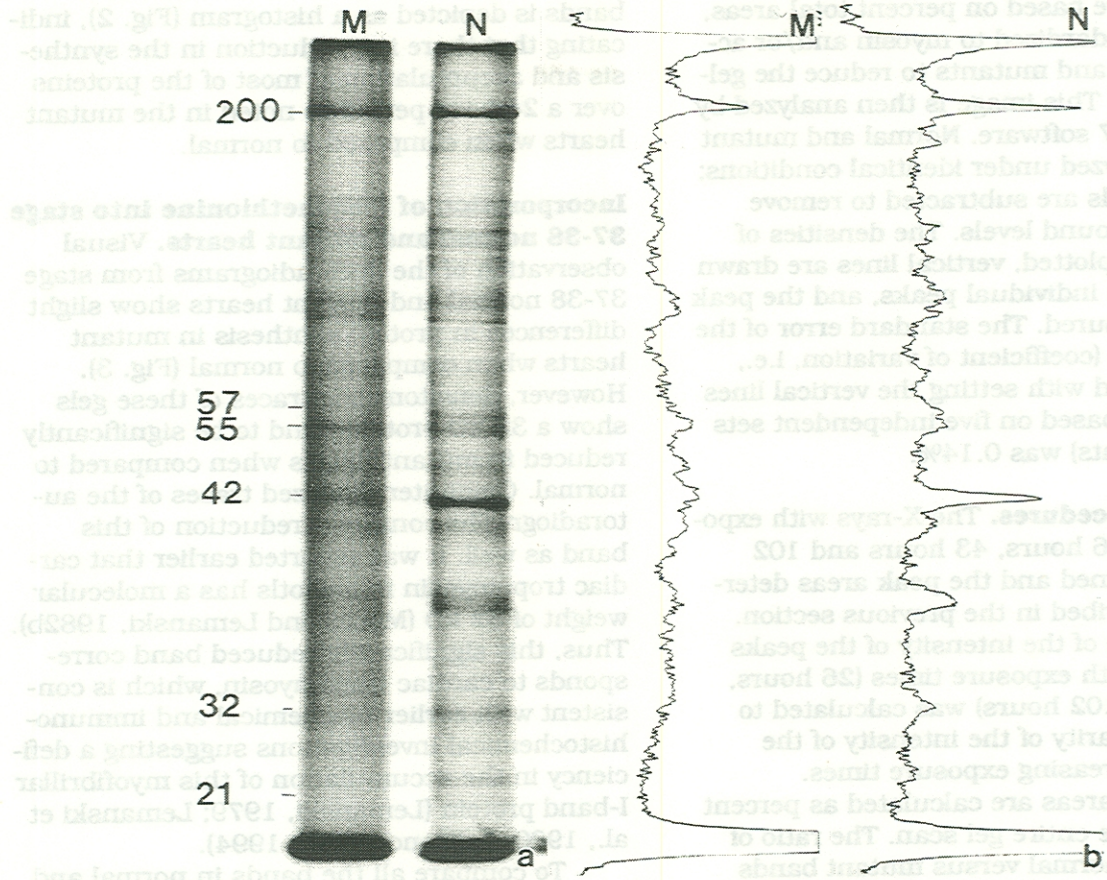

Figure 5. Autoradiogram of ^{35}S-methionine labeled proteins synthesized by the normal and mutant axolotl embryonic hearts at stage 41-42. Axolotl hearts are metabolically labeled with ^{35}S-methionine and electrophoresed on a 7% SDS polyacrylamide gel. M: mutant and N: normal, MW = Molecular weight b) Densitometer traces of the mutant (M) and the normal (N) lanes. Most of the bands are not detectable in the mutant lane (arrows). Actin is significantly reduced in mutant hearts.

tions are based upon the widely accepted premise that the proteins in question separate and show these migration patterns under the electrophoretic conditions used.

