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CLONING, SEQUENCING AND EXPRESSION OF AN ISOFORM OF CARDIAC C-PROTEIN FROM THE MEXICAN AXOLOTL (*AMBYSTOMA MEXICANUM*)+

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C-protein, a myosin binding protein, is thought to regulate and stabilize thick filaments during assembly of sarcomeric A-bands. Multiple isoforms of C-protein have been characterized in avian and mammalian systems. We now report the isolation and the nucleic acid sequence of a partial C-protein cDNA clone from an axolotl heart cDNA expression library in lambda gt11. The clone was isolated by screening the library with a heterologous monoclonal anti-C-protein antibody (MF1). Sequence comparison revealed that $CPRO_{Axocard}1$ has an average sequence identity of 62-68% at the nucleic acid and 72-78% at the amino acid levels respectively to human and chicken sequences. We could not detect any significant differences at the levels of expression of the cardiac isoform of C-protein ($CPRO_{Axocard}1$) in normal and non-beating heart tissues of the double-recessive cardiac lethal mutant (c/c) axolotl, Ambystoma mexicanum. This is the first report of a C-protein sequence from an amphibian species.

The Mexican axolotl (Ambystoma mexicanum) is an excellent model for studying heart development because it carries a recessive mutation in gene c. Double recessive (c/c) mutants form defective hearts which do not beat. The mutant embryos can survive until stage 42 provided that sufficient oxygen diffuses into their body from the medium. Eventually the embryos die due to an absence of circulation. The defective hearts cannot beat due to a lack of organized myofibrils (1); however, most of the sarcomeric proteins, except tropomyosin (2,3), are present in the mutant hearts in concentrations comparable to those of normals. Recently, we have undertaken a detailed molecular approach to understand more about the gene c mutation. We plan to compare the relative expression of various genes of myofibrillar proteins in normal and mutant hearts. For such analyses, precise information of the nucleic acid sequence of the genes of interest is an essential prerequisite. Unfortunately, very few cDNAs have been isolated and sequenced from the axolotl (4-7). In fact, no axolotl gene has been sequenced fully.

C-protein is a myosin binding protein of unknown function and is present in the A-bands of striated muscle (8-10). Myosin is the most abundant component of the thick filament and C-protein is thought to stabilize the thick filament by associating with myosin (11). In addition to its

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primary biological function, C-protein has recently been suggested to be of significant clinical importance; Kasahara et al. (12) proposed that C-protein is the primary antigen which induces autoimmune myocarditis. C-protein is known to have different isoforms which have been characterized mainly in avian and human systems (13,14). Using heterologous anti-chicken antibody, we have recently established the presence of at least two C-protein isoforms in striated muscle of the mexican axolotl (15). The monoclonal antibody MF-1, which was raised against chicken fast skeletal muscle C-protein isoform (15), can recognize specifically the C-protein isoform present in adult heart of the axolotl (16). The cDNAs of C-Protein from chicken muscle (17), human skeletal muscle (18), bovine skeletal muscle (14) and mouse heart (12) have been cloned and sequenced. In the present study, we have cloned a 1.4 kb C-protein cDNA from the lambda gt11 cDNA expression library of the adult axolotl heart using MF-1 antibody. We have determined the nucleic acid sequence of the cDNA clone. We also used a nucleic acid probe, labelled with [^{32}P] by means of polymerase chain reaction (PCR), to screen the same cDNA library. We isolated two more cDNA clones that overlap the original clone and obtained an additional ~150 bp of new sequence at the 5'-end. It should be noted that this is the first report of a C-protein sequence from amphibian striated muscle and the second cardiac isoform from the entire vertebrate world. In addition, we have studied the expression of the cardiac isoform of C-protein at different stages of development in normal and mutant embryos by the reverse transcription polymerase chain reaction (RT-PCR) using specific primer-pairs and a detector oligonucleotide probe.

Materials and Methods

Procurement and Maintenance of Axolotls - The axolotl colonies at Indiana University and the SUNY Health Science Center at Syracuse provided both adult and juvenile animals for the studies. The staging system of Bordzilovskaya et al. (19) for *Ambystoma mexicanum* was used throughout the study.

Antibody screening of the juvenile axolotl heart cDNA library - Using monoclonal antibodies against chicken C-protein fast isoform (MF1) a juvenile axolotl heart specific cDNA library in a λgt11 expression vector was screened to isolate clones of $CPRO_{Axcard}1$. The screening protocol was according to Mierendorf et al. (20).

