EXPRESSION OF A VACUOLAR PROTON ATPase AND TRANSCRIPTION FACTOR mRNAS IN LOBED AND LOBELESS EMBRYOS OF *ILYANASSA OBSOLETA*

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The fertilized egg of the invertebrate marine gastropod mollusc, *Ilyanassa obsoleta* Stimpson, sequesters factors located in its vegetal cortical cytoplasm into a polar lobe (PL) during first and second mitotic cleavage, and delivers these PL-factors first to the CD blastomere and then to the D blastomere. These D cell-contained PL-factors subsequently specify the dorsal-ventral (D/V) axis of *Ilyanassa* embryos and determine cell fates directly within D cell descendants and indirectly in other cell lineages within the embryo. Within 8-9 days, a veliger embryo forms that is bilaterally symmetric and contains muscular velar lobes, a foot with retractor muscles, statocysts and an operculum, a digestive system with stomodeum, esophagus, stomach, style sac, intestine and digestive glands, 2 eyes, a beating heart, a kidney, a mantle, and an external shell. If the PL is removed at first cleavage, either by incubation of the fertilized egg in nanomolar concentrations of Ag^+ during first cleavage (Conrad, A.H., et al., *Cell Motil. Cytoskel.* 27:117-132, 1994) or by experimental manipulation of the eggs at trefoil stage of first cleavage, the resulting veliger embryo loses D/V polarity, becomes radially symmetric, and fails to form a beating heart, eyes, an external shell, statocysts, an operculum, or an intestine, although muscle cells, shell-secreting cells, pigment cells, and digestive gland cells do differentiate (Atkinson, J.W., *J. Morphol.* 133:339-352, 1971). In no other well-described organism are D/V polarity determinants localized in the fertilized egg in a location so easily removed without loss of any nuclei. Thus *Ilyanassa* offers a unique, easily manipulatable system for elucidating possible effects of Ag^+ on embryogenesis, and for identifying molecular interactions critical for establishing D/V polarity and inducing heart, eye, shell, statocyst, operculum, and intestine development in molluscs.

We have previously isolated total RNA from adult tissues using the Promega RNAgents system and from fertilized eggs and normal embryos of various ages using the LiCl method (Sambrook, J., Fritch, E.F., and Maniatis, T. Molecular Cloning. A Laboratory Manual. Second Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 11724; 17.16-17.17, 1989), and used RT-PCR with degenerate and *Ilyanassa*-specific primers to sequence and identify *Ilyanassa* cDNA fragments for (1) myosin heavy chain II (ILMHCII), a cytoskeletal protein used for cell shape changes and muscle contraction, (2) tropomyosin (ILTrop), a cytoskeletal protein that interacts with actin filaments, (3) a sodium/proton transmembrane antiporter (ILNp), used for pH regulation, (4) a transmembrane vacuolar H^+ATPase (ILVH), also used for pH regulation, (5) an ILEts1/2 transcription factor, frequently used for regulating cell division and differentiation during early morphogenesis, (6) an engrailed (ILEn) homeodomain transcription factor, often associated with reiterated neuronal development along the central nervous system, but expressed exclusively in the shell gland in *Ilyanassa* embryogenesis (Moshel, S.M., et al., *Dev. Genes Evol.*, 208:135-401, 1998), (7) an ILHox1 homologue, closely related to the *Drosophila* homeotic gene *labial* by Blast, (8) an ILHox2 homologue, closely related to the *Drosophila* homeotic gene *proboscipedia* or the polychaete parahox gene *Xlox* by Blast, (9) an ILHox3 homologue, closely related to the *Drosophila* homeotic gene *sex-combs-reduced* by Blast, (10) an ILHox4 homologue, closely related to *Xenopus Xgbx2* by Blast, a homoedomain gene that may be involved in mesoderm differentiation, (11) an ILPax2/5/8 homologue, a paired-box homeodomain protein associated with brain segmentation and placode formation, and (12) an ILPax6 homologue, a paired-box homeodomain protein often associated with eye development (Conrad, A.H., et al., *Bull. MDIBL,* 37:2-3, 1998). We demonstrated by relative PCR quantitation that *Ilyanassa*

embryos express a constant level of ILVH mRNA from fertilized egg to hatching veliger, but variable levels of ILMHCII mRNA over this developmental period, from very low levels at fertilization through cleavage and gastrulation, to high levels of ILMHCII mRNA during velum and foot formation, organogenesis, and veliger hatching.

