Expression of a Novel Protein Associated with Heart Development in the Mexican Axolotl

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Summary

Recessive mutant gene *c* for "cardiac nonfunction" in the Mexican axolotl, *Ambystoma mexicanum*, results in a failure of affected embryos to develop contracting hearts. Mutant embryos survive approximately four weeks after fertilization, but eventually die from a lack of circulation. Morphological studies show that mutant hearts lack organized sarcomeric myofibrils. This abnormality can be corrected by co-culturing early mutant hearts with normal anterior endoderm/mesoderm tissues, by culturing them in a medium "conditioned" by this normal tissue, or by RNA isolated from normal endoderm/mesoderm. Additionally, RNA isolated from normal anterior endoderm/mesoderm conditioned medium corrects the mutant hearts in a dose-dependent manner. A cDNA library is constructed using this RNA. On the basis of sequence analyses on this cDNA library, it was estimated that 56% of the total RNA present in the conditioned medium is rRNA, while 44% is non-ribosomal RNA. One of the non-ribosomal RNAs that showed no significant homology with other known sequences in the Genebank was examined further. An RT-PCR analysis showed that this RNA (designated "N1") is expressed in juvenile skeletal muscle, brain, and heart in significant amounts, less in the lung, and not at all in the liver tissue. Affinity-purified polyclonal anti-peptide antibodies were produced against the most antigenic portion of the polypeptide which was deduced from this RNA. Western blot analyses of adult heart homogenates, using these antibodies, showed a specific doublet staining at 67 kDa and 65 kDa. These doublets were purified and analyzed for their amino acid composition, which showed that both bands most likely belong to the same protein. The N1 protein was further investigated to determine its localization in normal isolated hearts at embryonic stages 35, 38, and 41 and on cross-sections through the heart regions of whole normal embryos at stages 16, 33-34, 37-38, and 41-42 using immunohistochemical techniques and confocal microscopy. In addition, mutant embryos at stage 37-38 were studied for the presence and distribution of the N1 protein on cross-sections through their heart regions. The N1- protein staining was significantly reduced in mutant hearts when compared to normal.

Introduction

A naturally occurring recessive mutation in an imported stock of Mexican salamanders, *Ambystoma mexicanum*, results in an absence of normal heart contractions in affected animals (Humphrey, 1972). The gene that causes this mutation was designated *c* for "cardiac lethal." At stage 34, a week after fertilization, the normal hearts first start to contract. This is the earliest stage at which the mutant embryos (*c*/*c*) with non-beating hearts can be distinguished from their normal siblings (+/+ or +/*c*) in heterozygous spawnings (+/*c* x +/*c*). The mutant embryos develop for approximately three weeks after fertilization but eventually die from a lack of circulation (stage 41-42).

Electron microscopy reveals that normal cardiac cells contain well-organized sarcomeric myofibrils at stages 34 through 41. Mutant heart cells, however, contain sparsely scattered thin (6 nm) and thick (15 nm) filaments and an occasional Z-body, but sarcomeres generally do not form (Lemanski, 1973; Lemanski et al., 1976).

Immunohistochemical studies confirm that mutant ventricular myocardial cells contain most of the major myofibrillar proteins examined including myosin, actin, alpha-actinin, and the troponins; however, many of these proteins accumulate in the form of amorphous collections or randomly arranged filaments rather than as components 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; Erginel-Unaltuna et al.. 1994a). One of the major myofibrillar proteins, tropomyosin, appears to be significantly reduced in quantity in mutant hearts when compared to normal (Lemanski, 1979; Lemanski et al., 1980). Quantification of alpha-tropomyosin in

Figure 1. Nucleotide sequence of the N1 cDNA. The sequence of the primer pair used for RT-PCR analysis is indicated as Primer-1 (+) and Primer-2 (-). The sequence of the oligonucleotide used as a detector is indicated by Primer-1 (+) and Primer-2 (-). Bottom: Deduced amino acid sequence of the N1 cDNA. The peptide used for raising anti-N1 antibody is underlined.

NUCLEOTIDE SEQUENCE OF N1-cDNA

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                                                          Primer-1(+)
  1  GCAGGGGAAT TTCACAGACC ATTTCTACAG CCCCCATGCC TCTATGGCAT

 51  CTGGAGGTAT CAATGTCCAA AACTCCAATG TAGCACTGAT TCTAGATGAT

                                                    Detector
101  TTGATGGGGT CTCGACGCCC AGAAGCAGCG ACACCGCATC ACTGCATACA

151  CAAGCAAGAA GATTCCCGGG TGGGAAGATG TAAATGCTCT CTCTACAATG

                                                  Primer-2(-)
201  ATCTACCCCC ACAGTTACAG TTCTCCCAAG GCCATCTAAC CAATATGGAA

251  GCCAAAGCTT GCTAATCTCC ACAGTCATCG ATACACATCA CAAGGCATAC

301  CAGGCTCAGG GTAAACAACC AGATTTCGTC TCTCGGATGA GGGAGTCACA
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DEDUCED AMINO-ACID SEQUENCE OF THE N1-PROTEIN

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  1  QGNFTDHFYS PHASMASGGI NVQNSNVALI LDDLMGSRRP EAATPHHCIH

 51  KQEDSRVGRC KCSLYNDLPP QLQFSQGHLT NMEAKAC*
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normal and mutant axolotl hearts by using solid-phase radioimmunoassay confirms that tropomyosin is deficient in the mutant hearts (Moore and Lemanski, 1982). Recent radioactive amino-acid incorporation experiments have shown a reduced synthesis of this protein in mutant hearts (Erginel-Unaltuna et al., 1994). ^{35}S-Methionine incorporation studies for various contractile/cytoskeletal proteins in normal and mutant embryonic hearts suggest that there is an overall reduction in protein synthesis in the mutant hearts when compared to normals.

Humphrey (1972) transplanted mutant heart primordia into the heart regions of normal embryos at the early tailbud stages 29-30 and observed that the mutant hearts beat in this normal environment. When he transplanted the normal heart primordia into mutant hosts, no heartbeat was observed. Additionally, Humphrey (1972) parabiotically linked normal and mutant embryos at stages 24-25. The mutant hearts still failed to beat, and the normal embryos were not adversely affected by this procedure. These experiments suggested that there was something in the region of the heart that was abnormal in the mutants that did not permit the heart to differentiate into a contracting muscle. Moreover, it appeared not to be a general inhibitor that was carried in the blood circulation.

In salamanders, anterior endoderm is known as a potent inducer of heart differentiation (Jacobson and Duncan, 1968; Fullilove, 1970). To test the hypothesis that the deficiency in the mutant hearts might result from a deficiency of some normal induction process, Lemanski et al. (1979) co-cultured stage 29-30 normal embryonic anterior endoderm with mutant hearts at stage 34, when the mutants could first be distinguished from their normal siblings. Interestingly, the mutant hearts that were co-cultured with normal anterior endoderm for 48 hours acquired vigorous contractions, and electron microscopy revealed that the corrected mutant hearts contained well-organized sarcomeric myofibrils of normal morphology. In later studies, the mutant hearts were also rescued by a medium, which was "conditioned" by the anterior endoderm (Davis and Lemanski, 1987). In further studies to characterize the active components of the conditioned medium, Davis and Lemanski (1987) treated the media with several enzymes, including neuraminidase, trypsin, and ribonuclease. The ability of the conditioned medium to form contractile mutant hearts was abolished totally after the

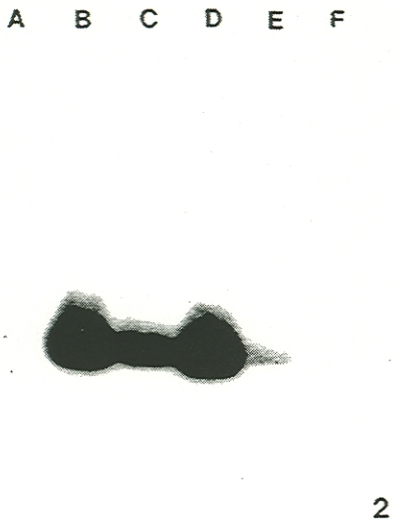


Figure 2. RT-PCR for detection of the *N1* gene in adult tissues. Reverse transcription-PCR amplification using primer pairs specifically designed for the *N1* gene shows that the gene is transcribed mostly in brain, skeletal muscle, and heart. Slight transcription is observed in lungs but not in liver. Lane A: Control, Lane B: Brain, Lane C: Heart, Lane D: Skeletal Muscle, Lane E: Lung, Lane F: Liver.

ribonuclease treatment, while the other enzymes tested had no effect. These initial studies indicated that the active material in the mutant heart rescue was RNA. This was corroborated in experiments wherein mutant hearts were corrected in organ cultures containing RNA isolated from normal anterior endoderm (Davis and Lemanski, 1987). In more recent studies, RNA was purified from normal endoderm conditioned medium, and this RNA has been shown to be effective in a dose-dependant manner (LaFrance et al., 1994). The number of corrected hearts increased in direct proportion to the quantity of RNA added to the culture medium.

