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Accumulation and localization of troponin-T in developing hearts of *Ambystoma mexicanum*

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SUMMARY

Troponin-T (Tn-T) expression in developing hearts of axolotls, *Ambystoma mexicanum*, was studied with the use of polyclonal and monoclonal antibodies and SDS-polyacrylamide gel electrophoresis. In precontractile hearts (stage 32/33), Tn-T was present in addition to myosin, actin and tropomyosin as evidenced by the presence of the protein bands in SDS-gels and by indirect immunofluorescence. Tn-T was localized in amorphous collections at the peripheries of these precontractile cells. Hearts of normal and cardiac lethal mutant siblings were also analysed for Tn-T expression. No detectable differences in the quantity of protein present was observed by gel electrophoresis or by indirect immuno-fluorescence. The most striking difference concerned the localization of the protein. In normal hearts, Tn-T was primarily localized in the I-bands of organized myofibrils; however, in mutant cells the Tn-T was localized in amorphous collections at the cell peripheries suggesting a reduction of myofibrillar organization in these cells. No differences were observed in the contractile protein composition between normal and mutant em-bryonic hearts by gel electrophoresis experiments.

INTRODUCTION

The temporal appearance of contractile proteins in differentiating myoblasts has received a great deal of attention in recent years. Results of various studies performed on skeletal myoblasts *in vitro* concur that a mechanism exists regulating the coordinate expression of both structural and regulatory contractile proteins (Devlin & Emerson, 1978, 1979; Allen, Stromer, Goll & Robson, 1979; Hastings & Emerson, 1982). Synthesis of these proteins is concurrent with myoblast commitment (irreversible withdrawal from the cell cycle) and fusion of myoblasts into myotubes, although these latter events are not required for the induction of contractile protein gene expression (Nguyen, Medford & Nadal-Ginard, 1983).

The differentiation of cardiac myocytes *in vivo* has not been studied as extensively. In differentiating cardiac myocytes, there is not a fusion of the cell

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membranes like that in skeletal muscle; instead, precardiac mesodermal cells individually begin to express the muscle phenotype. It is presently not clear what cellular events are associated with the initiation of contractile protein synthesis. It is also not known whether contractile proteins accumulate simultaneously as in the *in vitro* skeletal muscle system or, alternatively, in a sequential pattern. Recently, Wiens & Spooner (1983) reported that sarcomeric α-actin synthesis is initiated during stage 8 of chick heart development, a stage preceeding the ultrastructural appearance of thick and thin filament bundles at stage 9 and sarcomeric organization and onset of heart contractions at stage 10. However, whether the synthesis of other contractile proteins is also activated at this stage was not determined.

In the present study, monoclonal and polyclonal antibodies against troponin-T (Tn-T), the tropomyosin-binding subunit of the calcium-regulating troponin complex, were used to determine the presence and localization of Tn-T at various stages of developing hearts of *Ambystoma mexicanum*. These findings were correlated with the pattern of accumulation of other contractile proteins and with ultrastructural observations of myofibril assembly during development.

In the Mexican salamander, *Ambystoma mexicanum*, homozygosity for the cardiac lethal gene results in abnormal heart development. Mutant hearts display a heart beat but contract only in the conus and upper ventricular regions. Histological examinations of the embryonic hearts have revealed a reduced level of organization of myofibrils in mutant hearts when compared with normal hearts (Lemanski, 1973). In addition, it has been previously reported that the accumulation of tropomyosin is affected in the mutant hearts. Gel electrophoresis studies (Lemanski, 1976), radioimmunoassay studies (Moore & Lemanski, 1982), and immunofluorescent studies (Lemanski, Fuldner & Paulson, 1980) have reported a significant reduction of this protein in mutant hearts at all stages of development. Therefore, we undertook these studies to ascertain whether the presence of Tn-T might be similarly affected.

Our results reveal that Tn-T, in addition to other structural and regulatory contractile proteins, is present at a very early stage of normal heart development, just after the precardiac mesodermal sheets have migrated and fused to form the heart tube (stage 32). The Tn-T in normal hearts becomes increasingly confined to the I-bands of organized sarcomeres during development, but, for the most part, remains in amorphous collections at the cell peripheries in the mutant cells. The level of accumulation of Tn-T in normal and mutant hearts is similar; furthermore, no quantitative differences were noted for the other contractile proteins studied (myosin heavy chain, actin and tropomyosin).

MATERIALS AND METHODS

Procurement of tissues

Fertilized eggs were obtained from matings between adult axolotls maintained in our laboratory. The embryos were incubated at 20°C in 50% Holfreter's solution (Rugh, 1962) until needed for experiments. The staging system of Schreckenberg & Jacobson (1975) was used. Hearts were obtained from stage 32/33 normal embryos for electrophoresis and immunofluorescence studies of pre-heart-beat stages. Mutant embryos could first be distinguished from their normal siblings at stage 35 (one stage after onset of spontaneous heart contractions) by the absence of gill circulation and vigorous contractions throughout all regions of the heart. Stage-39 and -41 normal and mutant embryos were also used in the study.

