Expression of the Ca\(^{2+}\)-binding Protein, Parvalbumin, during Embryonic Development of the Frog, *Xenopus laevis*

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**Abstract.** A cDNA segment encoding the Ca\(^{2+}\)-binding protein, parvalbumin, was isolated with the use of antibodies, from a λgt11 expression library of *Xenopus laevis* tadpole poly(A)+ RNAs. The bacterially expressed beta-galactosidase-parvalbumin fusion protein of one lambda recombinant shows high affinity \(^{45}\)Ca\(^{2+}\) 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 *Xenopus* embryos.

**Materials and Methods**

**Cloning and Characterization of Parvalbumin cDNA**

A λgt11 tadpole cDNA library was prepared by transferring the inserts of a plasmid DNA library (12) into the Eco RI site of λgt11 (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 λgt11. 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 λgt11. The complex can serve as a source of antigens and mRNAs for the construction of monoclonal antibody and recombinant cDNA libraries.

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1. Abbreviations used in this paper: NNS complex, neural tube-notochord-somite complex.
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 λgtll phage the following protocol was used. 0.2 ml of Y1090 bacterial cultures was infected with λ 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 45Ca2+ binding to nitrocellulose-blotted proteins (34) was kindly performed by M. Krinks and C. Klee (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 phenylmethylsulfonylfluoride, 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 20% 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% formamide, 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 p71, a pBR322-eDNA 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 (1) 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 0.1% sodium azide for 1 d at room temperature, then washed with PBS, then treated with 0.2% Triton X-100 and 0.1% sodium azide for 1 d 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 λgtll 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 45Ca2+, 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

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Gel electrophoretic analysis of proteins encoded by recombinant λPV1. A displays the Coomassie Blue-staining pattern of a 10% polyacrylamide–SDS gel. B represents a Western blot with preimmune serum, C shows a similar blot with immune serum, and D exhibits an autoradiogram of 45Ca2+ binding to filter-bound proteins. In the four panels, the proteins synthesized by bacteria infected by the vector λgtll are shown in lanes marked λ, whereas proteins produced by bacteria infected by λPV1 are shown in lanes marked PV. The immune complexes on the filters were detected with secondary goat anti-rabbit IgG antibody coupled to horseradish peroxidase and chloronaphtho, whereas the 45Ca2+ binding was detected by film autoradiography. Lane M contains molecular weight standards with the molecular weights of the individual species listed in kilodaltons on the left.
Tadpole parvalbumin cDNA

Homology between Xenopus and carp parvalbumins

Figure 2. Nucleotide sequence of the APV1 insert, a portion of pPV2 recombinant cDNA plasmid, and the predicted parvalbumin protein.

The insert of APV1 was sequenced by the dideoxy method in both directions; the sequence is delineated with x's. The complete protein sequence was deduced by sequencing rightward from the Pvu II site of the recombinant pPV2. A protein encoded by the longest open reading frame in this sequence is shown, with a putative translation start site (Met) and the high affinity Ca2+-binding loop domains (24) of parvalbumin underlined, and the termination codon marked with asterisks. In this same interval, there are 6 and 11 translation stop codons in each of the other two forward reading frames. The regions of homology between carp beta-parvalbumin (10) and the Xenopus putative parvalbumin are shown below; identities and semiconservative changes between the two sequences (27) are noted by two and one dots, respectively.

supported by the findings that the amino acid sequence preceding the first Met residue does not match any known parvalbumin sequences, and that the nucleotide sequence surrounding the Met codon (ACTATGG) resembles the consensus sequence of eukaryotic translational initiation sites (PuCCATGG) (23). The putative frog protein sequence shows striking similarity with characterized parvalbumin proteins of other animal species. The best overall match (76%) was with carp beta-parvalbumin (10). The putative frog polypeptide also shows 67 and 51% amino acid identity with Rana and rat beta-parvalbumins (1, 6), respectively, according to the computer protein alignment programs (27). The similarity of the tadpole protein with known alpha-parvalbumins is much lower; for instance, it has only 53% homology with Rana alpha-parvalbumin (22). On the basis of the high degree of similarity between the tadpole clone and known beta-parvalbumins, we believe the recombinant that we have isolated encodes a Xenopus beta-parvalbumin. It should also be noted that the Xenopus parvalbumin sequence

Figure 3. Western blot with rabbit anti-parvalbumin antibodies to lysates from oocytes, eggs, and various Xenopus staged embryos. The lanes (from left to right) contain extracts from 1/4 equivalents of oocyte (stage VI), egg, stage 10 (gastrula), stage 16 (neurula), stage 22, stage 24 (tailbud), stage 31, stage 41 (tadpole) embryos, and dissected stage 41 head and tail segments. Proteins were resolved by electrophoresis in a 20% polyacrylamide-SDS gel and later transferred electrophoretically to nitrocellulose. Immune complexes were detected with 125I-labeled goat anti-rabbit IgG antibodies and film autoradiography.
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 polyadenylated, given its quantitative binding to poly U-Sepharose (data not shown). The size of the RNA is similar to one of the two parvalbumin RNAs (761 and 1,161 nucleotides long) that have been described in rat (14).

The parvalbumin RNA first becomes detectable in *Xenopus* embryos at about stage 24 (data not shown), coinciding with the appearance of the protein. Thus, the temporal pattern of parvalbumin RNA accumulation roughly matches the accumulation pattern for the protein (Fig. 3). The absence of hybridization to RNAs isolated from early embryonic stages is not due to extensive RNA degradation; strong hybridization signals can be found in these lanes when blots are reprobed with a nick-translated *Xenopus* calmodulin cDNA clone (data not shown). We are presently investigating the explanation for the broad appearance of the hybridizing parvalbumin RNA bands; it may be due to slight RNA degradation, 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, in the leg muscle extracts (Fig. 7) 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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-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. Ca^{2+}-binding protein genes are thought to have evolved through tandem duplications of a primordial Ca^{2+}-binding domain that gave rise to an ancestral four-domain protein (16, 18, 24). Calmodulin, with its four Ca^{2+}-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 untruncated 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 Ca²⁺-binding proteins by isolating the corresponding Xenopus parvalbumin gene(s).

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