A cDNA library was constructed from the conditioned medium RNA known to be active in rescuing the mutant hearts (Erginel-Unaltuna et al., 1994). Randomly selected clones from the library were partially sequenced and checked for their homology with other known sequences in the Genebank. Fifty-six percent of the RNA present in the conditioned medium was found to be ribosomal RNA while 44% consisted of other types of RNA. Among the non-ribosomal RNAs, 65% showed long open reading frames ranging from 54 to 261 bases, and most of them were not homologous with other known sequences.

The non-ribosomal RNA, with the longest open reading frame but not homologous with any other known sequences in the database, was examined further. RT-PCR studies showed that this RNA is present in significant amounts in brain, skeletal muscle, and heart, less abundant in the lungs, and absent from the liver. The amino-acid sequence was deduced from the nucleotide sequence to synthesize an oligopeptide of the most antigenic portion of the protein. An anti-peptide antibody was prepared against this oligopeptide. This new protein (designated N1 protein) was examined for its location in the normal whole heart tissue (stages 35, 38, and 41) and its tissue distribution on cross-sections through the heart regions of normal embryos at stages 16, 33-34, 37-38, and 41-42 using immunohistochemical techniques. In addition, mutant embryos at stage 37-38 were studied for the presence and distribution of the N1 protein on cross-sections through their heart regions.

Materials and Methods

Procurement and Maintenance of Axolotls. Normal and cardiac mutant axolotl embryos were obtained from matings between homozygous normal (+/+ x +/+) or heterozygous (+/*c* x +/*c*) animals from our colony at the SUNY Health Science Center at Syracuse or from the Axolotl Colony at Indiana University. The animals were maintained in aquaria in diluted Holtfreter's solution, fed commercial salmon pellets, supplemented occasionally with raw beef liver and live earth worms. The embryos were staged according to the methods of Bordzilovskaya et al. (1989).

Isolation of RNA from endoderm/mesoderm conditioned medium. The method of isolation of RNA was based on a rapid one-step procedure of Chomoczynski and Sacchi (1987) using a total RNA isolation kit purchased from Promega.

Electrophoretic separation of RNA. Isolated RNA from conditioned medium or from tissues was denatured and subjected to electrophoresis on 0.9-1.2% agarose gels containing formalde-

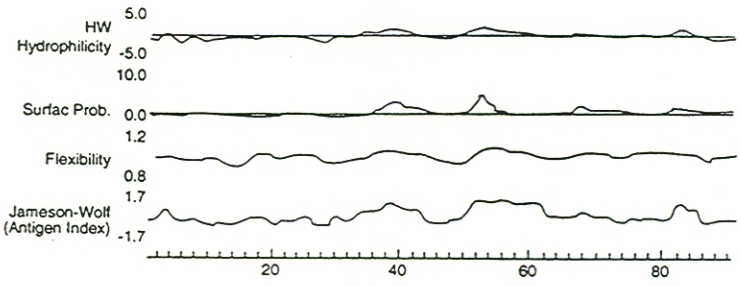


Figure 3. Hydropathicity, secondary structure, and antigenic index of N1 protein using plot-structure and GCG sequence analysis software package. The most antigenic site of N1 peptide is underlined. The method for calculating the antigenic index is described by Jameson and Wolf (1988).

hyde with appropriate molecular weight markers (RNA ladder, BRL or ribosomal RNA, BRL).

Construction of cDNA library in expression vector pcDNA II. The cDNA library was constructed from 1 µg of RNA isolated from normal endoderm-mesoderm conditioned medium which tested positively using the mutant heart bioassay. A librarian II kit (Invitrogen), which utilizes a prokaryotic expression vector, pcDNA II, was used to make the library following the instructions of the manufacturer. The average insert size was determined by PCR amplification of the inserts from several randomly selected clones. For PCR amplification, the universal primer [5'-GTAAAACGACGGCCAGT-3'] and M13 reverse primer [5'-CAGGAAACAGCTATGAC-3'] were used. The PCR amplified DNA was subjected to agarose gel electrophoresis with an appropriate marker DNA (Erginel-Unaltuna et al., 1994b).

DNA sequencing. Denatured double-stranded DNA was used for sequencing using the DNA Sequenase™ Kit version 2.0 (USB), which employs the dideoxy chain termination method (Sanger and Coulson, 1977). Reactions were electrophoresed on a 6% sequagel (BRL) at 60 watts for 2 or 4 hours. The gels were vacuum dried and exposed to Kodak X-omatic film overnight at room temperature.

Polymerase chain reaction (PCR). Inserts of the clones of the cDNA library were amplified using the GeneAmpR PCR Reagent Kit with AmplitagR DNA Polymerase purchased from Perkin Elmer Cetus. Specific primers designed for each of the DNA and Taq-polymerases were utilized according to the manufacturers instructions for the PCR amplification. Briefly, DNA, primers, and dNTPs were mixed with an appropriate buffer and amplified 45 or 60 cycles (Erginel-Unaltuna et al., 1994b).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from different tissues (brain, heart, skeletal muscle, lung, and liver) of juvenile axolotls was treated with 30 µl of RQ1 DNase (Promega, Madison, WI) at 37°C for 1 hour in the presence of 10 mM $MgCl_2$ and 40 µl of RNasin (Promega). The samples were then mixed with equal volumes of a 1:1 phenol/chloroform. After centrifugation, the top layer containing the aqueous layer was removed and precipitated overnight

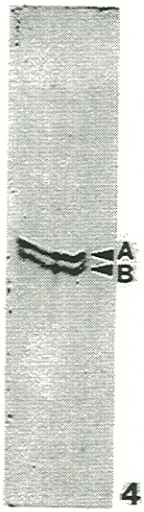


Figure 4. Western blot of whole heart homogenates with anti-N1 antibody. Antibody specifically stains a doublet of 67 kDa (Band A) and 65 kDa (Band B).