Preparation of muscle proteins

Partially purified tropomyosin was prepared from pectoralis major muscle of adult chickens by the method of Bailey (1948). Troponin subunits were extracted from back muscle of rabbit (Greaser & Gergely, 1971) or from bovine heart muscle (Brekke & Greaser, 1976). Purified actin was obtained either from Sigma Chemical Co. or prepared by the method of Spudich & Watt (1971). Purified myosin and C-protein were prepared by the method of Offer, Moos & Starr (1973). Cardiac myofibrils used in gel electrophoresis and immuneblotting experiments were prepared from axolotl cardiac muscle by homogenizing the tissue in six volumes of 50 mM-KCl, 20 mM-Tris, pH 8·0, 4 mM-$MgCl_2$, 2 mM-EGTA, 5 mM-ß-mercaptoethanol, 0·1 mM-phenylmethyl sulphonyl fluoride (PMSF) in a Dounce homogenizer. The homogenates were centrifuged at 3600*g* for 10 minutes. The pellet was rehomogenized in the original homogenization buffer (plus 1% Triton X-100) with a Polytron, centrifuged and then subjected to one additional homogenization and centrifugation cycle.

Production and specificity of monoclonal antibody

A crude tropomyosin–troponin mixture (40–60% saturated ammonium sulphate fraction) from adult chicken pectoralis was used as the immunogen. Female BALB/c mice were injected intraperitoneally with 500μg of the immunogen containing complete Freund's adjuvant. Isolated spleen cells were fused with myeloma cell line NS-1 (non-secreting) according to the procedure of Kohler & Milstein (1975). Hybridoma cells were maintained in culture for two weeks in HAT medium (60μM-hypoxanthine, 0·5μM-amniopterin, 20μM-thymidine, 20% foetal calf serum, 5μM-ß-mercaptoethanol in Dulbecco's MEM). Supernatants from the hybridoma cultures were initially screened in a microtitre plate using an ELISA procedure. Tn-T and other purified myo-

fibrillar proteins were individually suspended in 0·05 M-carbonate–bicarbonate buffer, pH 9·6 and placed in the wells overnight. For the assay, the wells of the plates were incubated in 50μl of hybridoma culture supernatant followed by alkaline-phosphatase-labelled rabbit anti-mouse IgG (Miles Labs.). Extensive washing of the wells with N6 buffer (0·15 M-NaCl, 50 mM-Tris, pH 7·4, 0·25 % gelatin, 0·05 % NP 40, 0·02 mM-$ZnCl_2$, 1·0 mM-$MgCl_2$, 0·13 % NaN_3) preceeded and followed the alkaline-phosphatase-conjugated rabbit anti-mouse incubation. The fluorogenic substrate, α-napthol AS-MX phosphate (Sigma) was added to the wells and the fluorescence monitored under U.V. illumination after an appropriate incubation period. Positive hybrids were expanded, frozen, and subcloned twice by a limiting dilution method. Antibody produced by one clone, 7:H3:C9:D10 was immunoreactive only with rabbit skeletal muscle Tn-T and bovine cardiac Tn-T when tested against various purified myofibrillar proteins (myosin, actin, C-protein, tropomyosin, troponin-I, troponin-C, rabbit skeletal Tn-T and bovine cardiac Tn-T) and was chosen for this study. Hybridoma culture supernatants were used as the source of antibody in all experiments of this study. Specificity of the antibodies was further tested by indirect immunofluorescence.

Production and specificity of polyclonal antibodies

Polyclonal antibodies against rabbit skeletal Tn-T were raised in goat. For the present study, the antiserum was affinity purified according to the procedure of March, Parish & Cuatrecasas (1974). An ethanol fraction of the antiserum was passed through a column containing rabbit skeletal Tn-T conjugated to Sepharose 4B. Specifically bound antibodies were eluted with 1·0 M-propionic acid, 0·15 M-NaCl and then neutralized with an equal volume of 1·0 M-K_2HPO_4. Fractions were pooled and dialysed against 0·9% NaCl, 20 mM-phosphate, pH 7·4. Specificity of the antiserum was tested by Ouchterlony double immunodiffusion carried out in 1 % agarose dissolved in 0·4 M-KCl, 20 mM-phosphate, pH 7·4, in order to maintain the solubility of Tn-T. Tn-T (0·5–1·0 mg/ml) and affinity-purified antibody (1·0 mg/ml) were dialysed versus 0·4 M-KCl, 20 mM-phosphate, pH 7·4 before immunodiffusion. Specificity of antiserum was further demonstrated by the specific staining of I-band regions of myofibrils.

SDS-polyacrylamide gel electrophoresis (PAGE)

