Expression of the Ca\textsuperscript{2+}-binding Protein, Parvalbumin, during Embryonic Development of the Frog, \textit{Xenopus laevis}

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Abstract. A cDNA segment encoding the Ca\textsuperscript{2+}-binding protein, parvalbumin, was isolated with the use of antibodies, from a \textit{\lambda}gt\textit{ll} expression library of \textit{Xenopus laevis} tadpole poly(A)+ RNAs. The bacterially expressed beta-galactosidase-parvalbumin fusion protein of one lambda recombinant shows high affinity \textsuperscript{45}Ca\textsuperscript{2+} binding. The sequence of the tadpole parvalbumin is highly similar to previously characterized beta-parvalbumins of other organisms. Data from protein and RNA blotting experiments demonstrate that parvalbumin is absent in oocytes, eggs, and early staged embryos, and only becomes expressed during embryogenesis at the time of myogenesis. The protein can be detected in individual developing muscle cells and in muscle fibers of tadpole tail muscles. A simple method is also described for the isolation of neural tube-notochord-somite complexes from \textit{Xenopus} embryos.

\textbf{Parvalbumin} is a Ca\textsuperscript{2+}-binding protein that has been identified in numerous types of vertebrates and that belongs to the troponin C superfamily of Ca\textsuperscript{2+}-binding proteins. Characterized parvalbumins are 104–109 amino acids long, have an acidic pI, and have two high affinity Ca\textsuperscript{2+}-binding sites (19, 24). The Ca\textsuperscript{2+}-binding sites also display high affinity for Mg\textsuperscript{2+} (17).

The distribution of parvalbumin in adult animal tissues is not uniform. In vertebrates it is most abundant in fast-twitch muscles where concentrations as high as 1 mM have been measured (42). In muscle, the protein is thought to be involved in muscle relaxation (5, 15, 21, 41). A mouse mutant (adr) with defects in muscle contraction has reduced parvalbumin levels (40). Parvalbumin has also been detected in developing bones, some endocrine glands, and gamma-aminobutyric acid–containing neurons of the brain (2, 8). The role of parvalbumin in these other tissues is unknown, although it is suspected that it participates in some type of cellular Ca\textsuperscript{2+} regulation (19).

Unfortunately, the developmental regulation of parvalbumin in early embryonic stages has not been previously examined. Since Ca\textsuperscript{2+} has been implicated in numerous embryonic processes (4, 13, 29), one may suspect that a Ca\textsuperscript{2+}-binding protein, such as parvalbumin, may play an important role in tissue differentiation, cell migration, and other events of early embryo development.

We have decided to study parvalbumin regulation during embryonic development in the frog, \textit{Xenopus laevis}. In this paper, we describe the cloning of a cDNA encoding a parvalbumin of tadpoles. Using the isolated cDNA segment and available antibodies as molecular probes, we find that parvalbumin and its mRNA are absent during early development. We infer from their absence that parvalbumin does not play an important role in oogenesis, fertilization, blastula formation, or gastrulation. The protein and mRNA are first demonstrable in later-staged embryos at the time of somite differentiation. These molecules accumulate significantly in the muscle fibers of tadpoles.

A simple procedure is also described for the isolation of the neural tube–notochord–somite (NSS) complex of frog embryos. This complex provides useful starting material for the study of the molecular biology of neural tube development. The complex can serve as a source of antigens and mRNAs for the construction of monoclonal antibody and recombinant cDNA libraries.

