

Expression of the Ca²⁺-binding Protein, Parvalbumin, during Embryonic Development of the Frog, *Xenopus laevis*

Brian K. Kay, Ami J. Shah, and William E. Halstead

Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27514

Abstract. A cDNA segment encoding the Ca²⁺-binding protein, parvalbumin, was isolated with the use of antibodies, from a λ gt11 expression library of *Xenopus laevis* tadpole poly(A)⁺ RNAs. The bacterially expressed beta-galactosidase-parvalbumin fusion protein of one lambda recombinant shows high affinity ⁴⁵Ca²⁺ 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.

PARVALBUMIN is a Ca²⁺-binding protein that has been identified in numerous types of vertebrates and that belongs to the troponin C superfamily of Ca²⁺-binding proteins. Characterized parvalbumins are 104–109 amino acids long, have an acidic pI, and have two high affinity Ca²⁺-binding sites (19, 24). The Ca²⁺-binding sites also display high affinity for Mg²⁺ (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 Ca²⁺ regulation (19).

Unfortunately, the developmental regulation of parvalbumin in early embryonic stages has not been previously examined. Since Ca²⁺ has been implicated in numerous embryonic processes (4, 13, 29), one may suspect that a Ca²⁺-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, *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 forma-

tion, 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)¹ 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.

Materials and Methods

Cloning and Characterization of Parvalbumin cDNA

A λ gt11 tadpole cDNA library was prepared by transferring the inserts of a plasmid DNA library (12) into the Eco RI site of λ gt11 (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 λ gt11. 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 λ gt11 library was screened with rabbit antibodies generated against HPLC purified *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 λ PV1. The 318-bp Eco RI insert of λ PV1 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.

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 λ gt11 phage the following protocol was used. 0.2 ml of Y1090 bacterial cultures was infected with 10^5 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 Na_2HPO_4 , and 1.5 mM KH_2PO_4) 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 ^{125}I -labeled goat-anti-rabbit IgG antibodies (gift of M. Beckerle, University of North Carolina, Chapel Hill, NC). Detection of $^{45}\text{Ca}^{2+}$ 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% formaldehyde, and then blotted to nitrocellulose (33). Parvalbumin RNA species were detected by molecular hybridization at high stringency with ^{32}P nick-translated pPV1 followed by XAR-5 film autoradiography. Hybridizations were also performed with nick-translated p71, 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 (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 exposed to antibodies diluted (1:200) in PBS 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 6% 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 λ gt11 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 λ PV1. The hybrid protein encoded by λ PV1 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 Ca^{2+} . 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 λ PV1 in Western blots. When comparable Western blots were probed with $^{45}\text{Ca}^{2+}$, a single band of calcium binding activity corresponding to the fusion protein was seen. Thus, it appears that the bacterially expressed λ PV1 fusion protein is both immunologically and functionally similar to parvalbumin.

The DNA sequences of the Eco RI insert of λ PV1 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

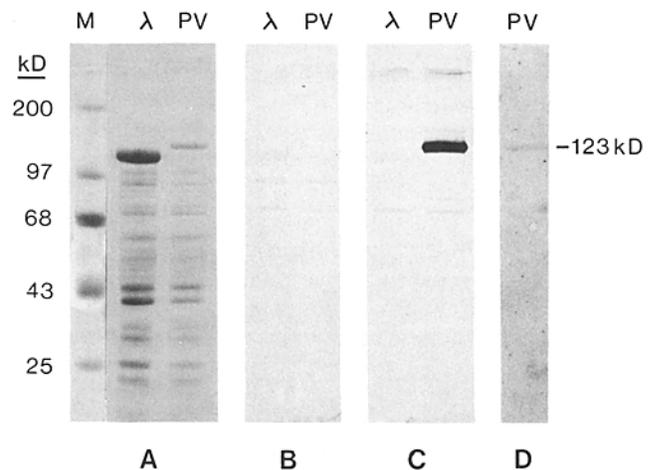


Figure 1. Gel electrophoretic analysis of proteins encoded by recombinant λ PV1. A displays the Coomassie Blue-staining pattern of a 10% polyacrylamide-SDS gel, B represents a Western blot with preimmune serum, C shows a similar blot with immune serum, and D exhibits an autoradiogram of $^{45}\text{Ca}^{2+}$ binding to filter-bound proteins. In the four panels, the proteins synthesized by bacteria infected by the vector λ gt11 are shown in lanes marked λ , whereas proteins produced by bacteria infected by λ PV1 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 chloronaphthol, whereas the $^{45}\text{Ca}^{2+}$ 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.