Embryonic axolotl hearts or adult hearts were dissected into 4 °C Holtfreter's solution containing 1·0 mM-PMSF. After an appropriate number had been collected, the hearts were pelleted, excess Holtfreter's solution was removed and the hearts were homogenized in a micro tissue grinder. An aliquot was removed to determine the protein concentration by the Bradford (1976) method using bovine serum albumin as the standard. The remainder of

the homogenate was placed in sample buffer consisting of 8·0 M-urea, 2·0 M-thiourea, 0·75 M-ß-mercaptoethanol, 0·0625 M-Tris, pH 6·8, and 3% SDS (Yates & Greaser, 1983), and heated for 2 min at 100 °C. The protein load found to achieve the best resolution was 1·5 μg/lane. One-dimensional SDS-discontinuous PAGE was performed in slab gels. The separating gel was 15% acrylamide containing a 200:1 ratio of acrylamide:bisacrylamide and 0·75 M-Tris, pH 8·8 as described by Thomas (1978). This procedure, which is a modification of the Laemmli (1970) procedure, was found to be useful in resolving low relative molecular mass proteins while still providing adequate separation of higher relative molecular mass proteins. The gels were stained with an ammonical silver method according to the procedure of Guilian, Moss & Greaser (1983) which was developed to provide a highly sensitive and reproducible staining of contractile proteins with a range of linearity between 2–70 ng/band. Contractile proteins in embryonic heart were identified on the basis of co-migration with purified standards or by co-migration with purified axolotl cardiac myofibrils. Tn-T in axolotl cardiac tissue was identified by immuneblotting as described below.

Immuneblotting method

Muscle homogenates from various developmental stages of embryonic axolotl heart and from adult heart of chick and axolotl were subjected to electrophoresis as described above. The proteins were then electrophoretically transferred to 0·45μm nitrocellulose sheets according to the method of Towbin, Staehelin & Gordon (1979). Nitrocellulose sheets were then reacted with hybridoma supernatant followed by incubation in alkaline-phosphatase-conjugated rabbit anti-mouse IgG. After extensive washing, the sheets were incubated with the enzyme substrate α-napthol AS-MX phosphate. The position of the reactive antigen was visualized as a fluorescent band with illumination under U.V. light (O'Connor & Ashman, 1982). Comparison with an adjacent nitrocellulose strip stained with amido black confirmed the position of the protein band on the original gel.

Indirect immunofluorescence microscopy

Glycerinated myofibrils to be used for immunofluorescence microscopy were prepared by stretching thin strips of chicken pectoralis muscle and immersing them in buffered glycerol solution (50% glycerol, 0·1 M-KCl, 2 mM-$MgCl_2$, 2 mM-EGTA in 10 mM-Tris-HCl, pH 6·8) at 4°C for 2 days. After the skeletal muscle strips were minced and homogenized, myofibrils were washed extensively in buffer by repeated cycles of centrifugation and resuspension. A drop was placed on albumin-coated slides and allowed to partially evaporate for 30 minutes. The myofibrils were incubated sequentially with primary antibody (hybridoma supernatant or affinity-purified polyclonal) and FITC-

conjugated secondary antibody for 30 min each. After thorough rinses, the slides were mounted in 2% n-propyl gallate in 50% glycerol, 0·9% NaCl, 20 mM-phosphate, pH 8·5 (Giloh & Sedat, 1982) and viewed with a Zeiss fluorescence microscope equipped with an epi-fluorescence attachment.

For frozen tissue sections, adult axolotl or embryonic hearts of various developmental stages were perfused with periodate–lysine–paraformaldehyde fixative (McLean & Nakane, 1974) at 4 °C for 30 min, followed by immersion in a fresh change of fixative for an additional 4 h. The tissues were washed for 2 h, each in 0·9 % NaCl, 20 mM-phosphate, pH 7·4 containing 7 % sucrose, 15 % sucrose and finally 25 % sucrose before embedding in O.C.T. compound (Tissue-Tek Labs) and freezing on to blocks. Frozen sections (1 μm) were prepared with a Sorvall FTS Frozen Thin Sectioning System (DuPont Instruments – Sorvall, Dupont Co.) and attached to albumin-coated slides prior to staining, mounting and viewing as described above. Paraffin embedment of whole fixed embryos were prepared according to the procedure of Sainte-Marie (1962) and 5 μm serial sections were cut and stained as outlined above.

RESULTS

Gel electrophoresis and immuneblotting experiments

Specificity and reactivity of the monoclonal antibody was tested further with immuneblotting. Purified bovine cardiac Tn-T was prepared, electrophoresed and transferred to nitrocellulose sheets (Fig. 1A). Incubation with the monoclonal antibody resulted in a positive reaction visualized as a fluorescent band (Fig. 1B). Whole adult axolotl heart homogenates were also tested in this manner (Figs 1C,D). Reaction with the antibody resulted in a reaction product localized at a protein band identified as Tn-T. The apparent relative molecular mass (M_r) of axolotl Tn-T was determined to be approximately 40000. A smaller relative molecular mass protein also reacted with the antibody in some preparations. This reaction is visible in the blot presented in Fig. 1D. Both the presence and relative intensity of a positive reaction for this lower relative molecular mass protein varied between sample preparations, which led us to believe this reactive protein is possibly a proteolytic fragment of Tn-T. Tn-T is known to be particularly susceptible to proteolysis (Dabrowska, Barylko, Nowak & Drabikowski, 1973; Ishura, Sugita, Suzuki & Imahori, 1979; Yosogama, Sanada & Katunuma, 1978). However, it is possible that the lower relative molecular mass protein is an isoform of Tn-T which exists in axolotl cardiac muscle. Chicken cardiac myofibrils tested in this manner exhibited a reaction to only one protein band which co-migrated with the axolotl Tn-T at M_r A 40000.