Nucleic acid screening of a juvenile axolotl heart specific library - The 5' region of a 1.3 kb C-protein clone which was previously isolated in the MF1 antibody screening of a λgt11 expression library was used to generated a specific [^{32}P] labelled nucleic acid probe (150 bp) using C-protein PCR (+2) 5'--ATC ACT GAC CTG AGG GGT CT--3' and PCR-1(-) 5'--TCC CAT TCT TCT TCC ACT T--3'. Screening of the library was performed by following the standard protocol as described by Sambrook et al. (21).

Determination of nucleotide sequence of cDNA clones - cDNA inserts in λgtll clones isolated by either antibody or nucleic acid probes were amplified using λgtll 5'(+) (GGTGGCGACGACTCCTGGAGCCC) and 3'(-) (TTGACACCAGACCCAACTGGTAATG) universal primers. The amplification procedure was carried out using the Perkin-Elmer/Cetus Amplitaq PCR Kit in a final volume of 100 μl for 30 or 45 cycles as described earlier (4,5). Amplicons were electrophoresed on 1.5% agarose gels with a 1 kb or a 100 bp molecular weight marker and the cDNA bands were extracted from the gels using the Qiagen DNA Gel Extraction Kit. The DNA was then subcloned into a M13mp18 (Gibco-BRL) and PCR™1000 vector (Invitrogen) which enabled us to sequence both single- and double-stranded DNAs by the dideoxy chain termination technique (22) using a Sequenase Kit version 2.0 (USB). For cloning into a M13mp18 vector, both amplified DNA and double-stranded vector DNA were digested with

EcoRI restriction endonuclease prior to the ligation using T4DNA ligase following the manufacturer's specification.

Total RNA Isolation - Total RNA from adult tissues were generous gifts from Dr. Nihan Erginel-Unaltuna and Mr. Arun Gaur . RNA from whole embryos as well as heart and skeletal muscle tissues from developmental stages (1 to 41) of normal and mutant embryos were isolated using an RNagents total RNA isolation kit from Promega. This isolation protocol is based on methods according to Chomczynski and Sacchi (23). RNA concentrations were determined by measuring samples at 260 nm and 280 nm in a Beckman spectrophotometer (model DU64).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) - First strand cDNA synthesis of developmental and adult tissue specific total RNA samples were performed using a Superscript Preamplification system from Gibco-BRL. All RNA samples used for reverse transcription were initially incubated with 2 units of RNase free deoxyribonuclease I (amplification grade, Gibco-BRL) in order to remove any possible contaminating genomic DNA. mRNA from 1 µg of isolated total RNA was then reverse transcribed into an initial strand of cDNA with the use of oligo dT primers and RNase H- Superscript II Reverse Transcriptase. The RNA from the RNA:cDNA hybrid was degraded by the addition of RNase H to each sample. PCR amplification of $CPRO_{Axcard}1$ using specific primer-pairs was done for 45 cycles with the Perkin-Elmer/Cetus Amplitaq kit under the previously mentioned amplification conditions as described earlier (5). Southern blots were carried out to verify the amplification of $CPRO_{Axcard}1$ by hybridization with [^{32}P] end-labelled oligonucleotide detector flanking the primer-pairs.

Results and Discussion

An axolotl heart λgt11 expression library was screened using monoclonal antibody against chicken C-protein fast isoform (MF1) and a 1.3 kb cDNA fragment of $CPRO_{Axcard}1$ transcript was isolated. For sequence analysis the cDNA clone was subcloned into M13mp18 and the nucleic acid sequence was determined following the strategies as presented in Figure 1. When compared to complete and partial nucleic acid cDNA sequences existing in the data bank, the axolotl 1.3 kb C-protein clone was found to be most homologous to the 5' region of chicken fast, human fast and human slow cDNAs. In an attempt to obtain the full length $CPRO_{Axcard}1$ cDNA, the axolotl heart cDNA library was screened again using a PCR-generated [^{32}P]-labelled specific nucleic acid probe. The 800 bp probe was designed from the 5' end of the $CPRO_{Axcard}1$ 1.3 kb molecule.