\textbf{Materials and Methods}

\textbf{Cloning and Characterization of Parvalbumin \textit{cDNA}}

A \textit{\lambda}gt\textit{ll} tadpole cDNA library was prepared by transferring the inserts of a plasmid DNA library (12) into the Eco RI site of \textit{\lambda}gt\textit{ll} (43). cDNA inserts were released from the recombinant plasmids by digestion with Hinc II, cleaved separately with Hae III or Alu I, modified by Eco RI methylase, and inserted with linkers into the Eco RI site of \textit{\lambda}gt\textit{ll}. All restriction endonucleases, DNA modification enzymes, and linkers were obtained from New England Biolabs (Beverly, MA) and used according to the directions of the manufacturer. Manipulations of the DNA and resulting recombinant phage were performed according to standard protocols (33). The tadpole \textit{\lambda}gt\textit{ll} library was screened with rabbit antibodies generated against HPLC purified \textit{Xenopus} leg muscle parvalbumin antibodies (gift of Y.-H. Chien, C. Klee, and I. Dawid), goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA), and chloronaphthol (Sigma Chemical Co., St. Louis, MO). Four recombinants with identical sized inserts were isolated; one recombinant was characterized further and was named pPV1. The 318-bp Eco RI insert of pPV1 was cloned in both orientations into M13mp8 and its nucleotide sequence determined (39). The Eco RI fragment was also cloned into the plasmid vector pUC8 as a hybridization probe; this recombinant is named pPV1. The fragment was also used

1. Abbreviations used in this paper: NNS complex, neural tube–notochord–somite complex.

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to screen a tadpole plasmid cDNA library (12) by molecular hybridization; one recombinant (pPV2) was isolated and partially sequenced.

**Protein and RNA Blot Analysis**

To analyze the protein products of bacteria infected with λgt11 phage the following protocol was used. 0.2 ml of Y1090 bacterial cultures was infected with λgt1 phage and then plated out in soft agar containing 10 mM isopropyl beta-d-thiogalactopyranoside onto petri plates (43). After confluent lysis occurred 6 h later, the plates were covered with PBS (consisting of 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4) for 3 h to allow diffusion of proteins into the buffer. The liquid was then clarified of bacterial debris by centrifugation and proteins were recovered from the supernatant by 10% TCA precipitation. The pellets were then washed three times with ice cold acetone, air dried, and boiled in gel loading buffer. Protein mixtures were resolved by electrophoresis in 8% polyacrylamide-SDS gels (25, 32). Western blots (41) were done with 121-labeled goat-anti-rabbit IgG antibodies (gift of M. Beckerle, University of North Carolina, Chapel Hill, NC). Detection of 32Ca2+ binding to nitrocellulose-blotted proteins (34) was kindly performed by M. Krimps and C. Kiecz (National Institutes of Health, Bethesda, MD).

Protein lysates were also prepared from full grown oocytes, unfertilized eggs, various embryonic stages (37), manually dissected tadpole heads and tails, and adult leg muscle by homogenization in 2% SDS, 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM EDTA, 1 mM leupeptin, 1 mM aprotinin, and 1 mM phenylmethylsulfonylfuoride, and then given a heat treatment in a boiling water bath for 3 min. Dyes were added to the heated homogenates and the mixtures were resolved by gel electrophoresis on a 10% polyacrylamide-SDS gel. Occasionally the gels were stained with Coomassie Blue stain to assure that the protein samples were not degraded.

RNA was extracted from oocytes, eggs, and various staged embryos by homogenizing material in a Dounce tissue homogenizer (model K-885300; made by Kontes Glass Co., Vineland, NJ) with 4 M guanidine thiocyanate, 1% beta-mercaptoethanol, 10 mM EDTA. Samples were then phenol-chloroform-extracted to remove the proteins and subjected to two 4-M LiCl precipitations to remove DNA. For each gel lane, 10 µg of total RNA was treated with formaldehyde, resolved by gel electrophoresis in 1.4% agarose, 6% formaldehyde, and then blotted to nitrocellulose (33). Parvalbumin RNA species were detected by molecular hybridization at high stringency with 32P nick-translated pPV1 followed by XAR-5 film autoradiography. Hybridizations were also performed with nick-translated p7L, a pBR322-cDNA recombinant containing the coding region for Xenopus calmodulin (9).

**Immunofluorescent Staining**

Parvalbumin was detected in tadpole tails by immunofluorescence techniques. Tadpoles were fixed for 2 h in Bouin's fixative (I) at room temperature, and the tails were isolated by dissection. Strips of tail were then treated in the following manner: they were first permeabilized overnight at 4°C in PBS with 2% Triton X-100, and exposed to antibodies diluted (1:200) in PBS with 0.2% Triton X-100 and 0.1% sodium azide for 1 h at room temperature, then washed with PBS for 1 h, incubated with goat anti-rabbit IgG antibodies coupled to fluorescein for 1 h, and finally, washed for 1 h with PBS. The specimens were then transferred to a drop of buffered glycerol and viewed by epifluorescence with a Nikon Optiphot microscope. Kodak Tri-X film was exposed at 800 ASA and processed accordingly.

