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Characterization and Expression of Evolutionarily Conserved Regulatory Genes in *Ilyanassa obsoleta*

by

Sharon M. Moshel

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1998

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Abstract

Characterization and Expression of Evolutionarily Conserved Regulatory Genes in *Ilyanassa obsoleta*

by

Sharon M. Moshel

Advisor: Professor Emeritus J. R. Collier

My strategy for studying the role of cytoplasmic determinants on gene expression in *Ilyanassa* obsoleta was to isolate evolutionarily conserved regulatory genes whose putative role in *Ilyanassa* development would be in polar lobe dependent structures.

Early experiments with an *engrailed* antibody showed *engrailed* localization in the shell gland, a polar lobe dependent structure. Because of these results, I attempted to isolate an *engrailed* homolog from the *Ilyanassa* genome. Using degenerative primers against the conserved homeobox region of *engrailed*, I identified an *engrailed* homolog in *Ilyanassa* which shares an 80% amino acid homology to the *Drosophila* gene. Using *in situ* hybridization and immunocytochemistry I have demonstrated *engrailed* localization solely to the shell gland. While the removal of the polar lobe disrupts the formation of an external shell, internal shell fragments are still present. These fragments

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stain positive for *engrailed*, illustrating *engrailed* expression is not polar lobe dependent. I propose the function of *engrailed* in *Ilyanassa* is to define shell gland cells, after their initial determination, which will lead to the terminal differentiation of the shell.

The second gene I isolated was *twist*, a helix-loop-helix gene, known to have a significant role in mesoderm differentiation in *Drosophila* and other organisms. Using the PCR, I identified an *Ilyanassa twist* homolog sharing an 82% identity with the bHLH region of *Drosophila twist*. *In situ* hybridization and immunocytochemistry have determined the role of *twist* as a mesoderm-specific transcription factor has also been conserved in *Ilyanassa*. In normal embryos, *twist* is expressed in the nuclei of the precursor cells of entomesoderm, a polar lobe dependent lineage. The removal of the polar lobe abolishes all staining of *twist* demonstrating that not only is the role of twist as a mesoderm-specific transcription factor conserved in *Ilyanassa*, the cytoplasmic factor(s) found in the polar lobe are essential for the translation of *twist*.

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Acknowledgements

I sincerely thank Dr. J. R. Collier for his dedication to both me and this project. Without his continual support and guidance, this work would not have been completed. Not only has he overseen this project to fruition, he was my constant source of stimulation.

I would also like to thank N. Patel for the *engrailed* 4D9 antibody and S. Roth for the *twist* antibody.

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General Introduction

The evolution of multicellularity has required metazoans to face the challenge of how to arrange their cells in three-dimensional space leading to the formation of a complex organism. Embryonic fates can be specified autonomously by localization of morphogenic determinants or inductively by cell-cell interactions. To solve this problem of organization, many organisms concomitantly use both the segregation of maternal determinants at the onset of embryonic cleavage and the positioning of cells causing diverse cell-cell interactions. The marine mud snail *Ilyanassa obsoleta* is a good example of an organism concomitantly using both mechanisms for establishing cell fate. Initially there is a localization of morphogenetic determinants in the D macromere which leads to autonomous cell fate specification in the D lineage. The derivatives of this lineage have an inductive effect on the offspring of the A, B, and C cells. (Clement, 1962, 1967, 1986a, 1986b; Render, 1991).

Spemann and Mangold (1924) were first to authenticate that the correct cellular orientation was essential for normal development. Their classic transplantation experiments in the newt embryos, *Triturus taeniatus* and *Triturus cristatus*, demonstrated that cells from the marginal zone of *T. cristatus* induced cells from *T. taeniatus*, which were originally fated to become ventral epidermis, to form a

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neural tube and dorsal mesodermal tissue. The phenomenon of cell-cell interactions has since been demonstrated in a variety of organisms including *C. elegans* (Greenwald, 1989), *Xenopus* (Nieuwkoop, 1969, 1973; Gimlich and Gerhardt, 1984; Henry and Grainger, 1990), sea urchin (Ettensohn and McClay, 1988; Henry *et al.*, 1989) and in the chick embryo (Waddington, 1933; Storey *et al.*, 1992).

The prelocalization of morphogenic factors in cytoplasmic regions of the egg is a leading factor of early development (Wilson, 1904). The sequestering of these maternal determinants into different regions of the developing embryo enables each cell to have a different fate depending on its cytoplasmic environment. Taking advantage of the colored egg cytoplasm of the ascidian *Styela partita* E. G. Conklin observed that each blastomere had a different fate depending upon what type of cytoplasm the cell is exposed to (Conklin, 1905). Whittaker (1980) transposed the cytoplasm of one cell into another cell of the embryo of *Styela plicata* and thereby changed the cell fate of the recipient cell. These observations suggested that there are morphogenic factors present in the egg cytoplasm that are essential for embryonic differentiation.

While the requirement for asymmetrical localization of maternal determinants has been established, what these maternal factors are and how they regulate embryonic development has only recently begun to be understood. Although the degree of maternal determinants varies depending

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upon the organism, localized regions of the cytoplasm direct the fate of specific parts of the embryo. This phenomenon has been shown most clearly in *Drosophila*, where four localized maternal determinants define the two major axes, anterior-posterior and dorsal-ventral, of the embryo. Although the axial organization is not apparent until gastrulation, the basic prepattern of both axes is established in the syncytial blastoderm (St Johnston and Nüsslein-Volhard, 1992). Once these maternal determinants, which in *Drosophila* are DNA transcription factors, set the polarity of the embryo, zygotic genes become activated and set up the necessary interactions leading to differentiation.