Silver-stained SDS gels of stage-41 normal and mutant hearts (Figs. 1E,G) revealed that a band co-migrating with adult Tn-T was present in both of the

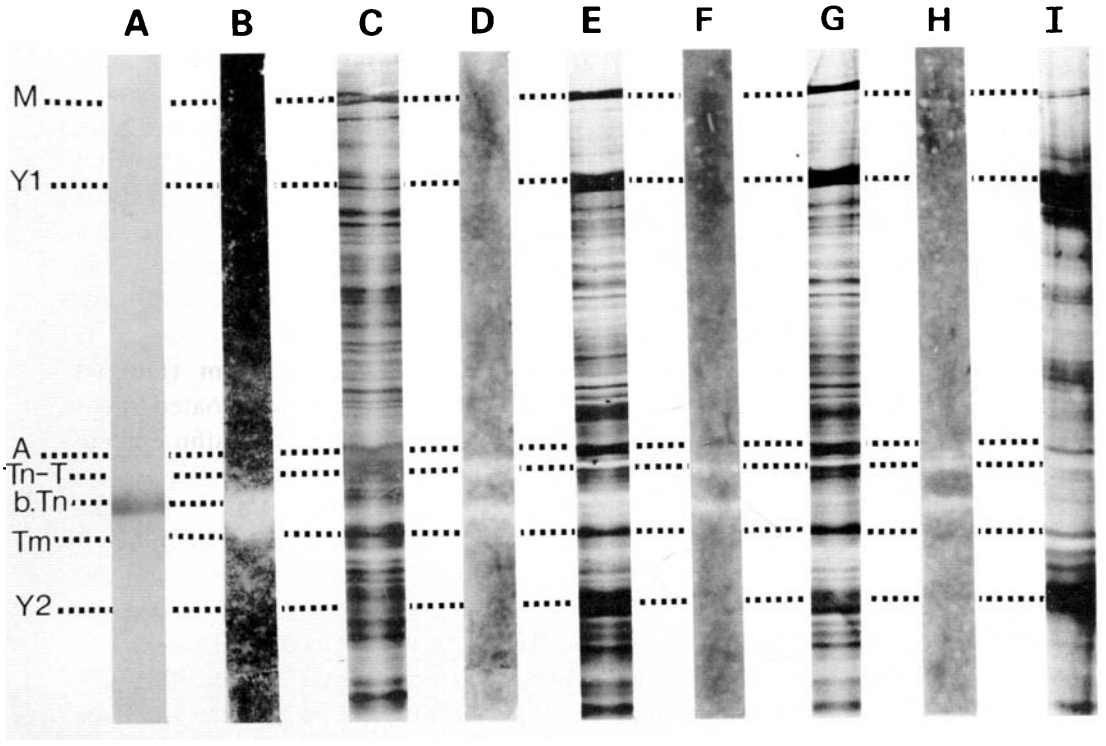


Fig. 1. Silver-stained SDS-PAGE and immuneblotting experiments with monoclonal antibody.

SDS-PAGE was performed and gels were silver-stained as described in *Materials and Methods*. The positions of the marked contractile proteins were determined by co-migration with adult axolotl cardiac myofibril standards. Proteins were electrophoretically transferred to nitrocellulose, reacted first with the monoclonal antibody, subsequently with a secondary antibody conjugated to alkaline phosphatase, and finally reacted with the fluorescent substrate.

(A) Silver-stained SDS-PAGE lane of bovine cardiac muscle Tn-T.

(B) Nitrocellulose transfer of (A) reacted with the monoclonal antibody. The antibody reacted with the bovine cardiac Tn-T.

(C) SDS-PAGE of adult axolotl heart homogenate (silver-stained).

(D) Nitrocellulose transfer of (C) reacted with monoclonal. Only Tn-T and a presumed smaller relative molecular mass proteolytic fragment reacted with the antibody.

(E) Silver-stained SDS-PAGE lane of stage-41 normal hearts.

(F) Nitrocellulose transfer of (E) reacted with monoclonal. Only Tn-T and a smaller relative molecular mass protein (same position as adult) reacted with the antibody. The position of embryonic Tn-T is identical to the adult protein.

(G) SDS-PAGE lane of stage-41 mutant hearts (silver-stained).

(H) Nitrocellulose transfer of (G) stained with monoclonal. The results are identical to the normal hearts of this stage.

(I) SDS-PAGE of precontractile stage 32/33 embryonic hearts (silver-stained).

M, myosin heavy chain; A, actin; Tn-T, troponin-T; b. Tn, bovine cardiac Tn-T; Tm, tropmyosin, Y1, high relative molecular mass yolk protein; Y2, low relative molecular mass yolk protein.

extracts. In order to confirm the identity of this protein band and to determine monoclonal antibody specificity in the embryonic heart tissue, 300 normal and mutant hearts of stage-41 embryos were excised, homogenized and subjected to electrophoresis. Results of the immuneblotting experiments revealed that the antibody reacted with this protein band in both normal and mutant heart extracts (Figs 1F,H). A positive reaction to a lower relative molecular mass protein was also seen in some preparations. This band had the same position as the lower relative molecular mass band observed in the adult axolotl cardiac muscle homogenate. However, contrary to earlier reports (Lemanski, 1976), we did not observe any differences in the amounts of the other contractile proteins (actin, myosin and tropomyosin) in viewing the silver-stained gels. In addition, a recent study performed on normal and mutant embryonic axolotl hearts at both stages 38 and 41 revealed no discernable differences in the relative quantities of contractile proteins between normal and mutant hearts as evidenced by scanning densitometry of Coomassie-blue-stained gels or by quantitation of contractile protein bands from gels of *in vitro* labelled embryonic heart extracts (Dr. John Armstrong, personal communication).