To begin examination of the expression of these cytoskeletal and transcription factor proteins in lobeless embryos compared to lobed embryos, we released fertilized *Ilyanassa* eggs from their capsules prior to first cleavage, separated them into control and lobeless pools, and incubated them in MFSW (Millipore-filtered-sea-water) containing 50 µg/ml gentamycin. As the "lobeless" eggs approached trefoil stage of first cytokinesis, they were rinsed twice in CMF-MFSW (MFSW made without calcium or magnesium), then gently sucked up in a Pasteur pipette and expelled quickly against the culture dish bottom to detach the PL from the animal hemisphere. PLs (which contain no nuclei) and animal hemisphere lobeless 2 cell embryos (which contain the two daughter cell nuclei) were pooled separately and returned to MFSW containing 50 ug/ml gentamycin. Eggs from 13-15 capsules (at 100-300 eggs per capsule) were combined for each sample, with matched control and lobeless groups from the same combined set. Total RNA was isolated by the LiCl method from PLs immediately after trefoil separation, and from matched control and lobeless embryos immediately after separation, and at 1 day (mesentoblast stage), 3 days (stomodeum invagination), 4 days (shell gland evagination), 5 days (velum and foot formation), 7 days (eye, statocyst, and intestine formation), and 8 days (hVel=hatched veliger) of incubation at 20°C. Total RNA from both control and lobeless embryos of all ages, isolated PLs, and fertilized eggs contained PCR-amplifiable ILVH transmembrane vacuolar H^+ATPase mRNA. However, 4-day and hVel control and lobeless embryos expressed ILEts1/2 and ILEn quite differently by PCR amplification. Control 4-day and hVel embryos expressed both ILEts1/2 and ILEn strongly, whereas lobeless 4-day and hVel embryos expressed ILEts1/2 strongly, but expressed ILEn weakly at hVel and at barely detectable levels on day 4. These results document weak expression of ILEn in lobeless embryos by PCR amplification, confirming the morphological observations of Atkinson (1971) and immunohistochemical staining of Moshel et al. (1998) showing that some shell-producing ILEn-positive cells do still differentiate in lobeless embryos even though the shell gland does not form properly, and establish for the first time that expression of the transcription factor Ets1/2 is not significantly disrupted by removal of PL-determinants in *Ilyanassa* embryos at trefoil of first cleavage.

We plan to make randomly labeled riboprobes for our cloned ILEts1/2, ILEn, ILHox1, ILHox2, ILHox3, ILHox4, ILPax2/5/8, and ILPax6 cDNA fragments and perform *in situ* hybridization on fixed control, lobeless, and Ag^+-treated *Ilyanassa* embryos to document for the first time in molluscs the expression patterns of *Ets1/2*, *Hox* and *Pax* genes in normal embryos and in embryos whose D/V axis has been disturbed by removal of the PL with its determinants at first cleavage. We will then isolate PL-totalRNA and inject this totalRNA back into one cell of a 4 cell lobeless embryo (thus simulating a D cell) to see if we can restore differentiation of lobe-dependent structures or lobe-dependent expression patterns of *En*, *Hox*, or *Pax* genes. If PL-totalRNA restores lobe-dependent differentiation, we will isolate PL-polyA$^+$RNA and repeat the injection experiments. If PL-polyA$^+$RNAs function as PL determinants, we will do subtractive suppression hybridization to identify specific PL-polyA$^+$RNAs and test them for their ability to restore D/V polarity and lobe-dependent differentiation in lobeless embryos. By these experiments we may identify new genetic pathways used to establish D/V polarity or specify specific cell types in molluscs.

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