Of the regulatory genes conserved throughout evolution the homeobox genes appear to play a major role in determining developmental fates. Many regulatory genes encode a homeodomain and have been found in parazoan and metazoan animals, plants and fungi. The presence of homeobox genes in porifera (Degnan, 1995) date the presence of homeobox genes prior to the parazoan-metazoan split between 600 and 1200 Mya (Valentine *et al.*, 1996). One homeobox gene identified in sponges is a hox-like gene, and since hox genes have been identified as metazoan-specific genes essential for anteroposterior patterning during embryogenesis, this suggests a monophyletic origin of the metazoans. Since homeobox genes have been found in so many organisms, one would expect to find their members in all metazoa, taking into account the loss of some of these genes by evolutionary

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divergence. Although identification of these genes in all phyla is important, understanding their role in development is essential to understanding why they have been retained in so many divergent organisms. The function of the homeobox gene appears to be that of a "selector gene" whose role is to activate the "realizer genes" which cause the development of anatomical structures of the adult (Tautz, 1996). Therefore, a full understanding of the evolution of regulatory genes requires their identification and understanding of their role in embryogenesis.

The neogastropod *Ilyanassa obsoleta* (Figure 1A) provides a natural experiment for studying the role of cytoplasmic determinants on gene expression in development. Prior to first cleavage, the *Ilyanassa* egg forms a large cytoplasmic protrusion, called the polar lobe, at its vegetal pole (Figure 1C). The polar lobe is an isolatable anucleate mass and its removal results in aberrant development. The control of development by conserved regulatory genes raises the possibility that the removal of cytoplasmic determinants in *Ilyanassa* may alter gene expression leading to the loss of structures.

H. E. Crampton was the first to discover that the removal of the polar lobe at the trefoil stage of first cleavage disrupted normal development (Crampton, 1896). Firstly, removal of the polar lobe altered the cleavage pattern. The polar lobe is normally absorbed into one of the first two blastomeres, the CD blastomere, which is then

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larger than the other cell, the AB blastomere. In lobeless embryos, equal division occurs and the two blastomeres are indistinguishable (Figure 1D). Later in development the content of the polar lobe is transferred into the D blastomere (recognized by its larger size), which does not occur in lobeless embryos. The fourth derivative of the D blastomere, the primary mesentoblast or the 4d cell, gives rise to mesodermal bands in normal embryos. Lobeless embryos form a 4d micromere different in structure from the normal 4d, and do not form mesodermal bands. Crampton was unable to analyze the final effects of removing the polar lobe because he could not rear embryos beyond early stages of development. Although erroneous in his conclusion that the yolk mass provided the stimulus for the D quadrant to be different, his experiments showed that the presence of cytoplasmic factors are essential for normal cleavage.

A. C. Clement extended Crampton's work using pasteurized sea water that enabled the embryos to survive long enough to produce veliger larvae (Clement, 1952, 1956, 1967, 1986; Cather, 1967). Clement saw that the removal of the polar lobe caused the D quadrant to appear like the other three quadrants, thus forming a "radial plan of organization" (Clement, 1952). The alteration of the D quadrant caused the derivatives of this cell line to also be affected. In normal embryos, 1d is smaller than the other micromeres (1a, 1b, and 1c), while 2d11 is larger than 2a11-2c11. There is also a precocious division of 3D resulting in the formation of the

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mesentoblast or 4d cell. In lobeless embryos none of these events occur. The mesentoblast demarcates the posterior pole of the future embryo. Since lobeless embryos do not form a typical mesentoblast cell, the anterior-posterior axis is absent. Therefore, the polar lobe directly or indirectly establishes the polarity of the embryo.

The removal of the polar lobe greatly affects larval differentiation. Lobeless embryos (Figure 1D and 1F) lack axial organization and fail to form eyes, foot, statocysts, operculum, external shell, heart and intestine. The lobeless larva does contain velar cilia, small cilia, an everted stomodeal structure, endodermal tissue, pigment, muscle fibers and internal masses of birefringent shell material.

Atkinson (1971 and 1986) made a histological study of normal and lobeless *Ilyanassa* embryos. He found that the lobeless embryo differentiated a variety of tissues (muscle, stomodeum, esophagus, digestive gland, stomach, and style sac) but that these tissues, though often juxtaposed as in normal embryos, were not organized into organs. These results show that the polar lobe functions in both cellular differentiation and in the organization of tissues into organs.

Thus it is clear that the polar lobe is essential for normal development, but the questions remain, what are the cytoplasmic determinants in the polar lobe and how do they control development? Though a number of investigators have compared the synthesis of nucleic acids and proteins in

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normal and lobeless embryos of *Ilyanassa* little progress has been made in understanding how polar lobe cytoplasm influences differentiation and the synthesis of these molecules. This earlier work has been reviewed by Collier (1976 and 1983) and Davidson (1986). Extensive studies (Collier and McCarthy, 1981; Collier, 1981, 1983; Brandhorst and Newrock, 1981) of the pattern of protein synthesis resolved by two-dimensional electrophoresis of radioactive proteins made by both normal and lobeless embryos failed to detect any qualitative differences in polypeptides made by these two classes of embryos. However, a significant finding of these studies was that the isolated polar lobe synthesized more than one hundred individual polypeptides; thus, the polar lobe contained at least this many maternal mRNAs.

Early studies by Davidson *et al.* (1965) and Collier (1977) showed that the rate of RNA synthesis in the lobeless embryo was significantly less than in the normal embryo; However, Collier (1975) found no significant differences between the content of polyadenylated mRNAs in normal and lobeless embryos.

My strategy for learning about cytoplasmic determinants in *Ilyanassa* is to study morphogenesis and gene expression related to polar lobe dependent structures. Because of the demonstrated role of the polar lobe in shell development and mesoderm formation, I am especially interested in studying genes related to differentiation of these structures. I have selected developmentally important

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genes that have been sequenced and well characterized. An early observation showed that the *engrailed* gene was expressed only in the shell gland of the *Ilyanassa* embryo and this observation initiated an interest in *engrailed*. Accordingly, *engrailed* was studied in relation to shell development and it became a prototypical gene in which I applied recombinant DNA technology to the study of *Ilyanassa* embryogenesis. The second gene I selected to study was *twist* because of its demonstrated role in mesoderm formation in various organisms. I have isolated, sequenced and determined the time and place of expression of these putative regulatory genes, *engrailed (en)* and *twist (twi)*.