Examination of our gels of tubular, precontractile embryonic hearts (stage 32/33) also revealed a band which co-migrated with the Tn-T from stage-41 normal and mutant embryonic hearts. Myosin, actin and tropomyosin, as well as Tn-T, were present in homogenates of precontractile hearts (Fig. 1I). Identification of these contractile proteins was based upon co-migration with contractile protein standards from adult axolotl cardiac myofibrils. Since the one-dimensional SDS-polyacrylamide gels do not discriminate between the non-muscle and muscle isoforms of actin and myosin, electrophoresis of non-muscle tissue (liver) was performed. The actin and myosin levels in the pre-heart-beat extracts were higher than those in the non-muscle tissue. No bands were observed to migrate to the same position as embryonic cardiac tropomyosin or Tn-T in the non-muscle extracts. Thus, the silver-stained SDS gels of the pre-heart-beat stage 32/33 normal heart reveal that Tn-T has already accumulated at this stage in addition to other regulatory (tropomyosin) and structural (myosin, actin) contractile proteins.

Indirect immunofluorescence microscopy

Specificity of the affinity-purified polyclonal antiserum was tested using Ouchterlony double immunodiffusion. The antibody formed a precipitin line only against rabbit skeletal Tn-T of the myofibrillar proteins tested (Fig. 2). Specificity of the antibody was further tested by indirect immunofluorescence with glycerinated, stretched skeletal muscle myofibrils. The I-bands of myofibrils reacted with the antibody, indicating the Tn-T located on thin filaments was reactive. There was no staining of myofibrils with antibody which had first been absorbed with excess Tn-T. Controls performed with the second antibody

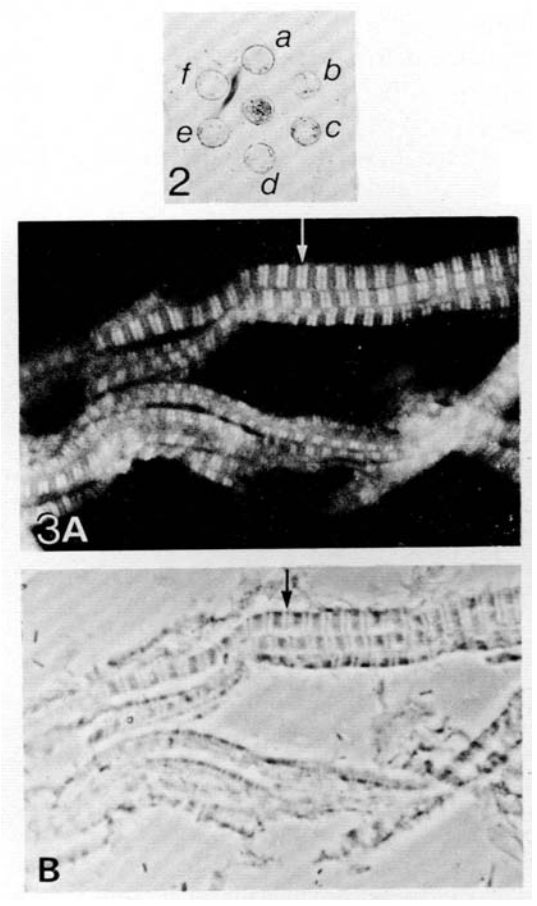


Fig. 2 Specificity of polyclonal antibody.
Ouchterlony double immunodiffusion plate stained with Coomassie-Blue. Centre well contained affinity-purified anti-Tn-T. The outer wells contained: *a*, Tn-C; *b*, Tn-1; *c*, skeletal muscle tropomyosin; *d*, bovine cardiac Tn-T; *e*, actin; *f*, rabbit skeletal muscle Tn-T. The antibody formed a precipitin line against rabbit skeletal Tn-T only. No reaction was evident with bovine cardiac Tn-T in this preparation.

Fig. 3A,B Immunofluorescence using polyclonal antibody
Corresponding fluorescence (A) and phase-contrast (B) light micrographs of fixed adult axolotl cardiac tissue stained with polyclonal antibody. The antibody specifically stains the I-bands of the myofibrils. Arrows indicate corresponding areas. (× 800)

alone revealed no fluorescence. The antibody also reacted with the I-bands of adult axolotl heart on frozen sections, although more weakly than with the skeletal myofibrils (Figs 3A,B).

Immunofluorescence microscopy performed with the monoclonal antibody of glycerinated, stretched chicken or axolotl skeletal myofibrils revealed a

specific I-band staining pattern. Prior absorption of hybridoma supernatant with either purified bovine cardiac Tn-T or rabbit skeletal Tn-T resulted in an elimination of the specific I-band staining pattern in myofibrils. In addition, immunofluorescence staining of frozen sections of fixed adult axolotl heart also revealed a specific I-band staining pattern with the monoclonal antibody (Figs. 4A,B).