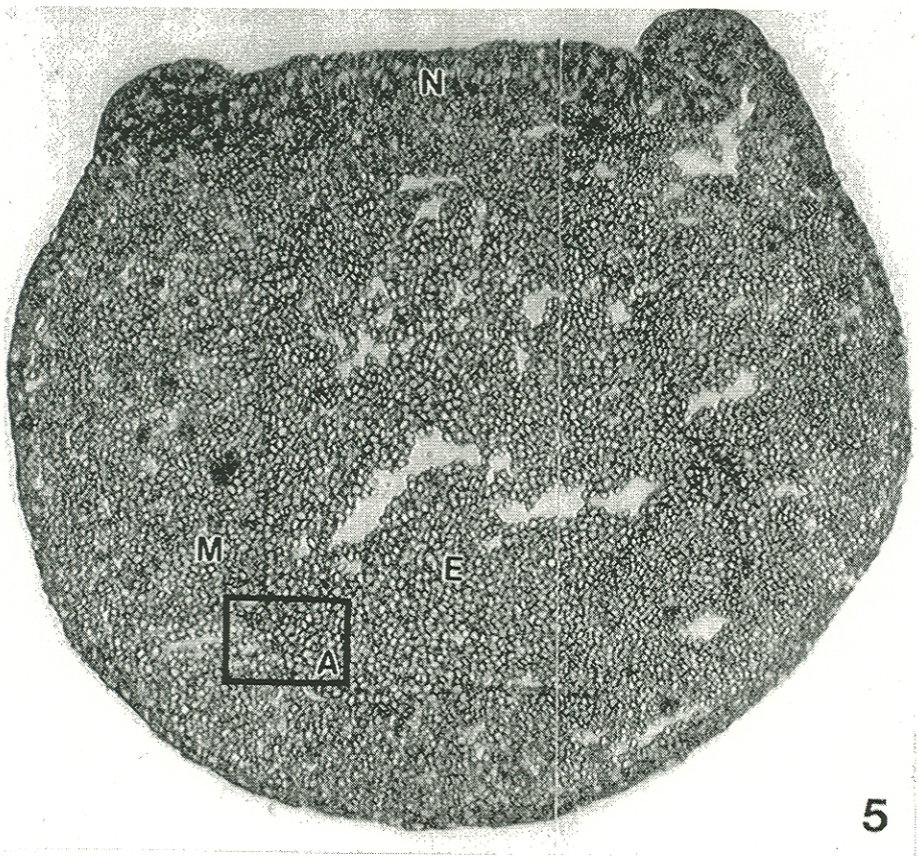


Figure 5. Overview of a cross-section of a normal stage 16 (+/+) embryo. This is a neurula stage embryo at which the precardiac mesoderm has already been induced to differentiate into heart tissue (N=neural plate, E=endoderm, M=mesoderm). Area A is enlarged in Figure 6.

in 0.3 M sodium acetate and 95% ethanol. Prior to RT-PCR, the samples were microfuged (12,000 g) for at least 45 minutes at 4°C.

Based on the partial sequence of N1 (Figure 1) we designed two sets of primers and one detector, a probe which flanks the two primers. Following are the sequences of
primer N-1(+):

5'-CTCTATGGCATCTGGAGGTATCAATGT-3';
primer N-2(-):

5'-AAGCTTTGGCTTCCATATTGGTTAGAT-3';
and detector/probe N-3(+):

5'-GACACCGCATCACTGCATACACAAGCAAGAAGAT-3'.

The oligonucleotides were custom made by American Synthesis, Inc. (Pleaston, CA).

RT-PCR Assay. Total RNA previously treated with DNase was first reverse-transcribed into cDNA and then amplified with specific primers using the DNA polymerase *rTth* (Roche Molecular Systems, Alameda, CA) as instructed by the manufacturer. The *rTth* enzyme is bifunctional, and, depending upon the cation present, copies either RNA only (in the presence of Mn^{2+}) or DNA only (in the presence of Mg^{2+}). DNase-treated, total cellular RNA was denatured at 95°C for 3 minutes and reverse-transcribed in the presence of Mn^{2+}, a single, strand-specific primer C14-2(-), and *rTth* at 70°C for 15 minutes. By chelation of Mn^{2+}, the enzyme shifts into its DNA-dependent mode. After addition of a second primer directed against the opposite strand, it copies the newly synthesized cDNA into double-stranded DNA by PCR amplification. The DNA products generated, depending upon their lengths, were subjected to 45 amplification cycles consisting of denaturation at 94°C for 1 minute followed by annealing at 53°C for 1 minute and by extension at 70°C for 2 minutes. Products were then detected by Southern blot hybridization on 1.5% agarose gels using the ^{32}P end-labeled probe c14-3(+), and subsequently autoradiography was developed following standard protocols (Figure 3). In the RT-PCR, we included a no primer control. In this control, no primer is added during the reverse transcription step. However, in the later stages or during the DNA dependent mode, both the primers are added. Negative results in the control suggest that indeed the amplification is due to the reverse transcription of RNA but not DNA.

Frozen sections.The heart cavities of the normal and mutant embryos were opened up by removing the skin ventral to the hearts. The embryos were fixed according to a modified method of Bell et al. (1987). This method allows simultaneous permeabilization and fixation using the cross-linker Dithiosulfylproprionate (DTSP, Pierce) and DMSO in the initial fixation step. Following a 20 minute fixation, the reaction was stopped by 0.1 M glycine. The fixed embryos were then washed in 7% sucrose in 0.1 M phosphate buffer, pH 7.4, two changes 30 minutes total and several hours with 15% sucrose in 0.1 M phosphate buffer to cryoprotect the tissue. Each embryo was then mounted on a mold and rapidly frozen in liquid nitrogen. Ten-micrometer-thick frozen sections were cut using a Sorvall MT-6000 ultramicrotome and mounted on albumin-coated glass slides for staining.

Confocal microscopy. Tissue used in this study was fixed according to a modified method of Bell et al. (1987) as described in the previous section. All steps took place at room temperature with gentle agitation on an orbital shaker. The procedure for staining whole hearts and normal and mutant whole embryo cross-sections through the heart regions was the same throughout the procedure. After blocking with 3% BSA in Steinberg's solution containing 0.05% Tween-20 for 1 hour, the hearts and cross-sections were incubated in 100% affinity-purified anti-N1 antibody for 40 minutes, washed several times and incubated again 30 minutes in 3% BSA in Steinberg's solution containing 0.05% Tween-20 to block non-specific binding. Hearts and the cross-sections were then incubated in FITC-conjugated anti-rabbit antibody at a 1:75 dilution for 45 minutes. Control hearts were not incubated in primary antibody but were incubated in secondary fluorochrome-conjugated antibody only. Following several washes in buffer, the hearts and sections were post-fixed in 2% paraformaldehyde for 30 minutes and quenched in 0.1 M glycine for 15 minutes. Finally, hearts and sections were mounted on slides in 50% glycerol in phosphate buffered saline containing 2% n-propyl gallate. The specimens were viewed on a BioRad MRC-600 Confocal Laser Scanning Microscope. Fluorescence was excited at 488 nm. A z-series was made for each sample, and system software was used to display the final image on a monitor. A Focus Graphics Imagecorder was used to store the record on Kodak TMAX 100 black and white print film or Kodak Ektachrome 100 color film. Color photographs were printed from the latter on Ilfochrome Classic Deluxe CLM 1k paper.

Gel Electrophoresis. Adult heart, skeletal muscle, brain, lung, and liver were homogenized in a gel electrophoresis sample buffer containing 0.80 M Tris HC1, 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 was then centrifuged at 12,000 g for 5 minutes and the insoluble precipitates discarded. Aliquots of the supernatant were loaded onto the gels for SDS-PAGE analysis according to the procedure of Laemmli (1970).

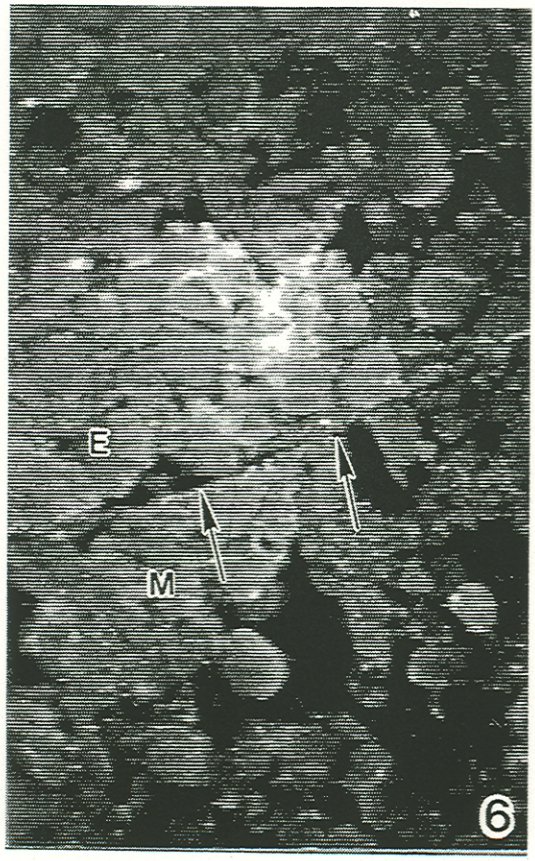


Figure 6. Confocal 3-dimensional image of the enlarged area marked A from Figure 5. Endoderm (E) and mesoderm (M) are distinctly separated on this 10 μM frozen section. The protein is localized primarily in that portion of the endoderm adjacent to the mesoderm (arrows). Some punctate staining is observed in the mesoderm as well.