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Figure 1. Development of normal and lobeless *Ilyanassa* embryos. (A) Female snail depositing egg capsules. (B). Egg capsule containing fertilized eggs, close examination will detect the presence of a polar lobe on these eggs. (C) and (D) normal and lobeless egg, respectively. (E) and (F) normal and lobeless larvae, respectively. (E) and (F) are from Atkinson, 1971.

Abbreviations: Bi. M., birefringent mass; D. Gl., digestive gland; e., eye; Es., esophagus; I. intestine; F., foot; op., operculum; Mes., mesenchyme; Sh., shell; S. S., style sac; Sto., stomach; V. L. velar lobe.

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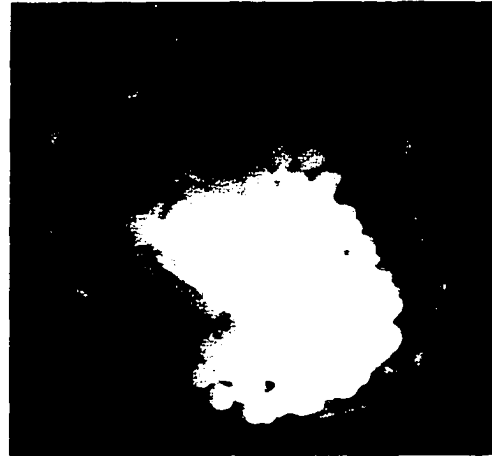

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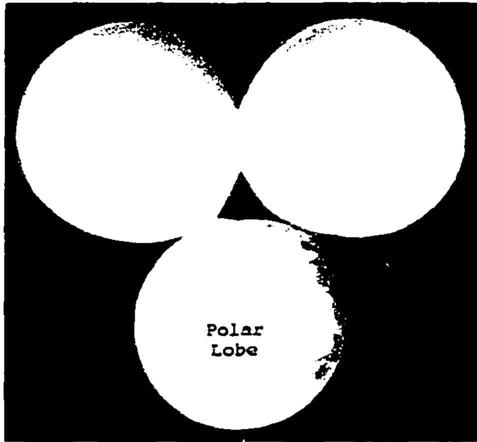

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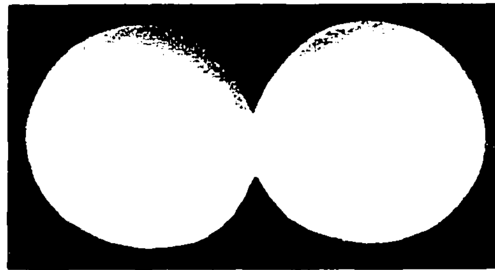

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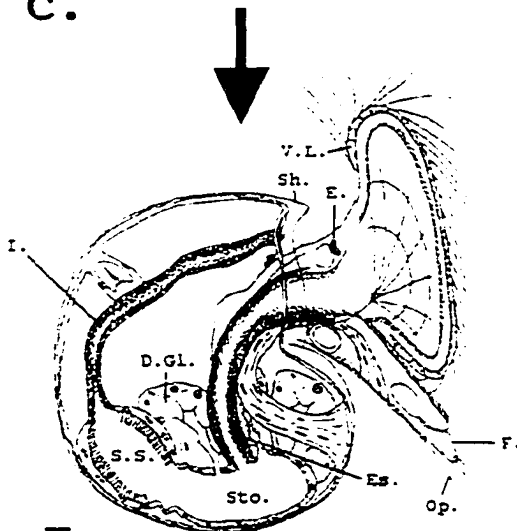

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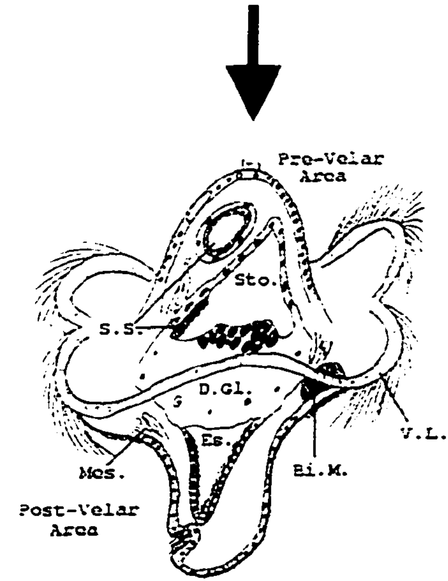

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Figure 1

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Shell Development and Mesoderm Formation

Shell Formation.

The external shell is formed by the shell gland and it is one of the early organs to differentiate in the post-gastrular embryo. The shell gland, formed from ectodermal cells derived from the 2d micromere, the primary somatoblast (Figure 2A), lies on the posterior-dorsal surface of the embryo (Figure 2B and 2C). The primary somatoblast gives rise to the "posterior growing-point" which will form the embryonic shell. Both the 2d and 2c micromeres are required for complete shell development. In the lobeless embryo, an organized external shell does not develop, only internal shell fragments are formed. Thus, differentiation of an organized external shell is polar lobe dependent.

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Figure 2. Gastropod shell differentiation. (A) 16-cell stage of *Crepidula* showing the primary somatoblast (2d) whose offspring give rise to the shell gland. (B) A mid-sagittal section of the shell gland of a 4.5 day-old *Ilyanassa* embryo. (C) A frontal section of the *Ilyanassa* shell gland. (A) redrawn from Conklin, 1897.