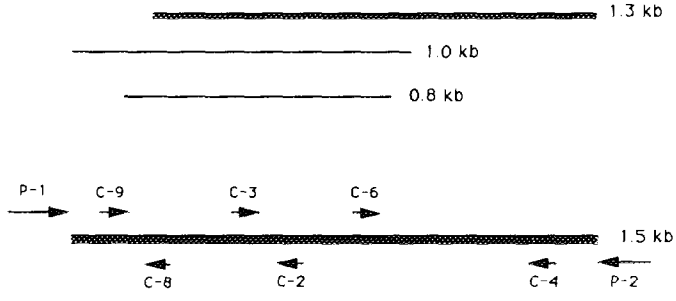

Figure 1. Strategies for sequencing and amplification of axolotl C-protein cDNA clones by polymerase chain reaction. The sequences of different primers used for sequencing are as follows. Primers P1 and P2 : Either T7: 5'-TAATACGACTCACT-3' or SP6: 5'-ATTTAGGTGACACTATA-3' or M13 universal primer: 5'-GTTTTCCCAGTCACGAC-3'; C2: 5'-TCCCATTCTTCTTCCACT-3'; C3: 5'-ATCACTGACCTGAGGGGTCT-3'; C4: 5'-TTGAGTTTGGCAGAAGGTTG-3'; C5: 5'-AATTTAAGCTGGTGATGGT-3'; C6: 5'-AGGACCAGATGGTGATGGT-3'; C8: 5'-GTCAAACTTGTCCTT-3' and C9: 5'-GTGAGCCAAACACCAAGATT-3'.

Two overlapping pairs (0.8 and 1.0 kb) were subsequently isolated, which gave an additional 225 bp of new sequence to the 5' end of the axolotl C-protein cardiac transcript (Figure 1). Figure 2 shows both nucleic and amino acid sequences of the compiled axolotl C-protein 1.5 kb cDNA fragment. The sequence provided an open reading frame (ORF) of a total of 1,500 bp. Sequence comparison revealed that $CPRO_{Axcard1}$ has an average sequence identity of 68% at the nucleic acid and 78% at the amino acid level to human and chicken sequences.

Primary structure analyses of $CPRO_{Axcard}1$ demonstrated the presence of two types of repetitive motifs as found in other C-proteins. One motif is similar to the fibronectin type-III and the second one is related to the Ig C2 repeats. As we do not have the entire cDNA sequence, it is not meaningful to calculate the number of each type of repeats in the axolotl cardiac C-protein. Sequence conservation in the C-protein family is not restricted to type-III or C2 modules. In fact, when all known C-protein sequences along with those of the axolotl are aligned, the longest region in identity with 18 identical residues in all four proteins, is located in the 5-kDa region underlined in Figure 2. In axolotl, however, one methionine has been changed to leucine which is a conservative substitution.

All primer-pairs and detector oligonucleotides used for RT-PCR were pedigreed first with $CPRO_{Axcard}1$ (1.3 kb) cDNA. Of the many tissues (skeletal, smooth muscle, liver, brain and lung) examined in the neotenous adult axolotl, C-protein cardiac 1 gene expression occurs only in the heart (Figure 3A). The expression of $CPRO_{Axcard}1$ is also detected in the back skeletal muscle of juvenile axolotl (3-4 months old). However, the level of expression in the juvenile skeletal muscle is significantly lower compared to embryonic skeletal muscle (Figure 3B). In the adult skeletal muscles, the expression of mRNA of $CPRO_{Axcard}1$ can hardly be detected by RT-PCR. A wide range of embryonic stages were analyzed (stages 1 to 41) in order to determine the onset of $CPRO_{Axcard}1$. Total RNA from whole embryos was isolated from "first cell cleavage" stages through stage 30 when the hearts begin to differentiate. Stages 35 to 41 heart and back skeletal muscles were collected separately and examined for their expression of either type of transcript. The results depicted in Figure 3B show that $CPRO_{Axcard}1$ gene expression is virtually undetected even at stage 30 (whole embryos). The inability to detect the $CPRO_{Axcard}1$ transcript is not due to a lack of integrity of the RNA preparation because the same RNA preparation yielded positive results when used for the amplification of myosin heavy chain and alpha tropomyosin (results not shown). Expression has begun between stage 30 and 35/36 in the heart as demonstrated in the over-exposed photograph of the samples in Figure 3C. $CPRO_{Axcard}1$ expression in skeletal muscle at these later stages (35-41) appears stronger in comparison to levels observed in the normal developing heart.

Figure 4 demonstrates that $CPRO_{Axcard}1$ gene expression at stage 41 of mutant striated muscle (cardiac and skeletal) appears to be similar to normal. A similar pattern was found in the case of myosin heavy chain expression (results not shown). The expression level appears to be higher (as judged by the stronger signal) in skeltal muscle than in cardiac muscle. Our RT-PCR results are consistent with those of biochemical, immunohistochemical and confocal microscopic (1-3, 24) analyses which suggest that although mutant axolotl hearts are deficient in alpha-tropomyosin, the levels of expression and accumulation of other myofibrillar proteins including C-protein and myosin are unaffected (16).