Immunoblotting. After electrophoresis, the peptides were transferred to nitrocellulose paper according to the method of Towbin et al. (1979). Upon transfer, part of the nitrocellulose was stained with amidoblack to show all of the proteins transferred. The sheets were treated with the anti-N1 antibody at a 1:100 dilution with immunoblot buffer followed by peroxidase-conjugated sheep anti-rabbit antibodies. The staining was visualized using 40 mg of 4-chloro-1-naphthol dissolved in 15 ml cold methanol, 75 ml PBS, and 100 µl of 30% hydrogen peroxide.

Purification of the protein

Direct method: covalent linkage of the primary antibody to tosylactivated beads. The primary antibody was covalently linked to the tosylactivated magnetic beads (Dynabeads M-280). Tosylactivated dynabeads (20 mg/ml in 0.05 M borate solution, pH 9.5) were mixed with 400 µg/ml anti-N1 antibody in 0.05 M borate solution and incubated for 24 hours at 37°C with slow tilting and rotation. The antibody coated dynabeads were collected using the magnetic particle concentrators purchased from the manufacturer. The procedure for protein isolation was the same for the two types of methods as described under the protein isolation section below.

Indirect method: Binding of the primary antibody to the beads covalently linked to the Sheep anti-Rabbit IgG. Anti-N1 antibody was added in equal amounts to the Dynabeads M-280 Sheep anti-Rabbit IgG. The mixture was incubated for 24 hours at 40°C providing gentle shaking. The beads coated with the primary antibody were washed in 0.1% bovine serum albumin (BSA) in PBS, pH 7.4, 4 times for a total of 30 minutes. The protein was isolated as described below.

Protein Isolation. Frozen skeletal muscle tissue was homogenized with the homogenization buffer containing 50 mM Tris, 0.5 M KCl, 0.5 mM DTT and 1 mM PMSF. Equal amounts of beads with attached anti-N1 antibodies in 0.1% bovine serum albumin (BSA) in PBS, pH 7.4, and skeletal muscle homogenates were mixed and incubated at 4°C in a shaker for 1 hour. The beads were then collected using the magnetic particle concentrators and washed 3

Figure 7. Confocal 3-dimensional image of a frozen cross-section through the heart region of a normal pre-heartbeat stage 33 (+/+) embryo. This section shows that the protein is present in the endoderm (E), and heart tube (H) at stage 33.

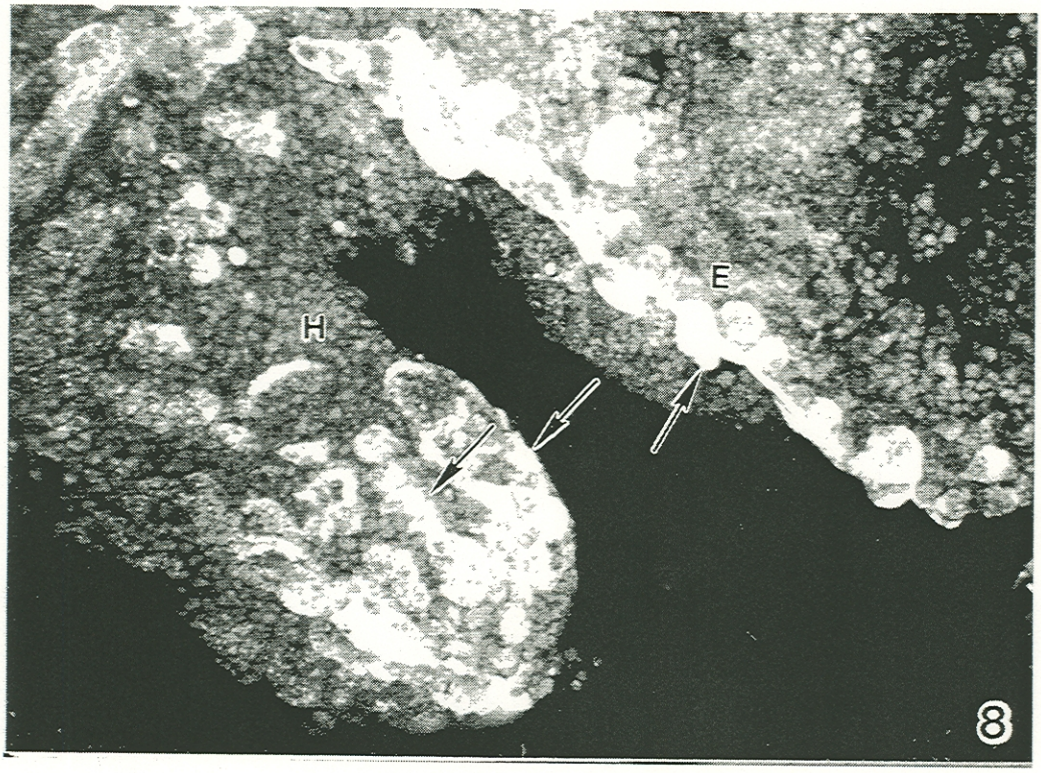


Figure 8. Whole-mount of a stage 34 (+/+) normal embryo stained with anti-N1 antibody. At this stage, the hearts first begin to contract. Anterior endoderm (E) and the heart (H) is seen on this confocal image. The protein is found only in specific cells of the anterior endoderm and heart, which form a mosaic pattern (arrows).

times with 0.1% BSA/PBS. The protein attached to the antibodies was then eluted out using a glycine/HCl buffer (0.1 M adjusted to pH 2.5 with 0.2 M HCl). The pH of the eluted protein was raised immediately to 8.5 with solid Tris.

Amino-acid composition analysis. The purified N1 protein was run on an SDS-gel and electroblotted to Problott™ membrane (Applied Biosystems) at 100 V for 4 hours in 192 mM glycine, 25 mM Tris-HCl, pH 8.3 (Towbin et al., 1979). The bands were cut with a razor blade and sent to LSUMC Core Laboratories, New Orleans, for amino-acid composition analysis.

Antibody production. The deduced amino acid sequence of the partial sequence of the clone containing the RNA for the N1 protein is as follows:

QGNFTDHFYS PHASMASGGI NVQNSNVALI
LDDLMGSRRPEAATPHHCIH
KQEDSRVGRC KCSLYNDLPP
QLQFSQGHLT NMEAKAC*.

The peptide sequence used for raising anti-N1 antibody is underlined. This portion of the amino-acid sequence was found to be the most antigenic region of the polypeptide using the GCG program, which established the most hydrophilic and hydrophobic regions of the polypeptide. The synthesis of the peptide and antibody production was done by Bio-synthesis, Inc., Lewisville, TX. After synthesis, about 20 mg of the peptide was coupled to Keyhole Limpet Hemocyanin (KLH) and injected with Freund's adjuvant into three rabbits. The serum was prepared and assayed using ELISA. Antibodies were then affinity-purified in a column to which the peptide is attached.

Results

A cDNA library was made from RNA isolated from the conditioned medium, which can initiate the non-beating mutant hearts to beat. The cDNA library was initially screened by radiolabeled conditioned medium RNA to pick the predominant RNA species represented in the library. After this initial screening, one of the clones with a relatively long reading frame was chosen for further analysis. The insert of this clone was partially sequenced (350 bases) and examined for its homology with other known sequences in the databases available to the Genebank using GCG software; no significant homology was observed. A homology search using the amino-acid sequence de-