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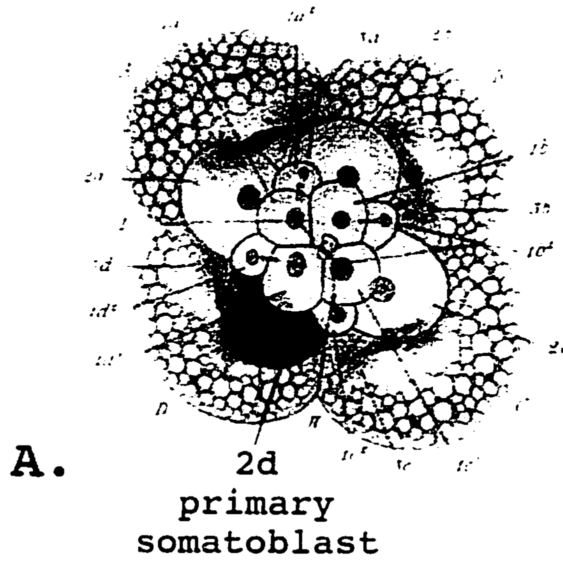

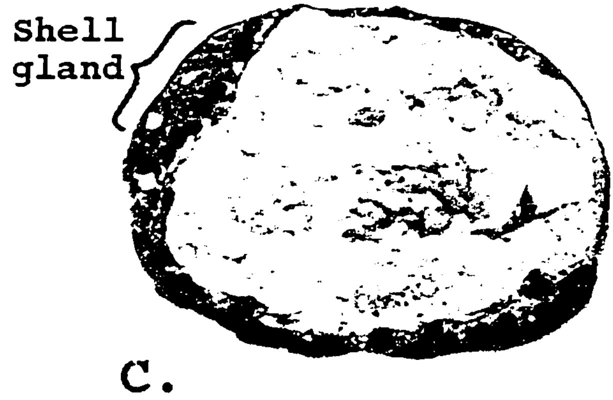

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Mesoderm Formation

Although the mechanism by which mesoderm is determined in early embryogenesis varies significantly among different organisms, the early mesoderm-specific genes, which are expressed after initial mesoderm determination, are highly conserved even between insects and vertebrates. Thus by comparing the genes used for mesodermal differentiation in *Ilyanassa* and other organisms I may be able to draw some conclusions as to whether there is a commonality of controller genes between these organisms which govern different mechanisms of differentiation for the same cell type.

In *Ilyanassa* there are two sources of mesoderm, ectomesoderm derived from the micromeres 3a, 3b, and 3c (Figures 3A and 4A) and entomesoderm derived from the primary mesentoblast or 4d cell (Figure 3B and 4B). The ectomesoderm, which is not polar lobe dependent, gives rise to larval mesenchyme that forms unicellular muscle fibers in the foot, head and velum. The larval mesenchyme degenerates at metamorphosis. The entomesoderm is formed from division products of the primary mesentoblast (Figure 3C) forming the left and right mesoblasts (Figure 3D) which divide into mesodermal bands (Figure 3E) giving rise to the larval mesoderm derivatives, including the heart and kidney.

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Figure 3. Mesoderm formation in *Ilyanassa.* (A) Micromeres 3a, 3b, and 3c which give rise to ectomesoderm. (B) Early cleavage stage (25-cells) showing the primary mesentoblast (4d) that will give rise to entomesoderm. (C) and (D) Derivatives of the 4d cell that will form the intestine (E^1 and E^2) and the mesodermal bands(ME^1 and ME^2). (D) ME^1 and ME^2 have divided in a dorsal-ventral plane to segregate the endodermal lineage (E^1 and E^2) from the mesodermal lineage (Me^1 and Me^2). The latter form right and left mesoblasts (also shown in D) that proliferate into mesodermal bands, shown in (E). A, B, C and D redrawn from Conklin, 1897.

Key: pink-mesodermal fate; purple-mixed lineage (both endodermal and mesodermal; yellow-endodermal;

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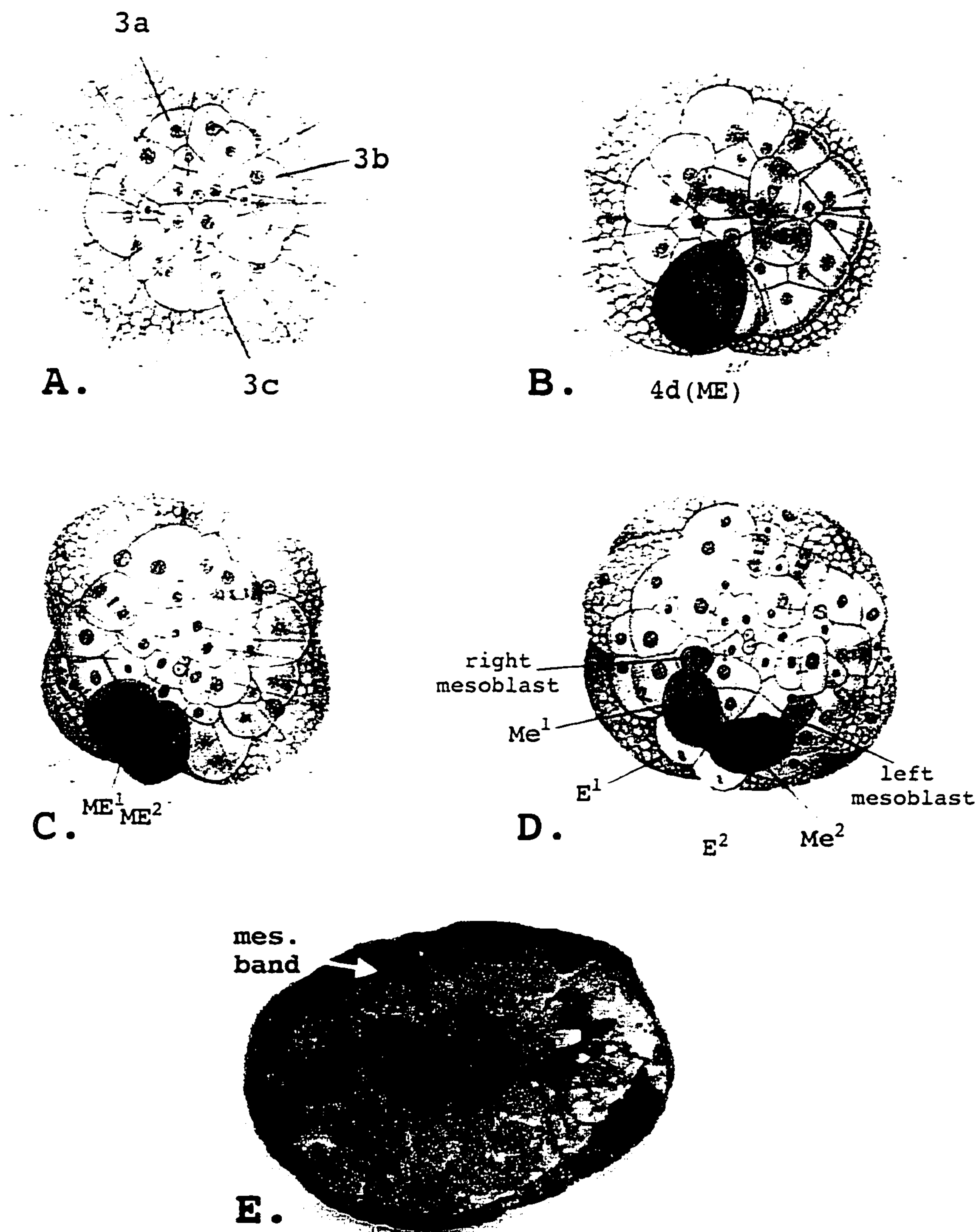