Densitometer tracing procedure. For densitometry, the autoradiograms of individual lanes containing normal and mutant heart homogenates are scanned under identical conditions. X-ray films at the three different exposure times are scanned at 570 nm in a spectrophotometer (Gilford model 25) equipped with a linear transport gel scanner (Gilford model 240) linked to a chart recorder (Gilford model 6051).

Computer-aided quantitation of autoradiograms. Autoradiographs of the gels containing normal and mutant heart homogenates are scanned with a Hewlett Packard Scanjet IIc flatbed scanner, which transfers a digitized image to a Macintosh II, 8MB memory computer. Even though equal amounts of radioactivity contained in the normal and mutant heart homogenates are loaded into each lane, unequal amounts of background staining for normal and mutant hearts at all of the stages is observed. In addition, streaks are present in all of the gels. The differences in background staining and occasional streaks probably result from uneven drying of the gels on the blot paper. For this reason, in addition to the densitometer tracing, we analyzed the autoradiograms using a scanner attached to a computer and utilized the NIH-Image 1.47 program to investigate the differences between normal and mutant proteins. This method provides an extremely sensitive and accurate analysis, since most of the very high, the very low or the uneven background staining is eliminated

by using the Image program. Moreover, our calculations are based on percent total areas, which are standardized to myosin and/or actin in normals and mutants to reduce the gel-loading-errors. This image is then analyzed by NIH Image 1.47 software. Normal and mutant lanes are analyzed under identical conditions; gel backgrounds are subtracted to remove uneven background levels. The densities of the bands are plotted, vertical lines are drawn to separate the individual peaks, and the peak areas are measured. The standard error of the measurements (coefficient of variation, i.e., error associated with setting the vertical lines for each peak based on five independent sets of measurements) was 0.14%.

Analytical procedures. The X-rays with exposure times of 26 hours, 43 hours and 102 hours are scanned and the peak areas determined as described in the previous section. The correlation of the intensity of the peaks (peak areas) with exposure times (26 hours, 43 hours and 102 hours) was calculated to assess the linearity of the intensity of the peaks with increasing exposure times.

The peak areas are calculated as percent total area of the entire gel scan. The ratio of peak areas of normal versus mutant bands that correspond to the molecular weight of myosin (or actin) is determined and the ratio used to normalize myosin (or actin) in normal and mutant hearts to equivalent peak areas. This procedure corrects for the slight discrepancy between the amount of protein among samples although total radioactivity for each sample is equivalent.

Results

Linearity of the bands. We have established that there is a linear relationship between band density and exposure time. Gels exposed for 26 hours were chosen to study the newly synthesized proteins since they have not reached the saturation levels of staining for any of the bands examined.

Incorporation of [^{35}S]-methionine into stage 35-36 normal and mutant hearts. Autoradiography of normal and mutant hearts in combination with conventional densitometric analyses of the bands show that the overall patterns for protein synthesis in mutant hearts at this stage are similar to the hearts of their normal siblings (Fig. 1). The differences in the normalized density plots of the individual bands is depicted as a histogram (Fig. 2), indicating that there is a reduction in the synthesis and accumulation of most of the proteins over a 24 hour period as noted in the mutant hearts when compared to normal.

Incorporation of [^{35}S]-methionine into stage 37-38 normal and mutant hearts. Visual observation of the autoradiograms from stage 37-38 normal and mutant hearts show slight differences in protein synthesis in mutant hearts when compared to normal (Fig. 3). However, densitometric traces of these gels show a 32 kD protein band to be significantly reduced in mutant hearts when compared to normal. Computer analyzed traces of the autoradiographs confirm a reduction of this band as well. It was reported earlier that cardiac tropomyosin in axolotls has a molecular weight of 32 kD (Moore and Lemanski, 1982b). Thus, this significantly reduced band corresponds to cardiac tropomyosin, which is consistent with earlier biochemical and immunohistochemical investigations suggesting a deficiency in the accumulation of this myofibrillar I-band protein (Lemanski, 1979; Lemanski et al., 1980; LaFrance et al., 1994).