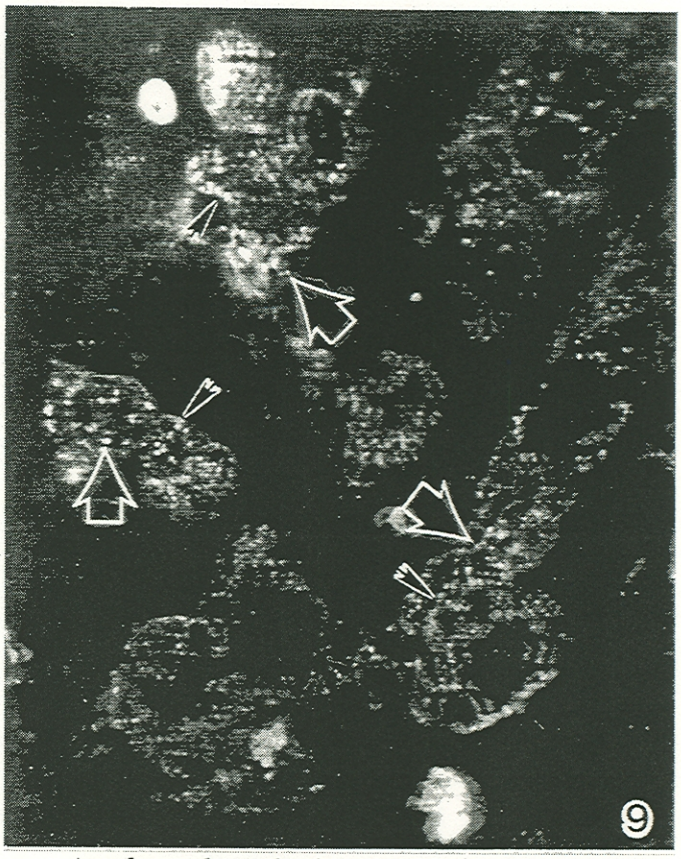


Figure 9. Enlarged area of anterior endoderm and heart from the confocal micrograph in Figure 8 (stage 34, +/+). The protein is localized on specific cells at or near the cell membranes (large arrows). Additionally, spots are observed throughout the cells (small arrowheads).

duced from the nucleotide sequence also indicated that this 88 amino-acid polypeptide was part of a novel protein; thus, we designated it as the N1 protein. Nucleotide sequence and the deduced amino-acid sequence of the *N1* gene is shown in Figure 1.

Total RNAs from juvenile axolotl skeletal muscle, heart, brain, lungs, and liver were isolated. By reverse transcription and PCR amplification using primer pairs specifically designed from the nucleotide sequence of the N1 cDNA as indicated in Figure 1, it was found that the gene is expressed mostly in brain, skeletal muscle, and heart. There was limited transcription in the lungs and none detectable in liver (Figure 2).

The deduced amino-acid sequence of the peptide was analyzed for its hydrophobicity and hydrophilicity to obtain the most antigenic region of the polypeptide (Figure 3). An oligopeptide was synthesized commercially from this portion of the polypeptide, and polyclonal anti-peptide antibodies were produced in three rabbits and the serum affinity purified. Western blot analyses of adult heart homogenates shows specific staining for the affinity purified anti-N1 antibody of a doublet at 67 kDa and 65 kDa (Figure 4). The protein was partially purified using two separate protocols as described in the Materials and Methods section. The bands that correspond to 67 kDa and 65 kDa were analyzed for their amino acid composition and were found to be very similar indicating that each band was a slightly different (phosphorylated?) form of the same protein (Table 1).

The localization of the N1 protein on normal embryonic sections and whole hearts. The distributions of the N1 protein on cross-sections of normal embryos at stages 16, 33-34, 37-38, and 41-42 were analyzed using immunohistochemistry. Additionally, whole hearts of stages 35, 38, and 41 were stained and examined on a confocal microscope for a 3-dimensional view of the protein. The presence and localization of the protein was also examined in cross-sections through the heart regions of stage 37-38 mutant embryos.

Cross-section of stage 16 (+/+) embryos. Figure 5 shows an overview of a cross-section through a stage 16 (+/+) normal embryo which was stained with the anti-N1 antibody. The embryo is undergoing neurulation at this stage. The neural folds, neural plate, endoderm, and mesoderm are clearly distinguishable on this section. Whole sections show sig-

nificant fluorescence in the areas adjacent to and between the endoderm and precardiac mesoderm (Figure 5) when stained with the N1 antibody. An area marked with a rectangle is enlarged to show the staining (Figure 6). The 3-dimensional confocal image shows an area where endoderm and mesoderm are clearly discernable. The N1 protein has accumulated in that portion of the endoderm located adjacent to the precardiac mesoderm as well as in the mesoderm itself.

Cross-section of stage 33 (+/+) embryo. At stage 33 (+/+), the heart tubes on both sides have not yet completely fused. The N1 protein is most prominent in three areas: myotomes, endoderm, and heart tubes. The neural tube does not exhibit staining for the N1 protein. However, the skin (ectoderm) and myotomes are heavily labeled (not illustrated). The endoderm region exhibits the protein homogeneously on the cells. As observed at stage 16, the protein remains in the skin (ectoderm) at stage 33. Heart tube, endocardium, anterior endoderm, and ectoderm are shown in Figure 7. The cells of the anterior endoderm, heart tube, and endocardium contain the protein mainly at their peripheries with the staining showing a spotted pattern throughout the section. Again, the ectoderm staining is clearly observable. Interestingly, the portion of the myocardium that is facing the anterior endoderm stains more intensely than the portion that is adjacent to the ectoderm.

Whole-mount of stage 34 (+/+) embryo. Stage 34 (+/+) whole-mount normal embryos were stained with the anti-N1 antibody after removal of the skin ventral to the heart regions (Figure 8). This is the earliest stage at which the hearts show contractions, and only a few myofibrils are present in the myocardial cells at this age. The heart and attached anterior endoderm are clearly seen in this confocal micrograph image at low magnification. Only specific cells are stained in both the heart and anterior endoderm forming a mosaic-like pattern with many of the stained cells in contact with each other. This same specimen is enlarged in Figure 9 and a 3-dimensional image has been created with the confocal microscope. The staining of certain cells on the heart and anterior endoderm is clearly seen. The protein seems to be localized primarily at or near the cell peripheries.

Whole-mount of stage 35 (+/+ or +/c) heart. A 3-dimensional confocal micrograph of a

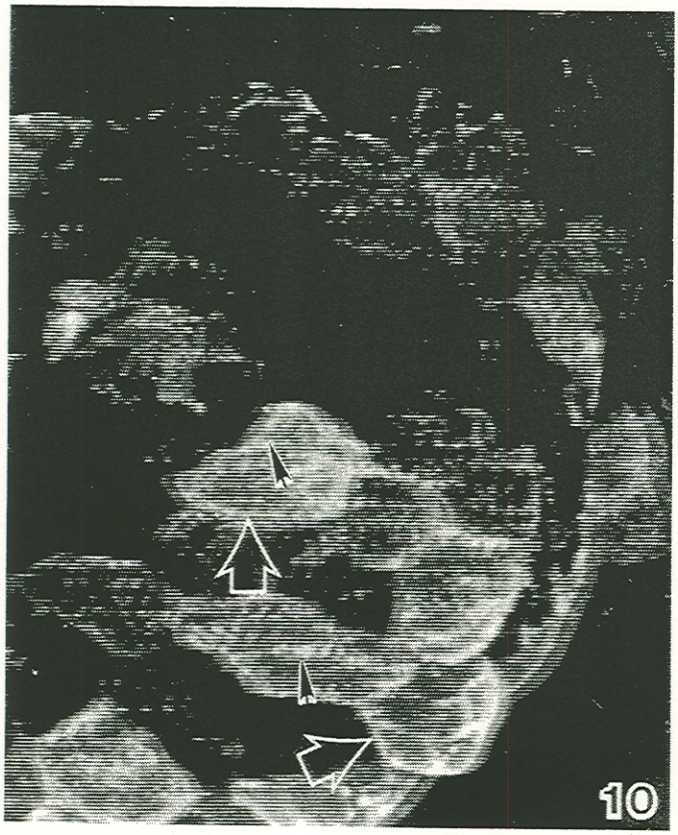


Figure 10. Three-dimensional confocal micrograph of a whole-mount preparation of a normal heart (+/+ or +/c) at stage 35 stained with the anti-N1 antibody. A mosaic-like staining pattern of cells containing the protein is seen in the heart (large arrows). The protein outlines the cell membranes in some areas of the heart, whereas there is punctate staining in other areas (small arrowheads).