Figure 3

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Figure 4. Two forms of mesoderm in *Ilyanassa*: ectomesoderm and entomesoderm. (A) Ectomesoderm is derived from micromeres 3a, 3b, and 3c. Ectomesoderm is not polar lobe dependent, and gives rise to larval mesenchyme that forms unicellular muscle fibers in the foot, head and velum which degenerate at metamorphosis. (B) Entomesoderm is derived from the primary mesentoblast (4d). 4d subsequently divides to form right and left mesentoblasts (ME^1 and ME^2). Each of these will divide to form two additional mesentoblasts, Me^1 and Me^2 and entoblasts E^1 and E^2. Me^1 and Me^2 divide to form the right (m^1) and left (m^2) mesoblasts. Each of these mesoblasts divide eight or nine times to make up the bilateral mesodermal bands. The mesodermal bands form the coelom (pericardial cavity) and mesenchyme cells of the posterior head region. The removal of the polar lobe causes an abnormal 4d cell to form which does not produce mesodermal bands.

Key: red-mesodermal fate; orange-mixed lineage (both endodermal and mesodermal; yellow-endodermal fate;

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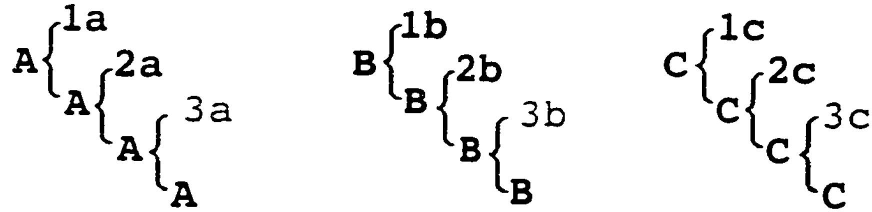
A.

ECTOMESODERM FORMATION



B.

ENTOMESODERM FORMATION

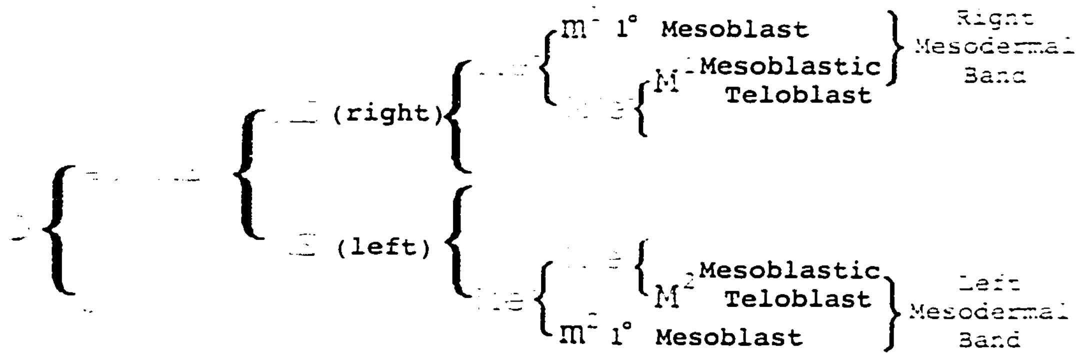


Figure 4

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Materials and Methods

Rearing of Embryos

Snails were collected from Plumb Beach in Brooklyn, New York and kept in the laboratory in a tank of recirculating sea water. When snails were fed a clam on alternate days they laid eggs daily. Embryos were reared at 18°C in Jamarin sea salts (Jamarin Laboratory, Osaka Japan) containing 50 μg/ml of gentamicin. All embryonic stages, for both normal and lobeless embryos, are days of development at 18°C.

Removal of polar lobes.

Polar lobes were isolated by removing the eggs from the capsule and placing them in 90% of calcium-magnesium free sea water and 10% sea water. When the eggs reached the trefoil stage, they were sucked into a capillary pipet and gently expelled to the bottom of the dish. This procedure detached the polar lobe from the rest of the egg. The lobeless eggs were then placed in 100% sea water containing 50 μg/ml of gentamicin and reared at 18°C.

Fixation of Embryos

Embryos were fixed in freshly prepared 3.7% formaldehyde diluted from concentrated stock with PBS. Embryos to be used for immunochemistry were fixed for 5 min at room temperature; longer fixation times gave variable results. For *in situ*

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hybridization embryos were fixed in the same solution for 1hr. In some cases embryos were processed immediately after fixation, other times they were dehydrated by two changes in 100% methanol and stored in methanol at -20°C. After fixation or storage embryos were washed in two changes of PBS followed by two changes in PBT (PBT is PBS containing 0.1% Tween 20).