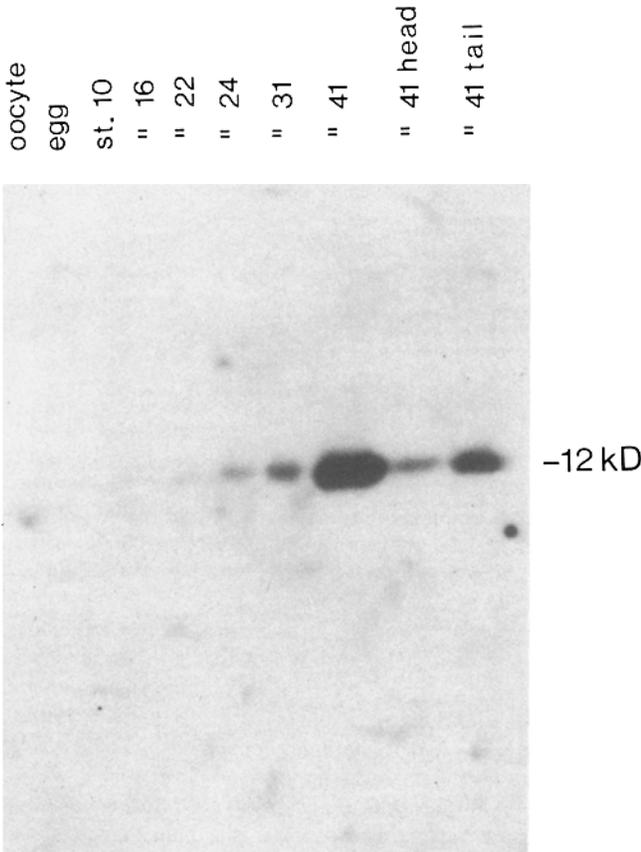
Tadpole parvalbumin cDNA

x
 20 40 60 80 100 120
 AGATTACTATGGCATTCGGTGGTATCCTGAGTGGCTGACATCTCTGCTGCCCTGCAGAACTGCCAAGCTGCTGACTCCTTCAACTTCAAAACTTTCCTTTGCCAGTCTGGTCTGAC
 ArgPheThrMetAlaPheGlyGlyIleLeuSerGluAlaAspIleSerAlaAlaLeuGlnAsnCysGlnAlaAlaAspSerPheAsnPheLysThrPhePheAlaGlnSerGlyLeuSer
 140 160 180 200 220 240
 AGCAAGTCCGCAGATGATGTGAAAAACGCTTTCGCCATCCTCGACCAGGACAGGACGGCTTCAATTGAGGAAGAGGAACTGAAGTTGTTCCTCCAGAACTTCAGCGCAAGTGCCAGGGCA
 SerLysSerAlaAspAspValLysAsnValPheAlaIleLeuAspGlnAspArgSerGlyPheIleGluGluGluGluLeuLysLeuPheLeuGlnAsnPheSerAlaSerAlaArgAla
 260 280 300 x 320 340
 TGACTGATGCTGAAACCAAGGCTTCTCTGGCAGCTGGTGACTCTGATGGTGAATGGAAATTCAGTCCCTAGTCAAACCTTGAAGAAGTAAGACCAA...
 LeuThrAspAlaGluThrLysAlaPheLeuAlaAlaGlyAspSerAspGlyAspGlyLysIleGlyValGluGluPheGlnSerLeuValLysPro***