Table 1. Amino Acid Compositions of Bands A (67 kDa) and B (65 kDa)

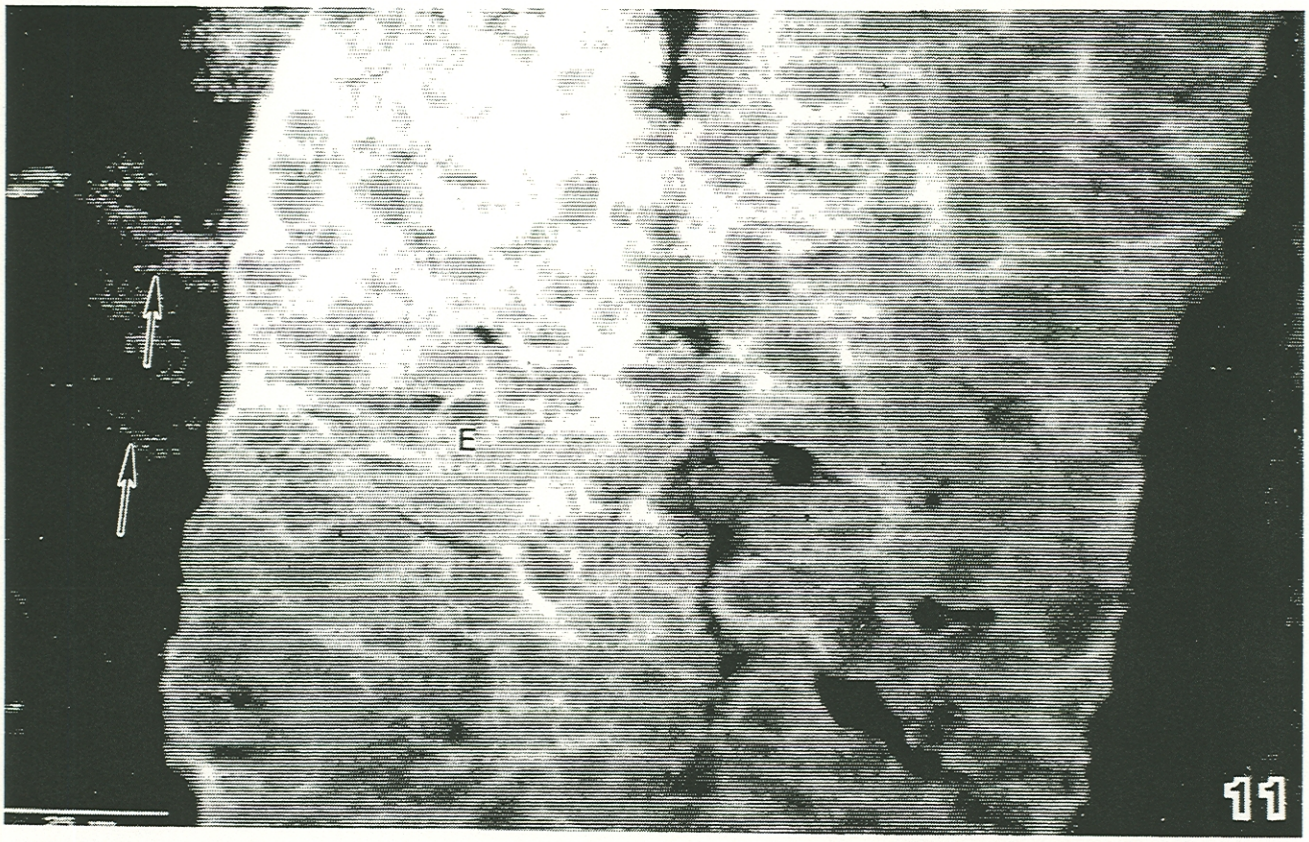
Amino Acids (Mol%)	Band A (Mol%)	Band B
Aspartic acid	7.92	7.25
Glutamic acid	13.97	13.41
Serine	7.09	8.39
Glycine	11.82	15.77
Histidine	2.18	1.91
Arginine	3.95	4.44
Threonine	4.58	4.97
Alanine	9.36	8.47
Proline	5.99	3.97
Tyrosine	2.26	2.20
Valine	8.93	6.56
Isoleucine	2.87	5.09
Leucine	10.12	8.15
Phenylalanine	3.73	2.74
Lysine	5.23	5.18
Methionine	—	0.51

Table 1. Amino-acid composition analysis of Band A (67 kDa) and Band B (65 kDa) isolated from the purified N1 protein. The compositions of Band A and Band B are very similar indicating that both bands belong to the same protein.

whole normal heart (+/+ or +/*c*) at stage 35 stained with anti-N1 antibody is shown in Figure 10. At this stage, the hearts beat vigorously. Again, a somewhat mosaic distribution of the heart cells staining for N1 is noticeable at this stage. The cells are outlined with the protein. In addition, smaller circular distributions of the stain is observed over the cells located at the surface of the myocardium, whereas the lumenal cells facing the endocardium show more staining on the cell peripheries.

Cross-sections of stage 37-38 normal (+/+ or +/c) embryos. At late stage 37, cross-sections through the heart regions of the embryos reveal that the N1 protein is present in large amounts in the heart, endoderm, and skin. Figure 11 shows a section through the endoderm region immediately above the pericardial cavity. An intense staining for the N1 protein is seen throughout the endodermal cells. Many spots appear to be distributed in the cells in random fashion. Figure 12 shows the heart in cross section and is immediately adjacent to the endoderm in Figure 11. There is significant staining for the N1 protein in the endocardium, myocardium, and cardiac jelly of the heart; connective tissue between the endocardium and myocardium also stains for the N1 protein. The myocardial cells show intense staining in a spotted pattern; the portions of the myocardial cells facing the endocardium exhibit more intense staining than the non-luminal areas of the cells. Additionally, the protein appears to be associated with the cardiac jelly; prominent "strands" of N1-positive material extend between the myocardial and endocardial layers (Figure 12). Some of the protein stains in the form of small spots, some is lined up along the cell surfaces, and some is diffusely distributed over the cells. Similar to what is observed between the endocardium and myocardium of the heart tube, thick "strands" of N1-positive staining extends from the surface of the endoderm adjacent to the heart, into the cardiac jelly dorsal to the forming endocardium (Figure 11).

Whole-mount of stage 38 normal (+/+ or +/c) heart. A 3-dimensional image of a whole-mount normal heart (+/+ or +/*c*) at stage 38, stained with the anti-N1 antibody using the confocal microscope, is shown in Figure 13. The 3-dimensional image clearly shows that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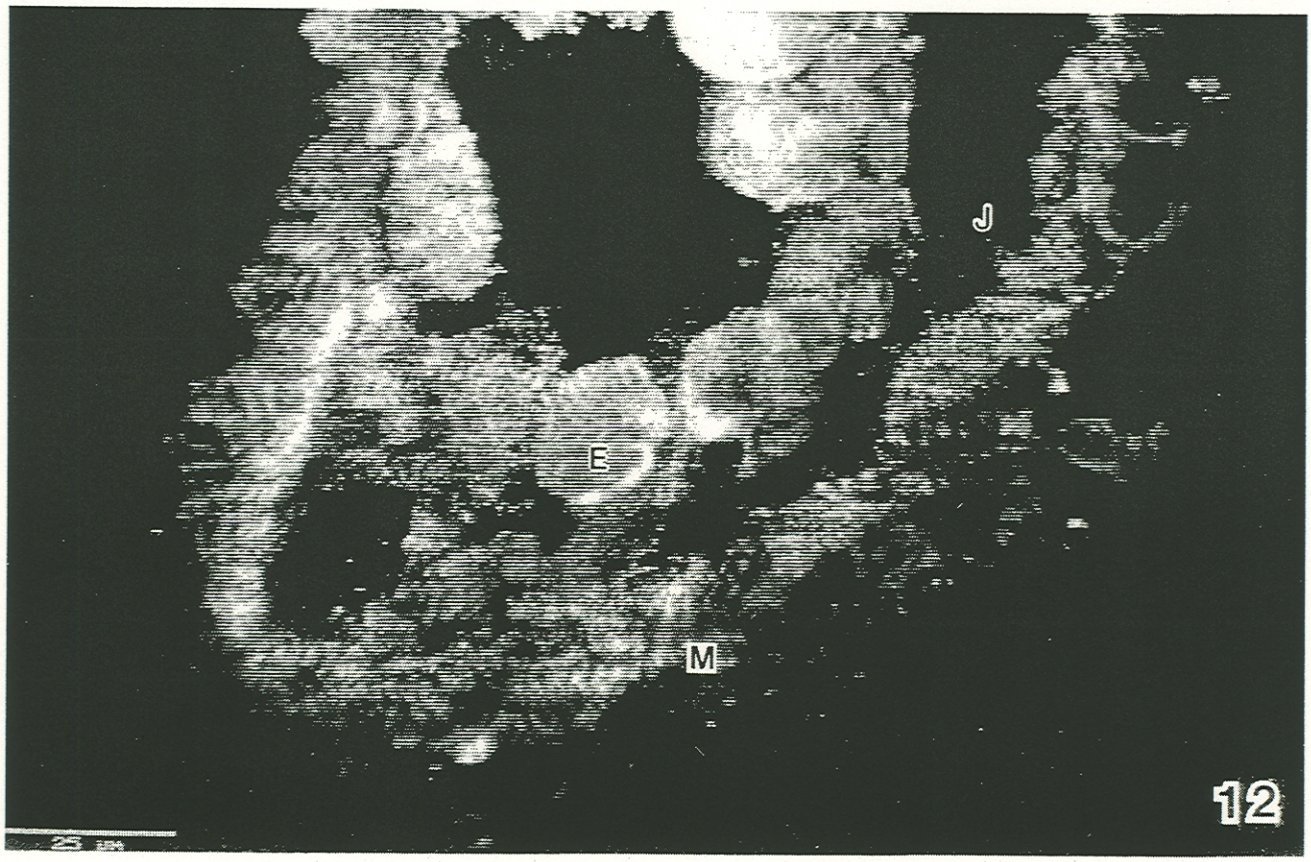
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the protein is localized at the cell peripheries of the myocardial cells. A spotted punctate pattern of staining is again observed. The pocket-like staining of the myocardial cells in these whole heart preparations indicate that the staining is primarily on the surfaces of the cells that lie next to the endocardium, which is consistent with the staining observed on the cross-sectioned material (Figure 12).