Identification of regulatory genes by PCR Amplification

To avoid polymorphic variations, genomic DNA was isolated from a testis of a single individual. Degenerate primers were used to amplify from *Ilyanassa* genomic DNA sequences homologous to *Drosophila en*. The primers for *Ily-en* (ProtoGeneLaboratories) were: upstream (5'GAGAAGCG[ATGC]CC [ATGC]CG[ATGC]AC[ATGC]GC[ATGC]TT3'), downstream 5'CA[ATGC]CTG CA[ATGC][ATGC]GCCAGCTC[ATGC]TT[ATGC]TT3').

For *twist*, primers were constructed flanking the helix-loop-helix domain of *Drosophila twist*, upstream 5'CA[AG][AC]G [ATGC]GT[ATGC]ATGGC[ATGC]AA[TC]GT3') and downstream (5' [ATGC][TG]CC[ATGC]CC[TC]TCCAT[ATGC]C[TG]CCA[ATGC]AC3'). PCR amplification was in a 100 μl reaction containing 1 μg of *Ilyanassa* genomic DNA, 0.5 μg of each degenerate primer, *TaqPlus* DNA polymerase (Stratagene), and optimization reagents (Invitrogen). Successful *engrailed* amplification required 60mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 2.0 mM [Mg^{2+}] and a pH 9.5. *twist* amplification required 60mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 2.0 mM [Mg^{2+}] and a pH 8.5. The amplification

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parameters included an initial incubation at 80°C prior to dNTP addition, followed by denaturation at 95°C for 2 min. These steps were followed by thirty cycles of 1 min denaturation at 95°C, 2 min primer annealing at 55°C, and 3 min primer extension at 72°C. The PCR was concluded by a final extension for 7 min at 72°C.

The amplified DNA sequences were resolved on 3.5% NuSieve GTG agarose gels (FMC BioProducts) and subcloned using Invitrogen's TA cloning kit. *engrailed* was subcloned into pCRII and *twist* was subcloned into pCR2.1.

DNA Sequencing

DNA sequencing was done by dideoxy chain-termination (Sanger *et al.*, 1977) using the U. S. Biochemical sequenase (version 2.0) kit. Sequencing was done from M13 and T7 primer sites in the pCRII and pCR2.1 vector containing the amplified DNA. Out of approximately 60 amplification products eight samples sharing the same squence were *engrailed.* Out of approximately 100 amplification products twelve *twist* samples were isolated sharing the same sequence.

In situ Hybridization

en sense and antisense riboprobes were transcribed *in vitro* from pCRII vectors containing amplified homeobox gene sequences using digoxigenin-labeled UTP and T7 or SP6 RNA polymerase. *twist* DNA probes were labeled with digoxigenin-dUTP using Klenow enzyme.

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In situ hybridization was according to Tautz and Pfeiffle (1989). After fixation embryos were digested with 10 μg/ml Proteinase K for 5 min, or treated with a detergent mixture (Riddle *et al.* 1993) for 1 hr. The detergent mixture (RIPA) was 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 150 mM NaCl, 1mM EDTA contained in 50 mM Tris buffer (pH 8.0). Both treatments were followed by a second fixation in 3.7% formaldehyde for 30 min.

Permeabilized embryos were washed in PBT and prehybridized at 45°C for 1 hr. Prehybridization buffer was 50% deionized formamide, 5 X SSC, 1 X Denhardt's solution, 0.1% Tween 20, 0.5% heparin and 100 μg/ml denatured sonicated salmon sperm DNA. Prehybridization buffer was replaced with fresh prehybridization buffer containing 50 μg/ml digoxigenin (DIG) labeled probe and hybridized at 45°C for 18 hr. Following hybridization embryos were washed three times in prehybridization buffer at 45°C. After washing with PBT they were incubated for 1 hr in 10% BSA blocking solution followed by 2 hr incubation at room temperature with anti-digoxigenin antibody conjugated with alkaline phosphatase. Embryos were then washed repeatedly with PBT containing 1 mM levamisole and transferred to alkaline phosphatase buffer (100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 1mM levamisole in 100 mM Tris buffer, pH 9.5). The digoxigenin antibody conjugate was visualized with chromogenic alkaline phosphatase substrates NBT and BCIP according to the manufacturers (Boehringer Mannheim Biochemicals) directions. Stained embryos were

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dehydrated in alcohol, cleared in xylene, mounted in Permount, and viewed with a Wild photomicrosope using bright-field optics. Photographs were taken with Kodak Ektachrome (Daylight, ASA 100) using an 80A filter with tungsten light.

Immunochemistry

After fixation non-specific protein-binding sites were blocked by incubation for 1 hr at room temperature or overnight at 4°C in 10% BSA in PT. After blocking with BSA the embryos were washed for 30 min in PT and then incubated with primary antibody (MAb 4D9 diluted 1:1 with PNBT or *twi* antibody diluted 1:200 in PNBT; PNBT is PBS containing 0.5 M NaCl, 1% BSA, and 0.1% Tween 20) for 4 hr at room temperature or overnight at 4°C. After incubation with the primary antibody embryos were washed for five changes of PNBT for two hr at room temperature, incubated with biotinylated secondary antibody, anti-mouse for MAb 4D9 and anti-rabbit for *twi* antibody, for 2 hr, washed sequentially with PNBT and PBT for 2 hr, incubated in Vector Laboratories (Burlingame, CA) avidin-biotin complex (ABC) for 1 hr, and then washed for 2 hr through four changes of PNBT. Staining was for 5 to 10 min in 0.3 mg/ml diaminobenzidine (DAB; Sigma Chemical Co.) reagent. Staining reactions were stopped by washing embryos in PT. Stained embryos were viewed and photographed as described above.