Somites were prepared from stage 28 embryos and evaluated by immunofluorescence for parvalbumin expression. Embryos were incubated in a calcium chelation medium (36) for 30 min and the NNS complex was isolated from the dissociated cells. The complex was freed of loosely attached cells by gentle pipetting up and down. The complex was then fixed in 2% paraformaldehyde in PBS for 30 min, transferred to a subbed slide with a drop of the same fixative, and squashed with a coverslip. The slide was then placed on a block of dry ice for several minutes. The coverslip was then removed with a razor blade and the slide placed into a Coplin jar of 95% ethanol for 5 min. Nonspecific protein-binding sites on the slides were blocked with 5% BSA in PBS for 15 min. For immunological detection, the specimens were treated as above.

**Results**

**Isolation and Characterization of Parvalbumin cDNA Clone from Tadpoles**

A λgt11 expression library of Xenopus tadpole cDNA inserts was screened immunologically with antibodies to Xenopus leg muscle parvalbumin proteins. Four recombinants producing cross-reactive antigen were identified and isolated for study. Since these recombinants all contained an identically sized Eco RI insert, one was chosen for further study and was named λPV1. The hybrid protein encoded by λPV1 was analyzed by SDS PAGE. As seen in Fig. 1 A, bacteria infected with this recombinant and grown in the presence of the inducer, isopropyl beta-d-thiogalactopyranoside, produce a new protein of 123 kD. This new protein appears to represent the fusion of beta-galactosidase and the product of the cDNA insert.

The fusion protein has been examined for its antigenicity and its ability to bind Ca2+. As seen in the Western blot shown in Fig. 1, preimmune antibodies react weakly with a few common antigens present in both lysates, but not with beta-galactosidase or the fusion protein. In contrast, the rabbit anti-parvalbumin antibodies produce a significant reaction with the fusion protein of λPV1 in Western blots. When comparable Western blots were probed with 32Ca2+, a single band of calcium binding activity corresponding to the fusion protein was seen. Thus, it appears that the bacterially expressed λPV1 fusion protein is both immunologically and functionally similar to parvalbumin.

The DNA sequences of the Eco RI insert of λPV1 and a portion of the isolated tadpole cDNA plasmid pPV2 are displayed in Fig. 2. From conceptual translation of the nucleotide sequences a polypeptide of 109 amino acids was deduced. In the original lambda recombinant it appears that beta-galactosidase is fused to the parvalbumin coding region upstream of the methionine start site. This interpretation is
Tadpole parvalbumin cDNA

AGATTT ACTATGGCATTCGGTGGTAT(~CTGAGTGAGGCTGACA~TGCCCTGCAGAACTGCcAA~TGAcTCCTTCAACTTCAAAAcTT~L-~A~~GC
ArgPheThrMetAlaPheGlyGlyIleLeuSerGlUAlaAspIleSerAlaGeInAlaAspSerPheAlaIleSerPheAlaGlnSer

140 160 180 200 220 240
AGCAAGTCCGCAC~ATGATGTGAAAAAC~T(3cTCGA~cAGGACAGGAGCGGCTTCATTGAGGAAGAGGAACTGAAGTTG~A~~~~
Set LysSer AlaAspAspVal LysAsnVal PheAlaIleLeuAspGlnAs~ArgSerGlyPheIleGlUPheGlUGluGluLeuLysLeuPheLeuGln

260 280 300 x 320 340
TGA~TGA~GGCCTTccTGGCAGCTGGTGA~TGGTGATGGCAAAATTGGAGTI~AAGAATTCCAGTCCCTAGTCAAA~GAAGTAAGAcCAA...
LeuThrAspAlaGluThrIleAlaPheLeuAlaAspGlySerGlyPheIleAlaGluGlnSerLeuValLysPro***

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Detection of Parvalbumin and Its RNA in Embryos

Western blots of frog embryonic proteins indicate that parvalbumin is developmentally regulated. From a survey of different developmental stages of *Xenopus* (Fig. 3), it is clear that parvalbumin is absent in oocytes, eggs, and early embryos, whereas it becomes detectable in whole extracts of stage 24 embryos and accumulates dramatically thereafter. In tadpoles, the parvalbumin protein is principally found in dissected tail fragments, although a significant amount can be detected in tadpole head fragments (Fig. 3).