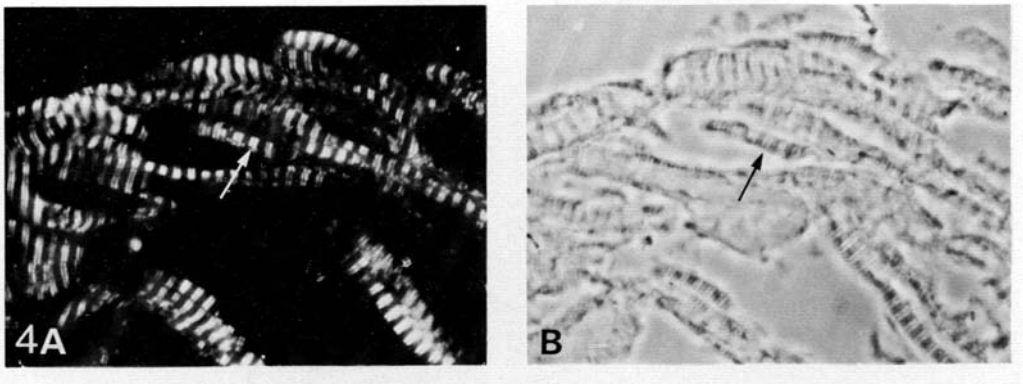


Fig 4A,B Immunofluorescence using monoclonal antibody

Corresponding fluorescence (A) and phase-contrast (B) light micrographs of fixed adult axolotl cardiac tissue stained with monoclonal antibody. The antibody specifically reacted with the I-bands of the myofibrils. Arrows illustrate corresponding areas. (× 800)

Fig. 5.(A) Pre-heart-beat stage 32/33 normal embryonic heart stained with monoclonal antibody. No detectable fluorescence is evident. (× 800)

(B) Pre-heart-beat stage 32/33 normal embryonic heart stained with polyclonal antibody. No sarcomeric pattern of staining is seen; however, staining is observed in amorphous areas at the cell peripheries. (× 800)

(C) Stage-35 normal embryonic heart stained with monoclonal antibody. A few organized I-bands are evident; however, the majority of staining is in amorphous areas at the cell peripheries. (× 800)

(D) Stage-35 mutant embryonic heart stained with monoclonal antibody. The staining is evident in amorphous areas at the cell peripheries. No sarcomeric pattern of staining is observed at this stage. (× 800)

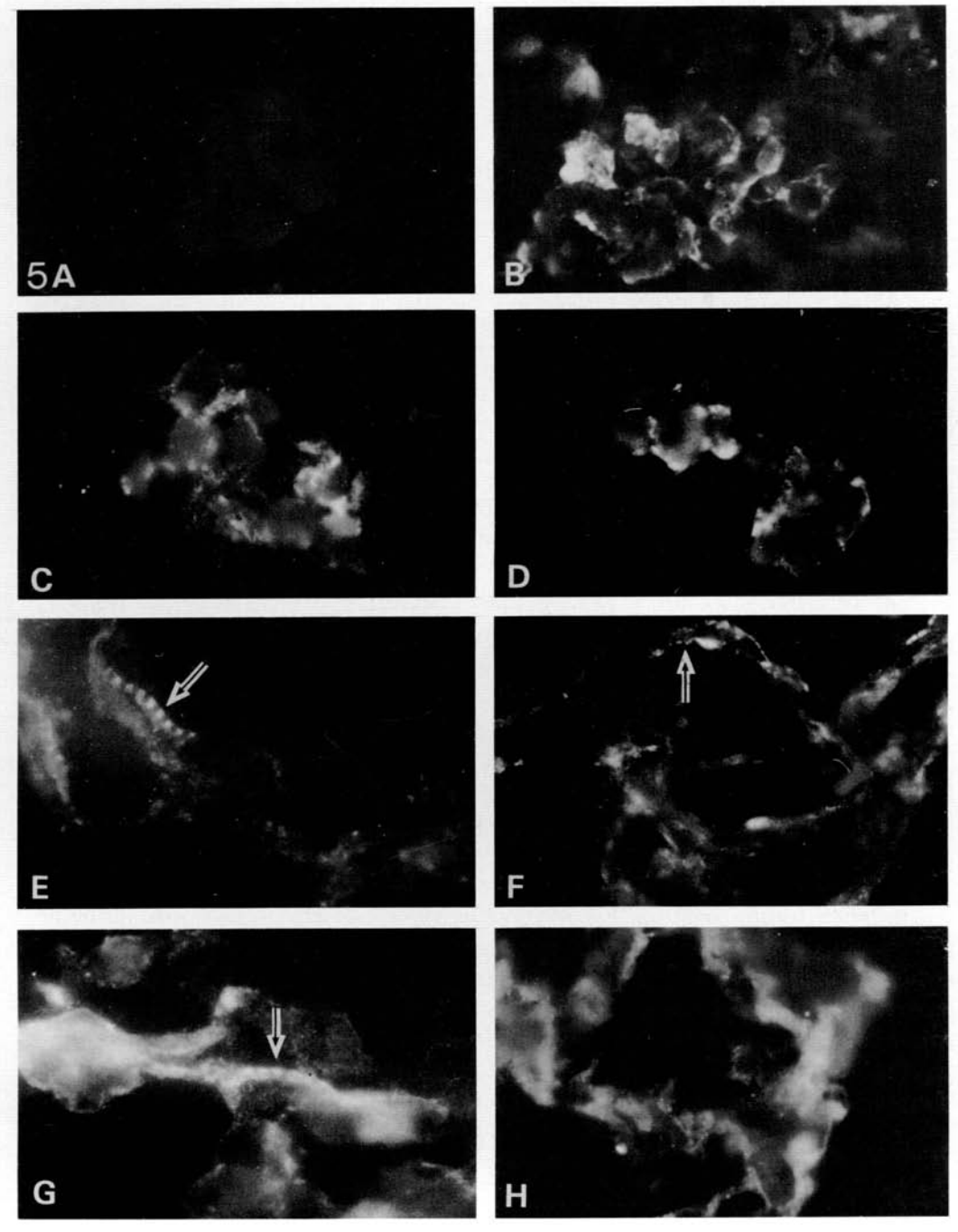
(E) Stage-41 normal embryonic heart stained with monoclonal antibody. At this stage of development, the majority of the staining is confined to the I-bands of organized myofibrils (arrow), although staining is sometimes viewed in amorphous areas at cell peripheries where myofibrils are likely to organize. (× 800)