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Introduction to *engrailed*

engrailed (*en*) is a helix-turn-helix homeobox gene which produces a transcription factor which regulates gene expression. Original interest in *engrailed* was based on homeotic transformations in *Drosophila* that occurred in homozygous mutants for the *engrailed* allele (Ecker, 1929). These mutants did not segment properly, causing *engrailed* to be called a segment polarity gene (Morata and Lawrence, 1975; Lawrence and Morata, 1976). While essential for establishing posterior compartments in *Drosophila* (Kornberg, 1981; Kornberg and Tabata, 1993; DiNardo *et al.*, 1985), later expression of *engrailed* revealed that it was also necessary for establishing the posterior region of the wing imaginal disc (Bower, 1986) as well as differentiation of both the adult and embryonic nervous system (DiNardo *et al.*; 1985; Bower, 1986; Lawrence and Johnston, 1994). These results along with the identification of various *engrailed* homologs in many organisms, including mouse (Joyner *et al.*, 1985), chicken (Darnell *et al.*, 1986), zebrafish (Fjose *et al.*, 1988), leech (Weisblat *et al.*, 1988), sea urchin (Dolecki and Humphreys, 1988) grasshopper, (Patel *et al.*, 1989) and human (Poole *et al.*, 1989; Logan *et al.*, 1989), has broadened the role of *engrailed* in development.

engrailed is the founding member of a distinctive class of homeobox genes (Poole *et al.*, 1985; Fjose *et al.*, 1985)

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which are expressed at several developmental stages in diverse tissues in all three germ layers (Holland *et al.*, 1997). *engrailed* has been cloned and sequenced in many organisms and its homeodomain is one of the best characterized DNA-binding domains in vertebrate (Fjose *et al.*, 1988; Joyner *et al.*, 1985) and invertebrate embryos (Fjose *et al.*, 1985; Kornberg *et al.*, 1985). Further, there is a specific monoclonal antibody (MAb 4D9; Patel *et al.*, 1989) available that recognizes the engrailed homeodomain in many species.

While the gene has been evolutionarily conserved, the diverse patterns of expression suggest that *engrailed* has many developmental roles (reviewed by Hidalgo, 1996) including neurogenesis (Condron *et al.*, 1994), axon targeting (Rétaux *et al.*, 1996; Friedman and O'Leary, 1996), establishment and maintenance of compartment boundaries (Logan *et al.*, 1993), cell cycle control and cyto-differentiation (Loomis *et al.*, 1996; Hidalgo, 1996). Accordingly, *engrailed* belongs to a special class of selector genes that establish a genetic address for determined cells and tissues (Lawrence and Struhl, 1996).

Because of the pleiotropic nature of *engrailed* the assignment of an ancestral function is difficult. The presence of *engrailed* in both segmented and nonsegmented organisms in protostomes and deuterostomes demonstrates the presence of *engrailed* before the divergence of protostomes and deuterostomes. If segmentation in protostomes and

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deuterostomes has evolved independently (Hyman, 1951; Willmer, 1990) the ancient function of *engrailed* would not be segment polarity; if segmentation arose only once during animal evolution, as suggested by Holland *et al.* (1997), then these two groups of animals probably shared a common segmented ancestor which may have had *engrailed* as a segment polarity gene.

Patel *et al.*, (1989) suggested that the ancestral function of *engrailed* was the control of neurogenesis. While *engrailed* expression differs among organisms there is a common pattern of neurogenic expression in annelids, arthropods, chordates and echinoderms. Because of the cell lineage homology between molluscs and annelids (see **Discussion**) the expression of *engrailed* in the *Ilyanassa* shell gland is consistent with this idea.

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Results

Sequence of *engrailed* Homeobox

Using PCR amplification with degenerate primers I have sequenced a 210 nucleotide fragment of *Ilyanassa* DNA (Figure 5A). This fragment of *Ilyanassa* DNA has a 79% identity to the homeobox of the *Drosophila engrailed* gene; its conceptual translation (Figure 5B) has 80% identity to the homeodomain of the *Drosophila engrailed* protein. These sequences have a 99% identity to the sequences reported for *Ilyanassa* by Wray *et al.*, (1995). Because the sequence of the *engrailed* homeodomain is unique among known homeodomain sequences (Scott, 1989) I conclude that *Ilyanassa* contains a homolog to the *Drosophila engrailed* gene. I designate this homolog as *Ily-en*.

The genomic sequence data predicts the amino acid sequence (LAAELGLTESQIKI) for amino acid residues 34-47 of the homeodomain of *Ily-en*. In *Drosophila engrailed* the corresponding region is (LSSELGLNEAQIKI) and is the epitope recognized by the monoclonal antibody 4D9 (Patel *et al.*, 1989).

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Figure 5. *engrailed* sequence homology (A) Nucleotide sequence of *Ilyanassa* and *Drosophila engrailed.* There is a 79% homology among the two organisms. (B) Deduced amino acid homology. *Drosophila* and *Ilyanassa* share an overall homology of 80%. Identical residues are highlighted in pink, conservative substitutions are highlighted in yellow. The small arrow demarcate the epitope recognized by the 4D9 antibody (Patel, *et al.*, 1989). The large arrow points to the glycine residue essential for the 4D9 antibody to bind.

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A.

engrailed DNA Homology

```
Consensus    GAGAAGCGTC CRCGCACSGC GTTCWCCAGC GARCAGYTRK CYCGYCTSAA  50
Drosophila   GAGAAGCGTC CACGCACCGC GTTCTCCAGC GAGCAGTTGG CCCGCCTCAA
Ilyanassa    GAGAAGCGTC CGCGCACGGC GTTCACCAGC GAACAGCTAT CTCGTCTGAA

Consensus    GCGRGAGTTY RACGAGWRTC GCTAYCTGAC SGAGMSGMGR MGSCRRSASC 100
Drosophila   GCGGGAGTTC AACGAGSSTC GCTATCTGAC CGAGCGGAGA CGCCAGGAGC
Ilyanassa    GCGAGAGTTT GACGAGTGTC GCTACCTGAC GGAGACGCGG AGGCGACACC

Consensus    TGRCSRSSGA GTTGGGCCTG AMSGAGKCSC AGATCAAGAT CTGGTTCCAG 150
Drosophila   TGACCAGCGA GTTGGGCCTG AACGAGGCGC AGATCAAGAT CTGGTTCCAG
Ilyanassa    TGGCGGCGGA GTTGGGCCTG ACGGAGTCCC AGATCAAGAT CTGGTTCCAG

Consensus    AACAAGCGGG CCAAGATCAA GAAGTCGWCG                        180
Drosophila   AACAAGCGGG CCAAGATCAA GAAGTCGACG
Ilyanassa    AACAAGCGGG CCAAGATCAA GAAGTCGTCG
```

B.