To compare all the bands in normal and mutant lanes, percent total proteins are plotted against the number of the bands (Fig. 4). Normal and mutant proteins are again normalized to myosin. With the exception of two bands, all the mutant proteins appear to be decreased from normal.

Incorporation of [^{35}S]-methionine into stage 41-42 normal and mutant hearts. At stage 41-42 the differences between normal and mutant heart proteins are substantial. Many of the proteins that are synthesized and present in normal hearts are missing in mutants, which is clearly seen on the autoradiograms and their densitometric traces (Fig. 5). The most striking difference is a significant reduction in the synthesis of actin; myosin is synthesized in near normal amounts.

A computerized profile of protein bands, normalized to myosin, shows a significant reduction of specific protein bands (Fig. 6). Ten bands in the mutant hearts are not detectable at this late stage of development (160, 145, 130, 95, 70, 50, 40, 34, 28 and 27 kD proteins). Significant reductions are observed on the bands with molecular weights of 63, 43, 37 and 32 kDs. Bands of 78 kD and 57 kD are slightly decreased. Proteins with 110

kD and 115 kD show a slight increase in the mutant hearts.

Comparison of selected myofibril-associated proteins at stages 35-36, 37-38 and 41-42. Individual bands on both densitometric traces and traces obtained by the scanner in combination with a computer were not completely resolved. Therefore, only the bands that are clearly resolved at all three stages, and have molecular weights of known myofibril associated proteins, are compared at stages 35-36, 37-38 and 41-42 (Table 1). Myosin (Band 1), actin (Band 3) and troponin I (Band 5) are resolved as individual bands; others are grouped together [vimentin and desmin (Band 2) and tropomyosin and troponin T (Band 4)]. Band 2 containing vimentin and desmin in mutants is approximately 50% of normal at stages 35-36 and 37-38, but shows a relative increase at stage 41-42 (70% of the normals). Interestingly, vimentin is virtually absent in the mutant lane at this stage, thus the 70% figure represents desmin only. Most surprisingly, actin is present at a concentration of 108% of the normal level in the mutants at stage 35-36 and drops to 26% of normal just before the mutant embryos die at stage 41-42. Mutant tropomyosin and troponin I containing Band 4 is 72% of normal at stage 35-36, reduces to 32% the normal level at stage 37-38 and virtually disappears by stage 41-42. Mutant heart troponin T is only 31% of the normal level, 62% at stage 37-8 and is virtually absent at stage 41-42.

Comparison of myosin- and actin-normalized data. In the studies described above, all of the proteins are normalized to myosin. Additionally, all of the bands are adjusted to actin (data not shown). At stage 35-36 the differences between normals and mutants remain the same when the proteins are normalized to actin and myosin. Similarly, at stage 37-38, the myosin-adjusted protein pattern for normal and mutant hearts is comparable to the bands that are standardized to actin. At stage 41-42, however, the myosin-adjusted proteins show a different pattern than the actin-adjusted bands. At this stage, it seems most reasonable to assume that the myosin-normalized data is the more valid one, since the autoradiographs clearly demon-