(F) Stage-41 mutant embryonic heart stained with monoclonal antibody. The amount of staining at this stage is similar to normal hearts; however, extensive myofibrillar staining is not seen. Most of the staining is exhibited in amorphous collections; occasionally a few sarcomeres are seen (arrow). (× 800)

(G) Stage-41 normal embryonic heart stained with polyclonal antibody. The pattern of staining with the polyclonal antibody is similar to that of the monoclonal; the staining is primarily seen in I-bands of organized myofibrils (arrow). (× 800)

(H) Stage-41 mutant embryonic heart stained with polyclonal antibody. The quantity of staining is similar to the normal hearts of this stage. A sarcomeric pattern of staining is seldom seen; the majority of the staining is observed in collections at the cell peripheries. (× 800)

Immunofluorescence studies were performed on frozen sections of embryonic hearts to determine the presence and cellular distribution of Tn-T during heart myogenesis. Staining of normal precontractile tubular heart



(stage 32/33) with the monoclonal antibody revealed no detectable fluorescent reaction (Fig. 5A). The affinity-purified polyclonal antibody, however, did react with cells of this same stage (Fig. 5B). The staining was present at the peripheries of the cells as diffusely stained collections; no sarcomeric pattern of staining was ever observed in any of the cells. Previous electron microscopic studies (Woodroofe & Lemanski, 1981) of this stage of heart development have revealed that the cardiac myocytes contain only a few thick and thin filaments sporadically arranged within amorphous proteinaceous collections of the cell peripheries. The pattern of staining observed in this study for Tn-T is similar to that observed for other contractile proteins (myosin, α-actinin and tropomyosin) previously studied at this stage of development (Lemanski *et al.*, 1980).

At stage 35, one stage of development after the onset of spontaneous contractions, the monoclonal antibody showed reactivity with both normal and mutant hearts (Figs. 5C, 5D). The localization was primarily observed as diffuse collections at the cell peripheries. A few organized sarcomeres were observed in normal hearts; however, no sarcomeres were observed in the mutants. Cardiac myocytes of stage 41 (approximately two weeks after onset of contractions) normal and mutant hearts were the last stage to be examined since the mutant embryos were not viable after this time. At this stage of development, the staining intensity with the monoclonal antibody had increased significantly in both normal and mutant cells. Normal cells exhibited the majority of the staining in I-bands of organized myofibrils, although a small proportion of the staining was localized in amorphous areas at cell peripheries (Fig. 5E). Mutant hearts did not display staining of well-organized myofibrils; instead, the majority of the staining was localized in diffuse collections. Occasionally, a staining pattern was observed which indicated a few sarcomeres had organized (Fig. 5F, arrow). These sarcomeres are most likely present in the conus region of the heart where contractions are observed in the mutant embryos. An intermediate stage of development (stage 39, not shown) was also examined and the pattern of staining was similar to that observed at stage 41. Staining with the polyclonal antibody of stage-41 hearts revealed that both the pattern of staining and relative intensity of fluorescence between normal and mutant hearts was similar to the results seen with the monoclonal antibody (Figs. 5G,5H).

Due to the difficulty of obtaining cryosections through the entire heart region, paraffin embedment of embryos was performed and serial sections were stained in order to compare staining patterns in different regions of the heart as well as to obtain a more complete assessment of staining patterns. Comparison of stained paraffin sections correlated well with the results of the frozen sections, revealing no significant differences in quantity of staining between normal and mutant hearts of similar regions of the heart (results not shown).

Thus, it appears Tn-T is present in the precontractile normal hearts just after the mesodermal sheets have fused to form the heart tube and prior to the formation of sarcomeres. This is evidenced by positive indirect immunofluorescence staining with the polyclonal antibody and the presence of the protein band in SDS-gels. There are no obvious quantitative differences in staining between normal and mutant siblings at all stages studied although clearly the mutant myocytes display a reduced level of myofibrillar organization.