Homology between Xenopus and carp parvalbumins

	10	20	30	40	50	60	70	80	90	100
<u>Xenopus</u>	MAFGGILSEADISAAALQNCQAADSFNFKTFFAQSGLSKSSKADSVKVFALDQDRSGFIEEELKLFQNF	SASARALTD	AEKAF	LAAGDS	GDGKIG	VEEFQ	SLV	KP		
carp	AFAGVLNDADIAAALEACKAADSFNHKAFFKAVGLTSKSADSVKVFALDQDKSGFIEEELKLFQNF	KADARAL	TDGET	KTF	LKAGDS	GDGKIG	VDEFT	ALVKA		
	10	20	30	40	50	60	70	80	90	100

Figure 2. Nucleotide sequence of the λ PV1 insert, a portion of pPV2 recombinant cDNA plasmid, and the predicted parvalbumin protein. The insert of λ PV1 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 Ca^{2+} -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 se-

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 ^{125}I -labeled goat anti-rabbit IgG antibodies and film autoradiography.

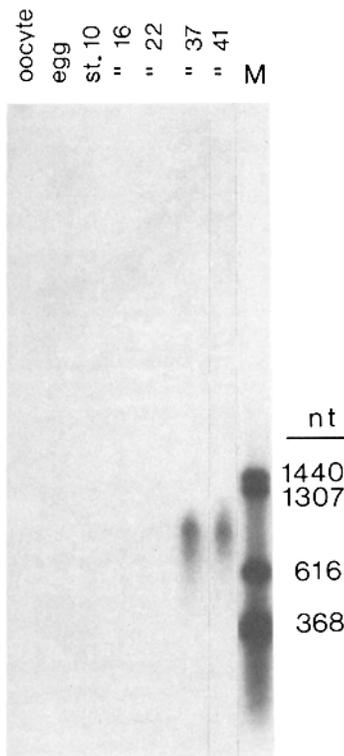


Figure 4. Northern blot analysis of parvalbumin homologous RNAs present in oocytes, eggs, and various staged embryos. The blot was hybridized with ^{32}P -labeled parvalbumin cDNA. The nucleotide (nt) lengths of some of the pBR322 DNA Taq I restriction fragments (lane M) are shown on the right of the autoradiogram. The lanes (from left to right) contain extracts from two oocytes (stage VI), eggs, stage 10 (gastrulae), stage 16 (neurulae), stage 22, stage 37, and stage 41 embryos.

quence contains two Ca^{2+} -binding sites (24) and they appear to be the only internally repeated regions within the protein (Fig. 2).

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 hy-

bridization signals can be found in these lanes when blots are reprobbed 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. 6B) from stage 28 embryos (Fig. 6A). When the complexes are squashed, the muscle cells become well dispersed (Fig. 6C) and are easily identified by their elongate shape and the presence of a few myofibrils (Fig. 6D). 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. 6E), indicating that the protein accumulates during myofibril formation. Nonmuscle cells do not stain with the antibody (Fig. 6E).

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.