The developmental pattern of parvalbumin RNA accumulation has been established by RNA gel blotting with labeled *pPV1* DNA, which contains only the parvalbumin coding region. A survey of total RNA populations in different embryonic stages is shown in Fig. 4. Parvalbumin homologous RNA is absent in oocytes, eggs, and early embryos, and is detectable in stage 37 and stage 41 embryos. The size of the parvalbumin transcript is 770 nucleotides; it is presumably detectable in stage 37 and stage 41 embryos. The parvalbumin transcript length heterogeneity, or the expression of similar sized transcripts from related genes. Additionally, we are currently quantitating the amounts of parvalbumin protein and RNA at the various embryonic stages to follow the accumulation of these molecules more precisely during embryogenesis.

To localize the parvalbumin antigen in the tails of stage 41 tadpoles, we performed indirect immunofluorescence on whole mount preparations of tadpole tail strips. Examination of the slides revealed a very strong fluorescence signal with individual tail muscle fibers (Fig. 5). Preimmune sera or antisera against traditionally nonmuscle proteins, such as serotonin antibodies, failed to stain these muscle fibers (data not shown).

Detection of Parvalbumin in Developing Muscle Cells

We have investigated the developmental appearance of parvalbumin during myogenesis with protein blotting and immunofluorescence experiments. To aid the detection, we have devised a simple purification protocol (see Materials and Methods) for the isolation of NNS complexes (Fig. 6 B) from stage 28 embryos (Fig. 6 A). When the complexes are squashed, the muscle cells become well dispersed (Fig. 6 C) and are easily identified by their elongate shape and the presence of a few myofibrils (Fig. 6 D). These cells appear to still contain yolk platelets and have only one observable nucleus. The myotome cells of *Xenopus* are unusual in that they develop to their fully differentiated state as mononucleated cells (35). These same cells stain well with anti-parvalbumin antibodies (Fig. 6 E), indicating that the protein accumulates during myofibril formation. Nonmuscle cells do not stain with the antibody (Fig. 6 E).

Protein blotting experiments (Fig. 7) also confirm that the NNS complex contains all, or the vast majority, of the parvalbumin present in the stage 28 embryo. At this level of resolution, the embryonic and adult leg muscle parvalbumins are similar in size (12 kD). The identities of the two additional cross-reactive species, 13.5 and 19 kD, are not known.

Discussion

In this paper we describe the expression of the Ca$^{2+}$-binding protein, parvalbumin, during embryogenesis of *Xenopus*. Neither the protein nor its mRNA appear to be maternally derived, but instead are found synthesized de novo during development. It can be inferred from parvalbumin's absence in oocytes, eggs, and early embryos that the protein plays no major role in oogenesis, egg fertilization, early cleavage, or gastrulation. Parvalbumin might have been expected to be expressed in the early embryo since a number of human and rodent carcinomas express parvalbumin-like Ca$^{2+}$-binding proteins (30, 31, 38). It has been speculated that these proteins may be involved in cell replication (3) and motility (38). Apparently, early *Xenopus* embryos do not require parvalbumin (or structurally related proteins) for either of these two processes.
Parvalbumin is first detected in *Xenopus* embryos at stage 24, which is coincident with the initiation of muscle development. At this stage, the muscle cells are active both in the expression of parvalbumin and the assembly of their contractile apparatus. Even though the embryonic muscle cells are not fully differentiated in comparison with skeletal muscle, they are clearly able to contract and relax, as demonstrated by the ability of stage 24 embryos to twitch rapidly upon external stimulation (35, 37). The coordinated expression of parvalbumin and the contractile proteins in frog muscle cells supports the belief that parvalbumin may play a role in muscle cell contraction-relaxation; a similar function has been postulated for the protein in adult vertebrate muscles (5, 17).