Cross-section of stage 41-42 normal (+/+ or +/*c*) embryo. The N1 protein is again localized primarily in the skin, heart and endoderm of stage 41-42 normal embryos (+/+ or +/*c*). A small amount of staining is observed on the myotomes as well (not illustrated). Figure 14 shows the heart region stained with the anti-N1 antibody. Again, the protein is present near the surfaces of the myocardial cells. In some areas of the myocardial cells, the spots appear to be distributed in linear patterns with regular periodic distributions. Endoderm which is differentiated into gut tissue is strongly stained at this stage as well; some areas show regularly spaced linear arrays of spots (Figure 15). In addition to the endoderm, the notochord is seen in Figure 15 where staining is very sparse. Part of the endoderm and cartilaginous tissue are visualized in Figure 16; although the endoderm stains intensely, the gill cartilage shows very little staining.

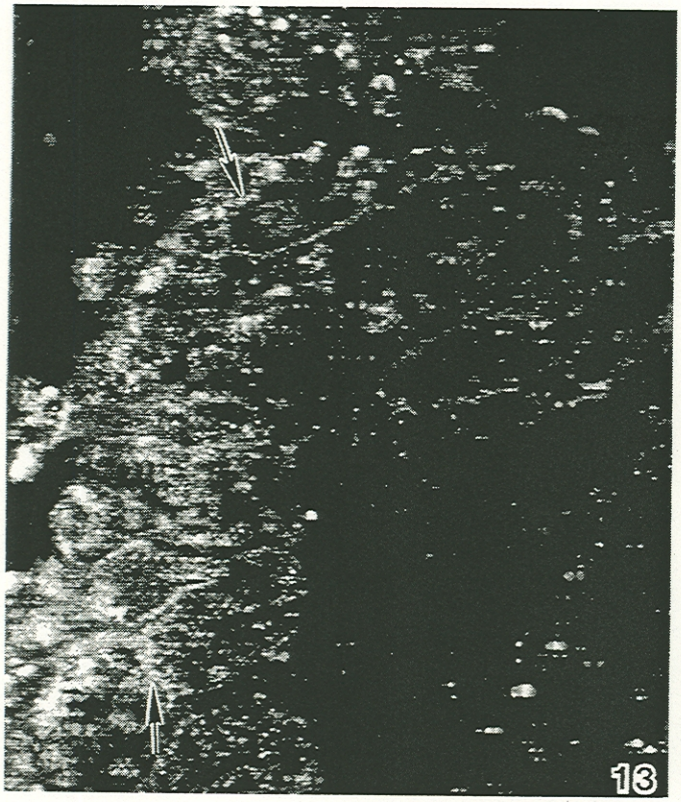
The localization of the N1 protein on mutant embryonic sections. The distribution of the N1 protein in the mutants is examined on cross-sections through the heart regions of mutant embryos at stage 37-38 (+/*c* and +/*c*). The mutants showed a significant reduction in staining of the N1 protein when compared to normal. A few spots are found at the cell surfaces of the mutant cardiac cells as well as some staining in the cardiac jelly (Figure 17); however, mutant staining is in significantly lower amounts than normal (compare with Figure 12). Although substantially reduced from normal, the surfaces of the mutant myocardial cells facing the endocardium contain slight staining throughout the heart. Mutant endoderm shows much less staining than normal endoderm at the same age (not shown).

Figure 11. Confocal image in 3 dimensions showing the endoderm (E) of a stage 37-38 normal (+/+ or +/*c*) embryo. The endodermal cells are strongly stained for the N1 protein. "Strands" of N1 protein extend from the surface of the endoderm towards the heart (arrows).

Figure 12. Three-dimensional confocal micrograph of a section through the heart region of a normal stage 37-38 (+/+ or +/*c*) embryo stained with anti-N1 antibody. The myocardial cells (M) and the endocardial cells (E) are strongly stained. The protein is found at or near the cell periphery in addition to its punctate form over the whole cells. The area between the endocardium and the myocardium, which appears to be the cardiac jelly (J), shows strands of staining as well.