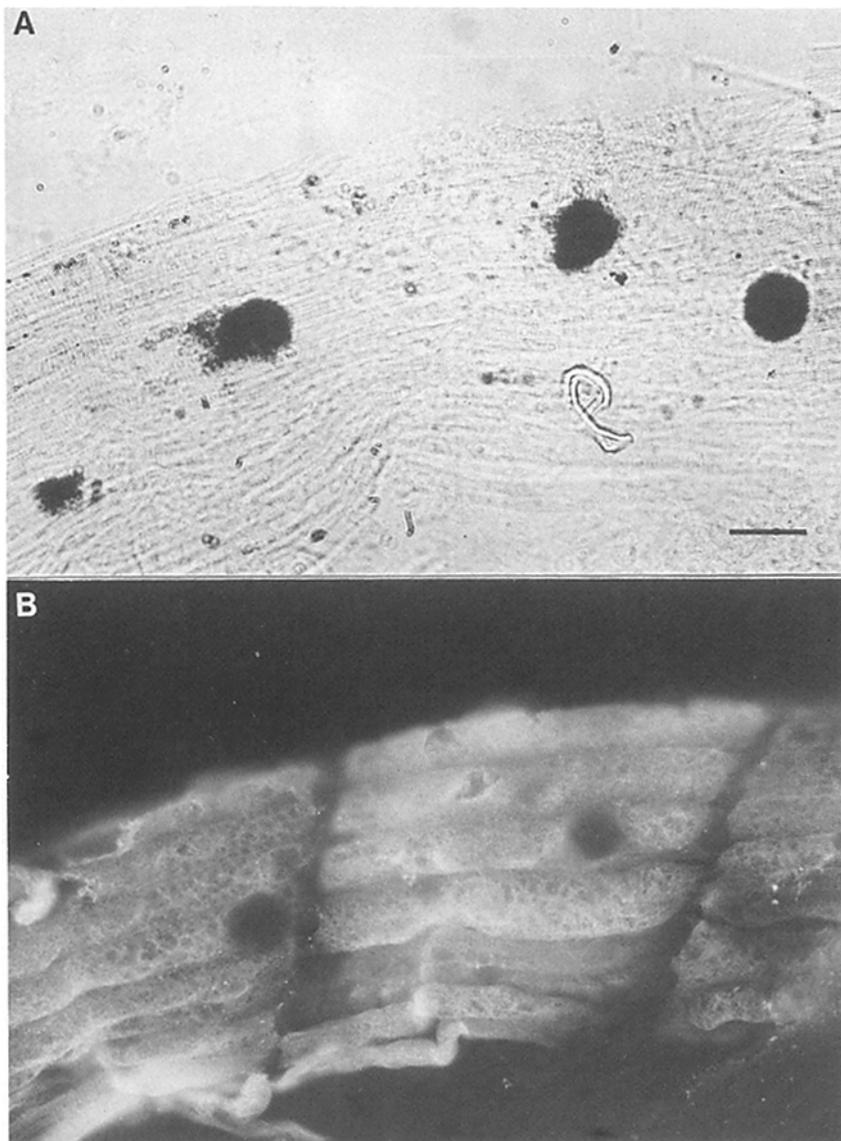


Figure 5. Immunological detection of parvalbumin in tadpole tail muscle cells. Strips of tail muscle from stage 41 tadpoles were permeabilized with Triton X-100 and incubated sequentially with anti-parvalbumin antibodies and goat anti-rabbit IgG antibodies coupled to fluorescein. *A* shows a phase contrast image of the tail muscle strip, whereas *B* shows the fluorescent image. The black spots in *A* are epidermal melanocytes. Bar, 30 μ m.

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 (11, 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