In stage 41 swimming tadpoles, parvalbumin is quite abundant in tail muscle fibers. This protein appears to be muscle specific in the developing embryos, and is restricted to fast-twitch muscle fibers (Kay, B. K., and L. Schwartz, manuscript in preparation). Parvalbumin expressing and nonexpressing muscle fibers have also been detected in the tadpole head.

The pattern of parvalbumin expression during myogenesis differs somewhat between frogs and chickens. Parvalbumin is not detectable in the leg muscles of chick embryos until just before hatching (26), whereas the majority of contractile proteins are synthesized upon myoblast fusion (II, 20). Delayed expression of parvalbumin in chick embryonic leg muscles may be a mechanism of preventing leg muscle contraction while the embryo is in the eggshell. The early expression of parvalbumin in frog myogenesis, on the other hand, might be an advantageous strategy for allowing frog embryos to respond to external stimuli before they attain their free swimming form.

During the metamorphosis of the tadpole to frog, the tail of the *Xenopus* tadpole degenerates and disappears without a trace, coincident with the development of legs (37). Thus, the parvalbumin present in the leg muscles of *Xenopus* tadpoles and adults is either due to reexpression of the embryonic parvalbumin gene or expression of a second member of a parvalbumin gene family. We are currently investigating these two possibilities. Preliminary evidence suggests that...
Figure 6. Immunofluorescent detection of parvalbumin in stage 28 developing muscle cells. A shows a stage 28 embryo, B displays an isolated NNS complex from a stage 28 embryo, C shows an azure blue-stained squash of the NNS complex, D shows a phase contrast image of the differentiating muscle cells, and E shows the fluorescence of these cells reacted with anti-parvalbumin antibodies. Bars: (A) 400 μm; (C) 85 μm; (D) 15 μm.

Figure 7. Western blot detection of parvalbumin in developing somites of Xenopus embryos. Protein lysates were prepared from adult leg muscle, the NNS complex of two stage 28 embryos, the remainder of two stage 28 embryos minus their NNS complexes, and one complete stage 28 embryo. The proteins were resolved on a 18% polyacrylamide-SDS gel by gel electrophoresis and blotted to nitrocellulose. The antigen complexes formed with rabbit anti-parvalbumin antibodies were detected with 125I-protein A and X-ray film autoradiography.

several genes in the Xenopus genome potentially encode for parvalbumin (data not shown).

Recently a set of Ca²⁺-binding proteins have been found to be developmentally regulated in sea urchin embryos. During sea urchin embryogenesis, the Spec proteins are synthesized de novo and accumulate in presumptive dorsal ectoderm cells (28, 38). These proteins are related in primary sequence to the troponin C superfamily (7), which includes calmodulin, troponin C, myosin light chains, and parvalbumin. Among this family of Ca²⁺-binding proteins, the sea urchin Spec proteins and vertebrate parvalbumins are not very similar; between Spec1 and tadpole parvalbumin there is only 30% identity in a 68 amino acid overlap. In sea urchins, the Spec proteins are thought to regulate the Ca²⁺-induced contraction of the dorsal ectoderm during larval development and metamorphosis.

It is of interest to compare the Xenopus parvalbumin coding sequence with Xenopus calmodulin and rat parvalbumin sequences. Ca²⁺-binding protein genes are thought to have evolved through tandem duplications of a primordial Ca²⁺-binding domain that gave rise to an ancestral four-domain protein (16, 18, 24). Calmodulin, with its four Ca²⁺-binding
domains, is thought to be closest to the ancestral four-domain protein, whereas parvalbumin appears to have evolved the most by losing two domains; one by deletion and the other by mutation (16). Between the Xenopus calmodulin (9) and parvalbumin proteins and mRNAs, the similarity is 30 and 40% at the amino acid and nucleotide sequence (coding region) levels, respectively. The similarity is even better between the Xenopus and rat parvalbumin proteins and mRNAs (14); the two proteins and coding regions are 56–57% similar. It should be noted that there is no significant homology between the untranslated sequences of the Xenopus tadpole cDNA and the corresponding regions of Xenopus calmodulin and rat parvalbumin cDNAs. In the future, we intend to characterize the developmental and cellular expression of the Xenopus parvalbumin family and elaborate the evolutionary relationship between parvalbumin and other Ca$^{2+}$-binding proteins by isolating the corresponding Xenopus parvalbumin gene(s).

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