Discussion

Heart differentiation is a gradual process which involves inductive and suppressive interactions between the heart mesoderm and surrounding embryonic tissues. There are three factors involved in regulating heart formation and differentiation (Jacobson, 1960; Jacobson and Duncan, 1968; Fullilove, 1970). The principal inductor tissue is the anterior endoderm. Apparently, a specific inductive substance in the anterior endoderm increases the rate and frequency of heart differentiation. The heart cannot differentiate without the interaction of the anterior endoderm (Bacon, 1945). Secondly, a general stimulatory effect of the epidermis increases the frequency of heart development. It has been proposed that there are three possible effects of the epidermis on heart differentiation (Jacobson, 1961). The first effect is probably a non-specific shielding of the mesoderm from the environment. The second role of the epidermis is to provide a proper interface for the migration of the mesodermal tissue. If the epidermis is removed at the neurula stage, the migration of the mesoderm stops. The third effect of the epidermis on heart determination is specific and appears to provide an influence that increases the frequency of heart development. The third factor is the inhibitory effect of the neural plate tissue. Interestingly, the N1 protein is mainly found in the heart and in the two major inductive tissues: epidermis and endoderm; N1 is essentially absent from neural plate and brain tissues in embryos. In the present study, a cDNA library was prepared using the RNA isolated from conditioned medium, which was made by culturing the anterior endoderm region of a stage 24 (+/+) normal embryo for 48 hours in Steinberg's solution and then removing the tissue. RNA purified from the same conditioned media had the ability to promote beating and to cause myofibril formation in mutant hearts. The N1 protein is deduced from a partial sequence ob-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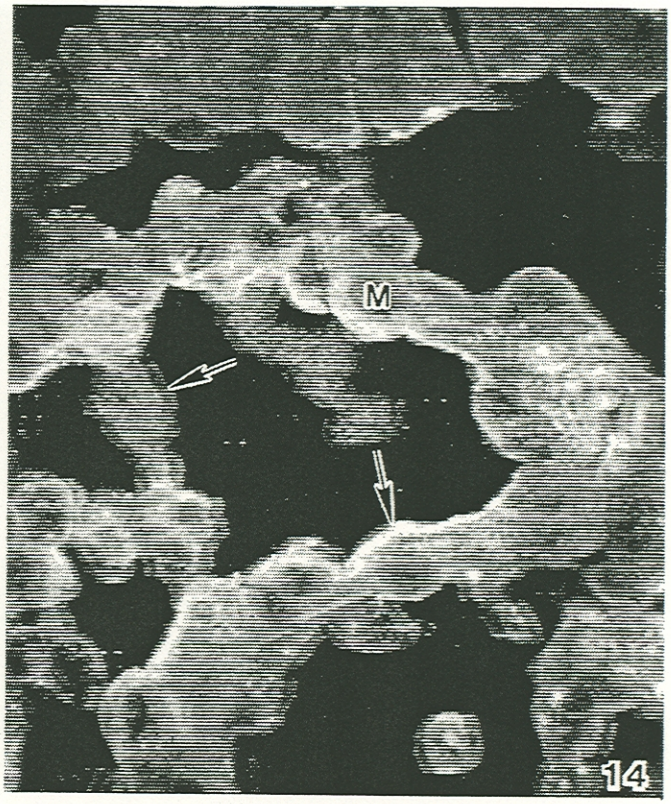
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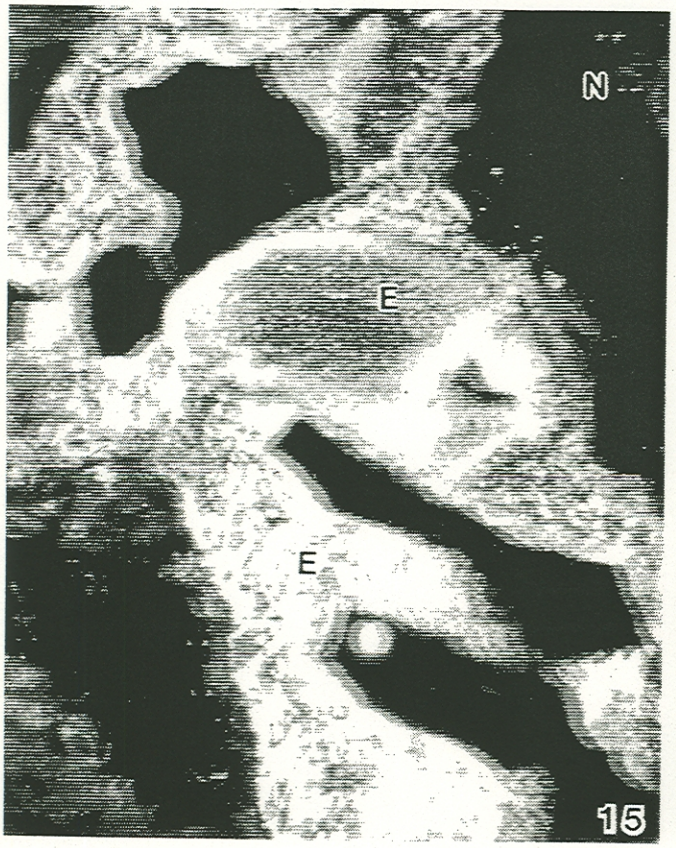
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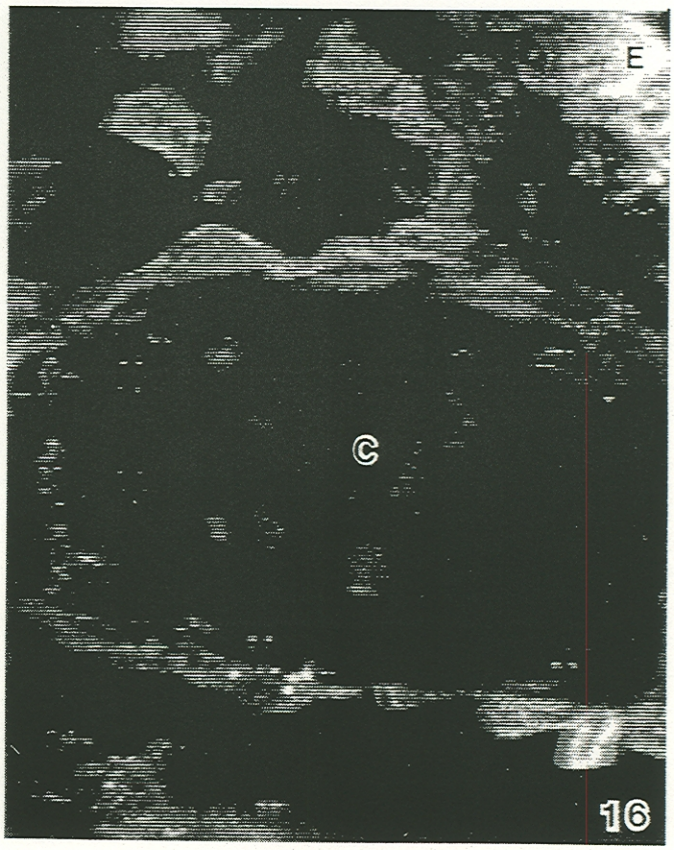
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Figure 17. Three-dimensional confocal image of a section through the heart of a stage 37-38 mutant (*c/c*) embryo stained with the anti-N1 antibody. There is only a small amount of staining along the cell surfaces at the myocardium. The staining for the N1 protein is very significantly reduced when compared with the normal sibling hearts (see Figure 12 for comparison).

tained from a clone of this cDNA library. The fact that the RNA from which the polypeptide is deduced originates from the anterior endoderm region of a stage 24 normal embryo, and that the protein is located mainly in inductive tissues, makes it inviting to speculate that the protein may be related somehow to the induction process. Moreover, the mutant embryonic sections through the heart regions exhibit the protein, but in significantly reduced amounts. This is an interesting observation which requires further detailed study.

The clone which contains the insert for the N1 protein was selected from among the various clones of the cDNA library that were hybridized with ^{32}P-labeled conditioned RNA. This initial screening of the library with the labeled conditioned medium RNA was done to

Figure 13. Three-dimensional confocal micrograph of a stage 38 normal (+/+ or +/*c*) heart stained with the anti-N1 antibody. The protein is found mainly at or near the cell peripheries and stains in a punctate pattern.

Figure 14. Confocal image in 3 dimensions of the heart region of a stage 41-42 normal embryo (+/+ or +/*c*). Many of the surfaces of myocardial (M) cells that are facing the endocardium are stained in continuous or punctate lines with the anti-N1 antibody (arrows).

Figure 15. Three-dimensional confocal micrograph of the endoderm (E) region of a stage 41-42 normal embryo (+/+ or +/*c*). This section shows strong staining for the N1 protein on the endoderm (gut). A portion of the notochord (N) shows sparse staining.

Figure 16. Three-dimensional confocal micrograph of an adjacent area of the same section shown in Figure 15. Cartilage (C) from the gill and part of the endoderm (E) are shown in this section. The endoderm shows intense staining, which is in a punctate form; however, there are only a few spots present within the cartilage.

select the most abundant species of RNA present in the conditioned medium. A majority of the clones we obtained contained inserts that belong to ribosomal RNAs. N1 was derived from one of the remaining clones which were non-ribosomal. Thus, it is likely that the RNA belonging to the N1 protein is one of the most abundant RNA species in the conditioned medium.

The results of Western blot analyses with tissue homogenates show the presence of a doublet of 67 kDa and 65 kDa suggesting the possibility of phosphorylation of the N1 protein. It is now well established that the configuration of a protein can be changed due to the phosphorylation in such a way that the native protein and phosphorylated form of the native protein move as a doublet in an SDS gel. Our hypothesis that these doublets are the same polypeptide is further supported by the fact that the amino acid compositions of these two polypeptides are very similar (Table 1).

We are currently in the process of getting the full length sequence of the RNA from which the protein is obtained. After getting more sequence information, we should be able to better understand the nature of the protein. A clone with 1400 bases has been isolated after antibody screening of a juvenile axolotl heart cDNA library. To ensure that the anti-peptide antibodies (N1) in fact recognize the protein that is expressed by the *N1* gene, polyclonal antibodies will be produced against the fusion protein which is expressed by the clone isolated after antibody screening. Thus, the present study opens up a whole new line of investigation to explore the nature of the cardiac lethal mutation.

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