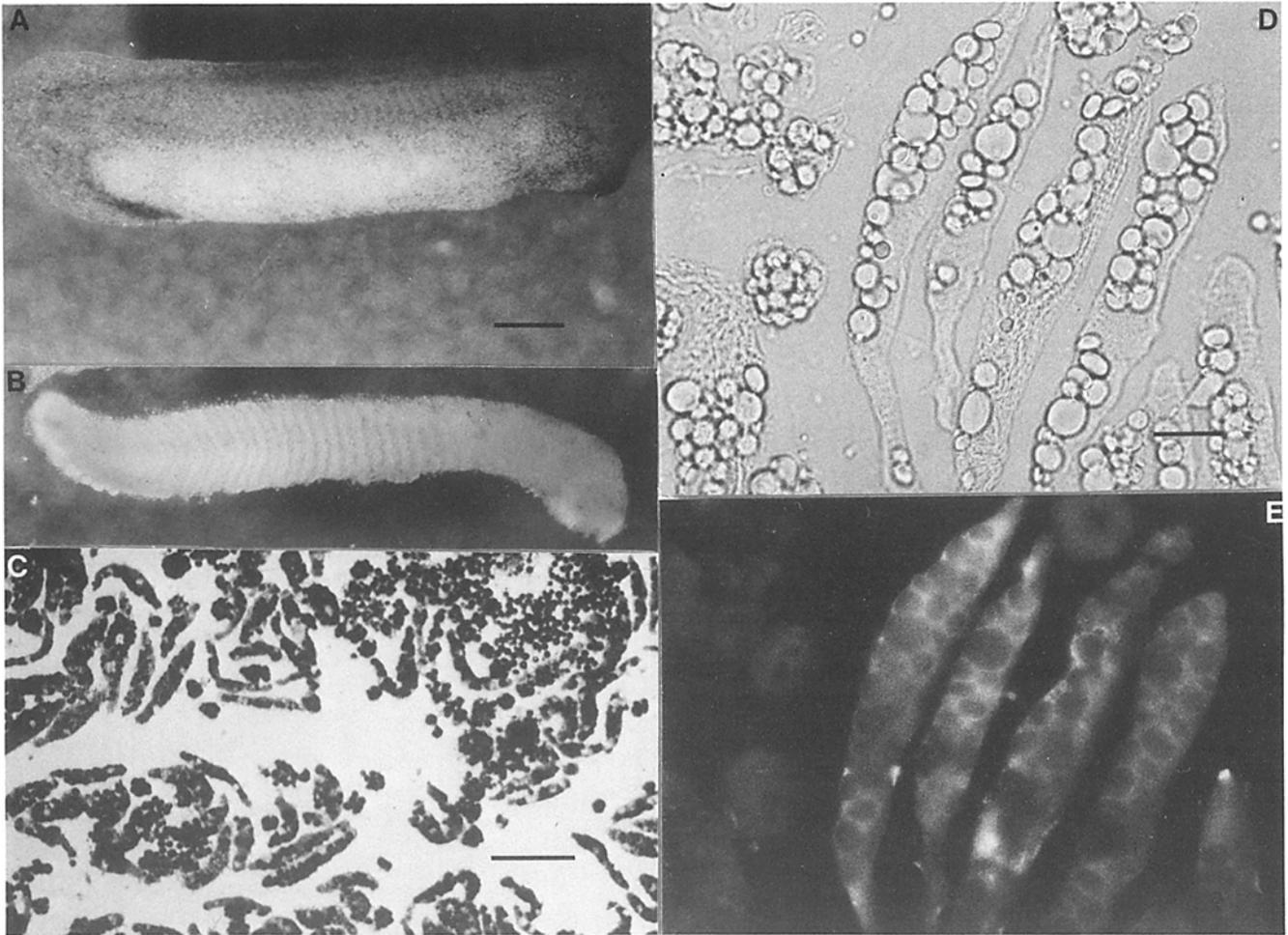


Figure 6. Immunofluorescent detection of parvalbumin in stage 28 developing muscle cells. *A* shows a stage 28 embryo, *B* displays an isolated NNS complex from a stage 28 embryo, *C* shows an azure blue-stained squash of the NNS complex, *D* shows a phase contrast image of the differentiating muscle cells, and *E* shows the fluorescence of these cells reacted with anti-parvalbumin antibodies. Bars: (A) 400 μm ; (C) 85 μm ; (D) 15 μm .

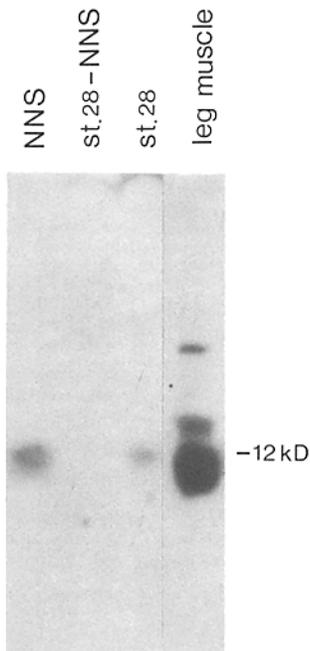


Figure 7. Western blot detection of parvalbumin in developing somites of *Xenopus* embryos. Protein lysates were prepared from adult leg muscle, the NNS complex of two stage 28 embryos, the remainder of two stage 28 embryos minus their NNS complexes, and one complete stage 28 embryo. The proteins were 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 ^{125}I -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 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 Ca²⁺-binding proteins by isolating the corresponding *Xenopus* parvalbumin gene(s).