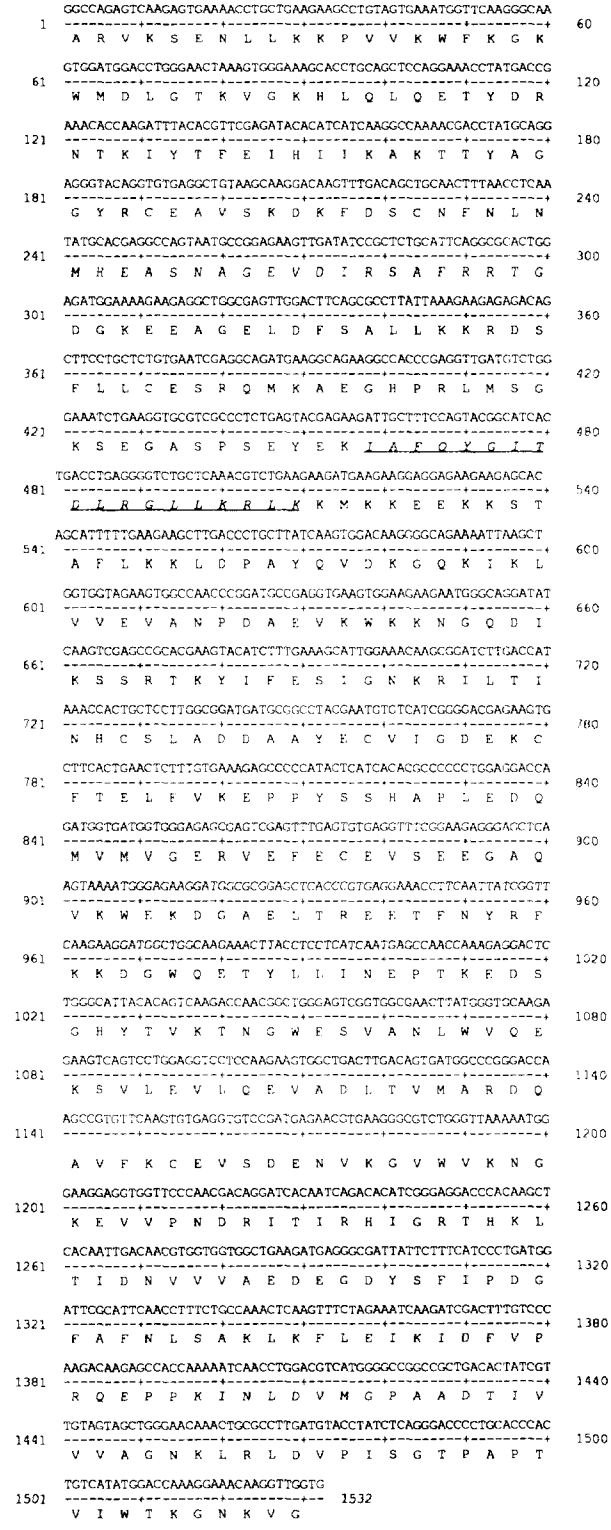

Figure 2. Nucleotide and deduced amino acid sequence of Axolotl C-protein cDNA. The underlined nucleotide indicates the conserved amino acid residues. The axolotl C-protein sequence has been submitted to the Database Genebank (accession code pending).