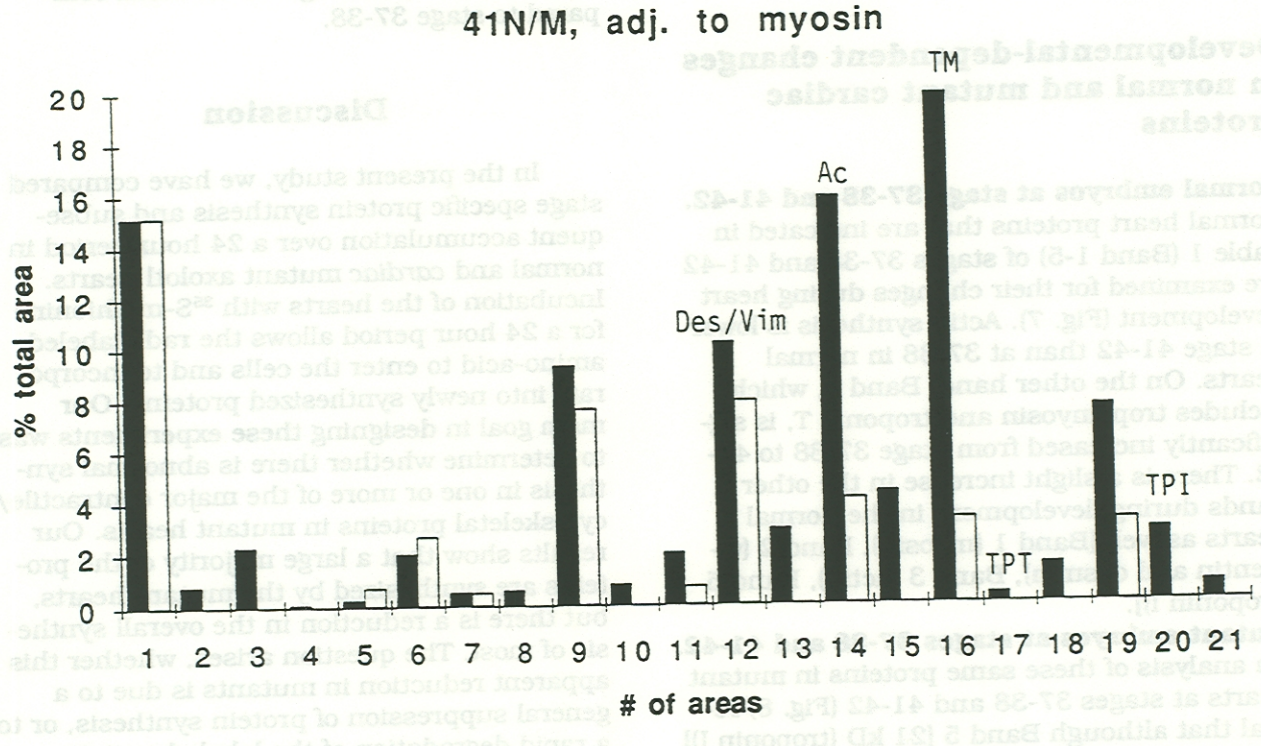

Figure 6. Chart showing a comparative analysis of the areas from computer analyzed plots of scanned autoradiograms from Fig. 5. Areas 1-21 are calculated as percent total areas, normalized to myosin and plotted against the numbers, which reflect the molecular weights from high to low. Myo = 200 kD (myosin), Des/Vim = 55 kD and 53 kD (desmin/vimentin), Ac = 42 kD (actin), TM = 32 kD (tropomyosin), TPT = 31 kD (troponin T), TPI = 21 kD (troponin I). Black bar = normal hearts, White bar = mutant hearts.

Table 1

Band # **	MW (Kds)	Protein	Stage* 35-6	37-8	41-2
1	200	myosin	100	100	100
2	55	vimentin	58.5	51.3	77.2
	53	desmin			
3	42	actin	108.8	88.1	26.4
4	32	tropomyosin	72.6	32.6	16.5
	31	troponin T			
5	21	troponin I	31.6	62.7	0

*Data expressed as a percentage of radioactivity in mutants compared to normals
**The bands include one or more proteins as indicated in the table.

Selected myofibril associated proteins (Bands 1-5) are compared in normal and mutant hearts at three different stages (35-36, 37-38 and 41-42) after adjusting them to myosin. Bands 2 and 4 contain more than one protein, because the peaks of individual proteins are summed up for those proteins at one or more stage and not resolved as a single band.
Band 1= 200 kD (myosin), Band 2 = 55 kD (vimentin) and 53 kD (desmin), Band 3 = 42 kD (actin), Band 4 = 32 kD (tropomyosin) and 31 kD (troponin T), Band 5 = 21 kD (troponin I).

strate that the myosin band in normals and mutants is in comparable amounts, whereas actin is significantly reduced.