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References

- Berchtold, M. W., M. R. Celio, and C. W. Heizmann. 1984. Parvalbumin in nonmuscle tissues of the rat. Quantitation and immunohistochemical localization. *J. Biol. Chem.* 259:5189–5196.
- Berchtold, M. W., C. W. Heizmann, and K. J. Wilson. 1982. Primary structure of parvalbumin from rat skeletal muscle. *Eur. J. Biochem.* 127:381–389.
- Boynton, A. L., J. P. MacManus, and J. F. Whitfield. 1982. Stimulation of liver cell DNA synthesis by oncomodulin, an MW 11500 calcium-binding protein from hepatoma. *Exp. Cell Res.* 138:454–457.
- Campbell, A. K. 1983. Intracellular Calcium: Its Universal Role as Regulator. John Wiley & Sons, Inc., New York. 556 pp.
- Cannell, M. B., and D. G. Allen. 1984. Model of calcium movements during activation in the sarcomere of frog skeletal muscle. *Biophys. J.* 45:913–925.
- Capony, J.-P., J. Demaille, C. Pina, and J.-F. Pechere. 1975. The amino acid sequence of the most acidic major parvalbumin from frog muscle. *Eur. J. Biochem.* 56:215–227.
- Carpenter, C. D., A. M. Bruskin, P. E. Hardin, M. J. Jeast, J. Anstrom, A. L. Tyner, B. P. Brandhorst, and W. H. Klein. 1984. Novel proteins belonging to the troponin C superfamily are encoded by a set of mRNAs in sea urchin embryos. *Cell.* 36:663–671.
- Celio, M. R. 1985. Parvalbumin in most gamma-aminobutyric acid-containing neurons of the rat cerebral cortex. *Science (Wash. DC)* 231:995–997.
- Chien, Y.-H., and I. B. Dawid. 1984. Isolation and characterization of calmodulin genes from *Xenopus laevis*. *Mol. Cell. Biol.* 4:507–513.
- Coffee, C. J., and R. A. Bradshaw. 1973. Carp muscle binding protein. I. Characterization of the tryptic peptides and the complete sequence of component B. *J. Biol. Chem.* 248:3305–3312.
- Devlin, R. B., and C. P. Emerson, Jr. 1978. Coordinate regulation of contractile protein synthesis during myoblast differentiation. *Cell.* 13:599–611.
- Dworkin, M. B., and I. B. Dawid. 1980. Construction of a cloned library of expressed embryonic gene sequences from *Xenopus laevis*. *Dev. Biol.* 76:435–448.
- Epel, D. 1982. The physiology and chemistry of calcium during fertilization of eggs. In Calcium and Cell Function. W. Y. Cheung, editor. Vol. II. Academic Press, Inc., New York. 356–379.
- Epstein, P., A. R. Means, M. W. Berchtold. 1986. Isolation of a rat parvalbumin gene and full length cDNA. *J. Biol. Chem.* 261:5886–5891.
- Gillis, J. M., J. Thomason, J. Lefevre, and R. H. Kretsinger. 1982. Parvalbumins and muscle relaxation: a computer simulation study. *J. Muscle Res. Cell Motil.* 3:377–398.
- Goodman, M., J.-F., Pechere, J. Haeich, and J. G. Demaille. 1979. Evolutionary diversification of structure and function in the family of intracellular calcium-binding proteins. *J. Mol. Evol.* 13:331–352.
- Haeich, J., J. Derancourt, J.-F. Pechere, and J. G. Demaille. 1979. Magnesium and calcium binding to parvalbumins: evidence for differences between parvalbumins and an explanation of their relaxing function. *Biochemistry.* 18:2752–2758.
- Hardin, S. H., C. D. Carpenter, P. E. Hardin, A. M. Bruskin, and W. H. Klein. 1985. Structure of the Speel gene encoding a major calcium-binding protein in the embryonic ectoderm of the sea urchin, *Strongylocentrotus purpuratus*. *J. Mol. Biol.* 186:243–255.
- Heizmann, C. W. 1984. Parvalbumin, an intracellular calcium-binding protein: distribution, properties and possible roles in mammalian cells. *Experientia (Basel).* 40:910–919.