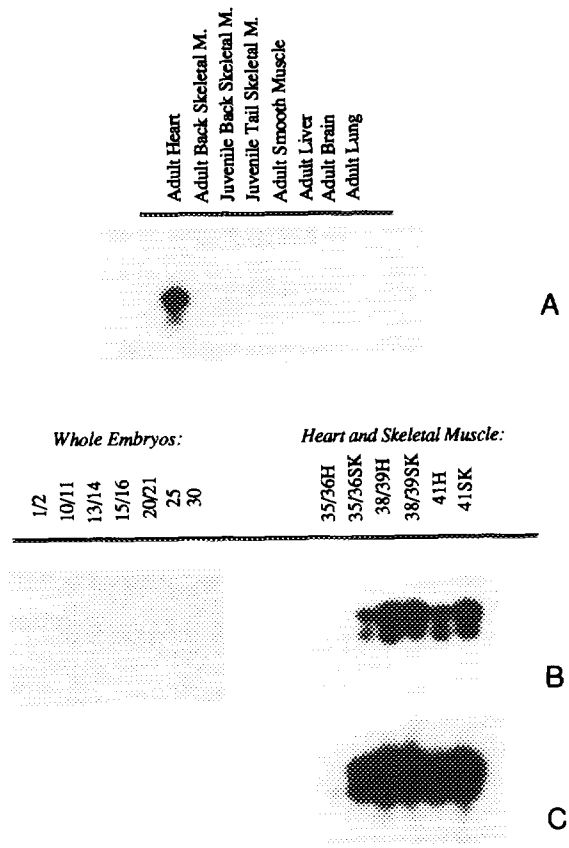

Figure 3. RT-PCR analysis of CPRO$_{Axcard}$1. A: Adult and juvenile axolotl tissues. B: Embryonic developmental stages. C: Overexposed autoradiogram. For each RT reaction, 1 µg of total RNA was used. The sequences of the primer-pairs and detector oligonucleotide used in this study are as follows: Primer-1(+): 5'-ATTAAGCTGGTGGTAGA-3' (593-609); Primer-2(-): 5'-TTGAGTTTGGCAGAAGGTTG-3' (1350-1332) and detector-3(+): 5'-AGGACCAGATGGTGATGGT-3'(834-852).

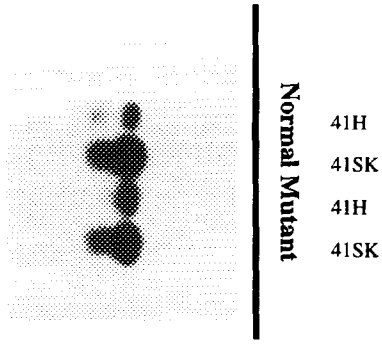

Figure 4. RT-PCR analysis of expression of CPRO$_{Axcard}$1 normal and mutant axolotl. Total RNA was isolated from heart and skeletal muscle of normal and mutant embryos (stage 41).

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References

1. Lemanski, L.F. (1973) Dev. Biol. 33, 312-333.
2. Lemanski, L.F. (1979) J. Cell Biol. 82, 227-238.
3. Lemanski, L.F., Fuldner, R.A., and Paulson, D.J. (1980) J. Emb. Exper. Morph. 55, 1-15.
4. Luque, L., Lemanski, L.F., and Dube, D.K. (1994) Biochem. Biophys. Res. Commun. 203, 319-325.
5. Gaur, A., Lemanski, L.F., and Dube, D.K. (1995) Gene (in press).
6. Whiteley, M., and Armstrong, J.B. (1990) Biochem. Cell Biol. 68, 622-629.
7. Busse, U., and Se'guin, C. (1993) Molecular analysis of the Wnt-1 proto-oncogene in *Ambystoma mexicanum* (axolotl) embryos. Differentiation 53, 7-15.
8. Offer, G., Moos, C., and Starr, R. (1973) J. Mol. Biol. 74, 653-676.
9. Craig, R., and Offer, G. (1976) Proc. R. Soc. Lond. B. Biol. Sci. 192, 451-461.
10. Offer, G. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 87-93.
11. Bandman, E. (1992) Dev. Biol. 154, 273-283.
12. Kasahara, H., Itoh, M., Sugiyama, T., Kido, N., Hayashi, H., Saito, H., Tsukita, S., and Kato, N. (1994) J. Clin. Invest. 94, 1026-1036.
13. Obinata, T., Reinach, F.C., Bader, D.M., Masaki, T., Kitani, S., and Fischman, D.A. (1984) Dev. Biol. 101, 116-124.
14. Fürst, D., Vinkemeier, U., and Weber, K.J. (1992) Cell Sci. 102, 769-778.
15. Takano-Ohmuro, H., Goldfine, S., Kojima, T., Obinata, T., and Fischman, D.A. (1989) J. Muscle Res. Cell Motil. 10, 369-378.
16. Ward, S .M. (1995) Ph.D. Dissertation: SUNY Health Science Center at Syracuse, New York.
17. Einheber, S., and Fischman, D. (1990) Proc. Natl. Acad. Sci. 87, 2157-2161.
18. Weber, F., Vaughan, K., Reinach, F., and Fischman, D. (1993) Eur. J. Biochem. 216, 661-669.
19. Bordzilovskaya, N.P., Dettlaff, T.A., Duhon, S.T., and Malacinski, G.M. (1989) In Developmental Biology of the Axolotl (J.B. Armstrong and G.M. Malacinski, Eds.), pp. 201-219, Oxford Press, New York.
20. Mierendorf, R.C., Percy, C., and Young, R.A. (1987) Methods of Enzymology 152, 458-469.
21. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
22. Sanger, F., Nicklen, S., and Coulson, A. (1977) Proc. Natl. Acad.Sci. USA 74, 5463-5467.
23. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
24. LaFrance, S., and Lemanski, L.F. (1995) Int. J. Dev. Biol. 38, 695-700.