Developmental-dependent changes in normal and mutant cardiac proteins

Normal embryos at stage 37-38 and 41-42. Normal heart proteins that are indicated in Table 1 (Band 1-5) of stages 37-38 and 41-42 are examined for their changes during heart development (Fig. 7). Actin synthesis is lower at stage 41-42 than at 37-38 in normal hearts. On the other hand, Band 4, which includes tropomyosin and troponin T, is significantly increased from stage 37-38 to 41-42. There is a slight increase in the other bands during development in the normal hearts as well [Band 1 (myosin), Band 2 (vimentin and desmin), Band 3 (actin), Band 5 (troponin I)].

Mutant embryos at stages 37-38 and 41-42. An analysis of these same proteins in mutant hearts at stages 37-38 and 41-42 (Fig. 8) reveal that although Band 5 [21 kD (troponin I)] is present at stage 37-38 in noticeable amounts, the proteins are not detectable at stage 41-42. Actin synthesis is significantly reduced in mutant hearts at stage 41-42 when compared to stage 37-38. Interestingly, the mutants show a slight increase in Band 2 and Band 4 (vimentin, desmin, tropomyosin and troponin T) at stage 41-42 when compared to stage 37-38.

Discussion

In the present study, we have compared stage specific protein synthesis and subsequent accumulation over a 24 hour period in normal and *cardiac* mutant axolotl hearts. Incubation of the hearts with ^{35}S-methionine for a 24 hour period allows the radiolabeled amino-acid to enter the cells and to incorporate into newly synthesized proteins. Our main goal in designing these experiments was to determine whether there is abnormal synthesis in one or more of the major contractile/cytoskeletal proteins in mutant hearts. Our results show that a large majority of the proteins are synthesized by the mutant hearts, but there is a reduction in the overall synthesis of most. The question arises, whether this apparent reduction in mutants is due to a general suppression of protein synthesis, or to a rapid degradation of the labeled proteins over the 24 hour period studied. Pulse-chase experiments will be necessary to unequivocally answer this question. If the synthesis of only one or a few myofibrillar protein(s) is depressed, and sarcomere formation is not suc-

cessfully completed, a rapid degradation of the other unused proteins in mutant hearts seems a possibility. Alternatively, the synthesis of all of the proteins might be affected by the hypoxic conditions that presumably exist in the non-beating mutant hearts.

The earliest stage examined is stage 35, when normal hearts contain newly-organized myofibrils and develop beating hearts (Lemanski, 1973b). Stage 35 mutant hearts fail to beat, although they exhibit staining for many of the myofibrillar proteins, most in the form of small amorphous collections (Lemanski, 1973a). Yolk platelets are abundant in both normal and mutant hearts at this earlier stage of development. For earlier SDS-Page analyses, the presence of the yolk platelets in embryonic hearts has been a limitation in resolving some of the proteins on the gels, since they are masked by the yolk platelet proteins. ^{35}S-Methionine labeling of the proteins overcomes this problem, because yolk platelet proteins are not being synthesized in the developing embryos and therefore do not incorporate the radioactive label.

Normal hearts at stage 37-38, an intermediate stage in development, have larger and more numerous well-developed myofibrils (Lemanski, 1973b). There are fewer yolk platelets in stage 37-38 normal hearts than in stage 35 normal hearts. On the contrary, yolk platelets continue to be abundant in mutant hearts at stage 37-38.

Normal hearts beat vigorously at stage 41-42 and the myocardium contains well-organized myofibrils. By stage 41, most of the yolk platelets disappear from the normal hearts, however, yolk is still abundant in mutant hearts. Also at this stage, the embryos hatch from their jelly coats and the mutants show ascites and microcephaly as well as other pathological conditions (Lemanski, 1973). These are secondary abnormalities resulting from a lack of circulation, since if the mutants are linked to normals parabiotically, all of these mutant abnormalities disappear (Humphrey, 1972). By stage 42, the final stage mutants survive, severe pathological symptoms appear in the mutants. The fact that protein synthesis in the hearts of mutant embryos at this late stage is severely depressed, and most of the proteins are no longer detectable, may also result from secondary defects caused by the mutation; most of the proteins in the mutant hearts are synthesized at earlier stages, although in reduced amounts.