- Heizmann, C. W., and E. E. Strehler. 1979. Chicken parvalbumin: comparison with parvalbumin-like protein and three other components. *J. Biol. Chem.* 254:4296–4303.
- Heizmann, C. W., M. W. Berchtold, and A. M. Rowleson. 1982. Correlation of parvalbumin concentration with relaxation speed in mammalian muscles. *Proc. Natl. Acad. Sci. USA.* 79:7243–7247.
- Jauregui-Adell, J., J.-F. Pechere, G. Briand, C. Richet, and J. G. Demaille. 1982. Amino-acid sequence of an alpha-parvalbumin, pI = 4.88, from skeletal muscle. *Eur. J. Biochem.* 123:337–345.
- Kozak, M. 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12:857–608.
- Kretsinger, R. H. 1980. Structure and evolution of calcium-mediated proteins. *CRC Crit. Rev. Biochem.* 8:119–174.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)* 227:680–688.
- LePeuch, C. J., C. Ferraz, M. P. Walsh, J. G. Demaille, and E. H. Fischer. 1979. Calcium and cyclic nucleotide dependent regulatory mechanisms during development of chick embryo skeletal muscle. *Biochemistry.* 18:153–159.
- Lipman, D. J., and W. R. Pearson. 1985. Rapid and sensitive protein similarity searches. *Science (Wash. DC)* 227:1435–1441.
- Lynn, D. A., L. M. Angerer, A. M. Bruskin, W. H. Klein, and R. C. Angerer. 1983. Localization of a family of mRNAs in a single cell type and its precursors in sea urchin embryos. *Proc. Natl. Acad. Sci. USA.* 80:2656–2660.
- MacManus, J. P., A. L. Boynton, and J. F. Whitfield. 1982. The role of calcium in the control of cell reproduction. In The Role of Calcium in Biological Systems. L. J. Anghileri and A. M. Tuffet-Anghileri, editors. CRC Press, Inc., Boca Raton, FL. 147–164.
- MacManus, J. P., D. C. Watson, and M. Yaguchi. 1983. A new member of the troponin C superfamily: comparison of the primary structures of rat oncomodulin and rat parvalbumin. *Biosci. Rep.* 3:1071–1075.
- MacManus, J. P., J. F. Whitfield, and D. J. Stewart. 1984. The presence in human tumors of a 11,700 molecular weight calcium binding protein similar to rodent oncomodulin. *Cancer Lett.* 21:309–316.
- Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5:180–190.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Maruyama, K., T. Mikawa, and S. Ebashi. 1984. Detection of calcium binding proteins by ⁴⁵Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem.* 95:511–519.
- Muntz, L. 1975. Myogenesis in the trunk and leg during development of the tadpole of *Xenopus laevis* (Daudin 1802). *J. Embryol. Exp. Morphol.* 33:757–774.
- Newport, J., and M. Kirschner. 1982. A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell.* 30:675–686.
- Nieuwkoop, P., and J. Faber. 1967. Normal Tables of *Xenopus laevis* (Daudin). 2nd ed. North-Holland Publishing Co., Amsterdam.
- Pfiffer, G. E., G. Haemmerli, and C. W. Heizmann. 1984. Calcium-binding in human carcinoma cell lines. *Proc. Natl. Acad. Sci. USA.* 81:6632–6636.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
- Stuhfauth, I., J. Reininghaus, H. Jockusch, and C. W. Heizmann. 1984. Calcium-binding protein, parvalbumin, is reduced in mutant mammalian muscle with abnormal contractile properties. *Proc. Natl. Acad. Sci. USA.* 81:4814–4818.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
- Wnuk, W., J. A. Cox, and E. S. Stein. 1982. Parvalbumins and other soluble high-affinity calcium-binding proteins from muscle. In Calcium and Cell Function. W. Y. Cheung, editor. Vol. II. Academic Press, Inc., New York. 243–278.
- Young, R. A., and R. W. Davis. 1983. Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA.* 80:1194–1198.