DISCUSSION

The data reported in this study demonstrate that Tn-T is present in normal precontractile cardiac myocytes in addition to other contractile proteins (tropomyosin, actin and myosin) prior to the assembly of sarcomeres. As development progresses, normal and mutant cardiomyocytes accumulate Tn-T. The protein is primarily localized in the I-bands of organized sarcomeres in normal hearts but remains, for the most part, in amorphous collections at the cell peripheries in mutant hearts. We found no differences in the composition of contractile proteins between normal and mutant hearts in silver-stained SDS gels.

The existence of isoforms for various myofibrillar proteins specific for muscle tissue type is well documented in cardiac and skeletal muscle (Hoh, Yeah, Thomas & Higginbottom, 1979; Whalen *et al.*, 1982). Recently, investigators have generated monoclonal antibodies specific for particular isoforms and used these as probes to determine their developmental expression (Crow, Olson & Stockdale, 1983) and also to demonstrate definitive embryonic isoforms *in vivo* (Bader, Masaki & Fischman, 1982).

Polymorphic forms of the two major calcium regulatory proteins (tropomyosin and troponin) are known to exist in skeletal and cardiac muscle (Roy, Streter & Sarkar, 1979; Dhoot & Perry, 1979; Dhoot, Frearson & Perry, 1979; Matsuda, Obinata & Shimada, 1981; Montarras, Fiszman & Gros, 1981). Using polyclonal antibodies which had been affinity-purified against various cardiac and skeletal muscle troponin subunit isoforms, Toyota & Shimada (1981), investigated the appearance of these subunits in developing chick cardiac muscle. No evidence of troponin subunit expression was found in chick embryonic hearts at stage 8 with indirect immunofluorescence. At stage 10 (onset of heart contractions), the embryonic heart tissue stained positively with all three polyclonal antisera against the cardiac troponin subunits and also with an antisera against the skeletal muscle form of Tn-I. Therefore, these workers provided some evidence that early developing chick cardiac tissue may synthesize both cardiac and skeletal isoforms or that a distinct embryonic isoform may exist in developing cardiac muscle which is capable of cross reacting with the skeletal muscle isoform as well. This is not surprising since it has been demonstrated with monoclonal antibodies which were specific for

either the fast or slow muscle isoforms of myosin light chains, that skeletal muscle myocytes *in vivo* initially express both slow and fast isoforms of myosin light chains, but later in development switch their synthetic patterns to include only those isoforms characteristic of the adult phenotype (Crow, Olson & Stockdale 1983). The monoclonal and polyclonal antibodies used in this study did not discriminate between the embryonic and the adult Tn-T. In addition, the embryonic Tn-T identified in our immuneblotting experiments migrated to the same position as the adult Tn-T in one-dimensional SDS-polyacrylamide gels. Therefore, we were not able to provide any evidence for the existence of a distinct embryonic isoform of Tn-T in the cardiac tissue of axolotl.

Our results are in disagreement with those of Toyota & Shimada (1981) concerning the presence of Tn-T in pre-heart-beat stages of development. The results of our gel electrophoresis experiments and indirect immunofluorescence with the polyclonal antibody indicate that the protein is present just after the heart tube has formed, a developmental stage comparable to the pre-heart-beat stage studied by Toyota & Shimada in chick heart in which they found no immunofluorescence with polyclonal antibodies. However, other investigators (Wiens & Spooner, 1983) have determined that onset of α-actin synthesis begins at stage 8 of chick heart development although other contractile proteins were not studied. The failure of our monoclonal antibody to recognize Tn-T in precontractile myocytes raises some interesting possibilities. Perhaps the antigen is simply masked and therefore unavailable to bind to the antibody at this particular stage of development or is present in too low a quantity to be detectable by a monoclonal antibody. Alternatively, the portion of the Tn-T molecule the antibody recognizes might be altered at this precontractile stage of development via binding to another molecule or by a post-translational modification and is therefore unrecognizable.

Nonetheless, our results indicate that Tn-T has accumulated at a very early stage of heart development just after heart tube formation, in addition to at least one other regulatory (tropomyosin) and two other structural (myosin, actin) contractile proteins as evidenced by gel electrophoresis and immunofluorescence data prior to the formation of sarcomeres. However, since this report does not directly examine the initiation of synthesis of these proteins, the possibility that the synthetic profile is consecutive rather than simultaneous cannot be ruled out.

In conclusion, the results of immunofluorescence and immuneblotting data on normal and mutant siblings of post-heart-beat stages indicate that the accumulation of Tn-T is not affected in the mutant hearts. Our gel electrophoresis results do not indicate a reduction of myosin or tropomyosin in the mutant hearts as has been previously reported by Lemanski (1976). It is possible that the tropomyosin in mutant hearts might be antigenically altered resulting in the inability of the antibodies to detect this protein in previous RIA and immunofluorescence studies (Moore & Lemanski, 1982; Lemanski *et al.*,

1980), even though this protein migrates to the same position in SDS-PAGE. Further studies are underway to answer this question and to understand why myofibril assembly is aberrant in the mutant cells which contain normal levels of the contractile proteins. In studying the cardiac mutant system, the regulatory mechanisms governing the proper assembly of sarcomeres from its protein components may be identified.

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