For quantitative comparison of all of the normal and mutant proteins, the percent total areas are calculated, adjusted to myosin, and plotted against the number of the bands with their respective molecular weights. In addition to myosin, the proteins are normalized to actin, to confirm the values obtained from the myosin-adjusted proteins. SDS-polyacrylamide gel electrophoresis (Lemanski et al., 1976) and radioimmunoassay (Lemanski et al., 1975; Lemanski, 1976) analyses of major contractile proteins in normal and mutant hearts show that myosin is slightly reduced from normal, however, actin is present in nor-

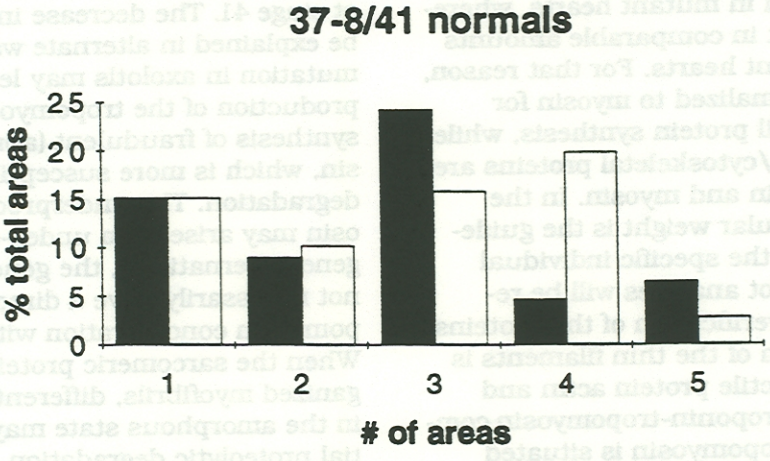

Figure 7. Comparison of normal heart proteins at stages 37-38 and 41-42. The bands (1-5) of normal hearts of stages 37-38 (black bar) and 41-42 (white bar), that are standardized to myosin, are plotted against their numbers, which indicate the molecular weights in decreasing order. Band 3 [42 kD (actin)] is reduced at stage 41-42, only a small increase is observed in Band 2 [55 kD (vimentin) and 53 kD (desmin)] and Band 5 [21 kD (troponin I)]; whereas Band 4 [32 kD (tropomyosin) and 31 kD (troponin T)] is increased tremendously at stage 41-42 in normal hearts.

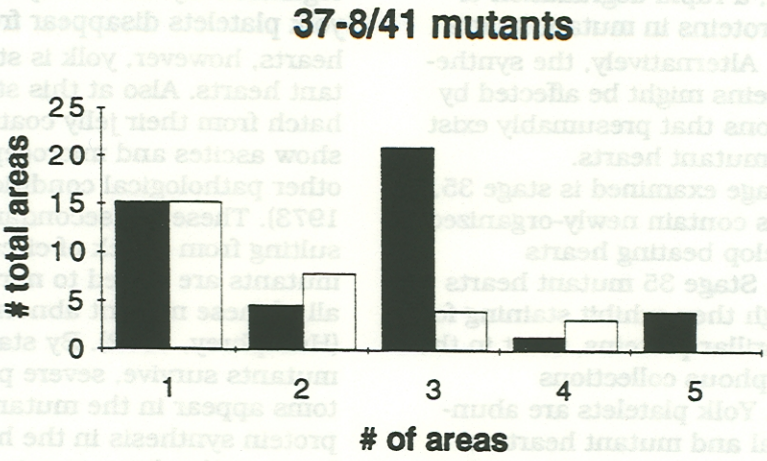

Figure 8. Comparison of mutant heart proteins at stages 37-38 and 41-42. The bands (1-5) of mutant hearts of stages 37-38 (black bar) and 41-42 (white bar), that are standardized to myosin, are plotted against their numbers, which indicate the molecular weights in decreasing order. Band 5 [21 kD (troponin I)] virtually disappeared at stage 41-42. Band 2 [55 kD (vimentin) and 53 kD (desmin)] and Band 4 [32 kD (tropomyosin) and 31 kD (troponin T)], increase as development progresses. Actin (Band 3 [42 kD]) is drastically reduced during later stages (41-42).

mal amounts in the mutant hearts at stage 35. The relative ratios of myosin to actin in normal and mutant hearts are found to be equal at stage 35, but increase during later stages. Therefore, myosin and actin are standardized for comparisons with the other protein bands. The pattern of protein synthesis is found to be equal for both, myosin and actin, at stages 35-36 and 37-38. However, stage 41-42 proteins show a large discrepancy after actin adjustment. This is not surprising, since the autoradiographs and the densitometric traces exhibit clearly that actin synthesis is significantly reduced in mutant hearts, whereas myosin is present in comparable amounts in normal and mutant hearts. For that reason, the proteins are normalized to myosin for comparison of overall protein synthesis, while selected myofibrillar/cytoskeletal proteins are adjusted to both actin and myosin. In the present study molecular weight is the guideline used to identify the specific individual proteins. Western blot analyses will be required for absolute verification of the proteins.

The composition of the thin filaments is complex. The contractile protein actin and four proteins of the troponin-tropomyosin complex are involved. Tropomyosin is situated along the helical groove of actin thin filaments and is also associated with troponin subunits I, C and T (I=inhibitory subunit; C=calcium-binding subunit; and T=tropomyosin-binding subunit). The troponin-tropomyosin complex is the major calcium-binding component regulating the interaction between actin and myosin in cardiac muscle. Tropomyosin has been a major protein of interest in our laboratory since the mutant embryonic hearts show reduced accumulations of the protein (Lemanski, 1979; Lemanski et al., 1980; Moore and Lemanski, 1982). Results of the present study confirm these earlier findings and suggest that tropomyosin synthesis and its subsequent accumulation is significantly reduced in mutant hearts. Compared to synthesis in normal hearts, tropomyosin in mutants is reduced 28% at stage 35, 58% at stage 37-38 and 84% at stage 41. The decrease in tropomyosin could be explained in alternate ways. The gene *c* mutation in axolotls may lead to either underproduction of the tropomyosin, or lead to the synthesis of fraudulent (abnormal) tropomyosin, which is more susceptible to proteolytic degradation. The underproduction of tropomyosin may arise from under-expression of the gene. Alternatively, the gene *c* mutation may not necessarily have a direct effect on the tropomyosin concentration within mutant hearts. When the sarcomeric proteins fail to form organized myofibrils, different proteins present in the amorphous state may undergo differential proteolytic degradation. Tropomyosin may be more susceptible to proteolysis than some of the proteins and, as a result, there is a preferential reduction of this protein in the mutant hearts. Future studies to determine the turnover of tropomyosin in mutant hearts by pulse and chase experiments followed by

quantitative immunoprecipitation will be required to verify this hypothesis. In addition, quantitation of tropomyosin mRNA in normal and mutant hearts at various stages of development will permit definitive conclusions to be made concerning the relationship of the gene *c* mutation and the lack of a normal concentrations of tropomyosin in mutant hearts. A few bands show an increase in mutant hearts when compared to normals. Such proteins may arise from proteolytic degradation of other proteins with higher molecular weights or else from the expression of a new gene or due to the over-production of some of the already expressed genes which ultimately prevent myofibrillar assembly in the mutant hearts. Obviously, the reduction in protein synthesis in *cardiac* mutant hearts requires further examination in order to fully understand the mechanism(s) involved and the relationship(s) of this reduction to recessive gene *c*.

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