Deduced Amino Acid Homology

```
                      10        20        30        40        50        60
             123456789012345678901234567890123456789012345678901234567890
Consensus    EKRPRTAF.SEQL.RLKREF.E.RYLTE.RR..L..ELGL.E.QIKIWFQNKRAKIKKS.
Drosophila   EKRPRTAFSSEQLARLKREFNENRYLTERRRQQLSSELGLNEAQIKIWFQNKRAKIKKST
Ilyanassa    EKRPRTAFTSEQLSRLKREFDECRYLTETRRRHLAAELGLTESQIKIWFQNKRAKIKKSS
```

Figure 5

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***Ily-en* Expression**

Because the micromeres 2d and 2c are essential for shell formation (Cather, 1967) I looked for the expression of *Ily-en* during early stages of development. There was no *in situ* hybridization or nuclear immunostaining by the 4D9 antibody at any time during early cleavage, i.e., from the 2- to the 29-cell stage which includes the first twenty-four hours of development. The first *in situ* hybridization and nuclear immunostaining by the 4D9 antibody occurred two days after gastrulation when embryos were 4.5 days old. Thus, the expression of *Ily-en* is not detectable when the determination of the shell lineage occurs.

After 4.5 days, the shell plate cells, precursors of cells that will form embryonic and larval shell, are present. The shell plate (stained with pyronin-methyl green) is shown in a mid-sagittal section in Figure 6A. Within a few hours the shell plate invaginates to form a shell gland which is shown in an oblique frontal section in Figure 6B (stained with pyronin-methyl green). After 5 days of development shell gland cells evaginate and multiply to form a shell field that spreads over the posterior area of the embryo (Figure 6G). In slightly older embryos the growing edge of the shell field has been displaced to one side of the embryo by the onset of torsion and has moved anteriorly over the embryo as shown in Figure 6H.

To determine the localization of *Ily-en* RNA whole-mount *in situ* hybridization was performed using digoxigenin-labeled

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antisense riboprobes transcribed *in vitro* from the *Ily-en* homeobox sequence. The corresponding sense riboprobes did not hybridize. Figure 6C shows that *Ily-en* RNA was exclusively localized in the shell plate of a 4.5 day embryo, Figure 6D shows the localization of Ily-*en* RNA in the shell gland.

To define the time and place of *engrailed* expression I immunostained whole-mount embryos with the monoclonal 4D9 antibody of the *engrailed* homeoprotein (MAb 4D9; Patel *et al.* 1989). Shell plate cells are shown (Figure 6E) in a whole-mount preparation immunostained with the 4D9 antibody. This is the earliest stage in which the *Ilyanassa* embryo is stained by the 4D9 antibody, and the nuclear localization of this immunostaining is evident here and in the shell gland in Figure 6F. The inset in Figure 6F is a fluorescent preparation of immunostaining with the 4D9 antibody.

A 5-day embryo immunostained with the 4D9 antibody is shown in Figure 6G, the staining by this antibody is exclusively localized in the nuclei of the cells of the shell field (Sh F). At this stage the foot primordium, head vesicle, and velar lobes have developed in the anterior part of the embryo, and show no staining with the 4D9 antibody.

Shown in Figure 6H is a slightly older embryo in which the growing edge of the shell field has been displaced to one side of the embryo by the onset of torsion. The embryo in Figure 6H has been immunostained with the 4D9 antibody which shows that the *Ily-en* homeoprotein is expressed only in the

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shell field. Thus, the *engrailed* homeoprotein is expressed early in shell plate cells and continues to be expressed in this lineage as it forms a shell gland and a shell field which forms shell matrix.

In early and late stages of organogenesis I have identified cells of the cerebral and pedal ganglia and have never seen any staining by the 4D9 antibody in these cells. I have not been able to identify other ganglia, commisures, or connectives but I have not seen the 4D9 antibody staining in any structures other than the cells of the shell gland and shell forming cells of the mantle.

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Figure 6. Shell gland formation and *engrailed* expression. (A) Mid-sagittal section of a 4.5-day old embryo stained with pyronin-methyl green. (B) Frontal section of an embryo stained with pyronin-methyl green. (C) *in situ* hybridization of a 4.5-day old embryo with an *Ily-en* riboprobe. (D) *in situ* hybridization of a 4.75-day old embryo with an Ily-*en* riboprobe. (E) Whole-mount of a 4.5-day old embryo immunostained with the *engrailed* monoclonal antibody (4D9). Compare with Figure 6C for mRNA staining of the shell plate. (F) Fragment of a 4.75-day old embryo stained with the 4D9 antibody. The inset in (F) is a rosette of shell gland cells seen in a whole mount after immunofluorescent staining with the *engrailed* antibody. Compare with Figure 6D for staining of mRNA the shell gland. (G) A 5-day old embryo stained with the 4D9 antibody. (H) A 5.5-day old embryo stained with the 4D9 antibody. Note the displacement of the shell field to one side which has resulted from the onset of torsion.

Abbreviations: AP, apical plate; Es, esophagus; Ft, foot; HV, head vesicle; Ma, mantle; Sh F, shell field; Sh G, shell gland; Sh P, shell plate; Sto, stomadeum; VL, velum;

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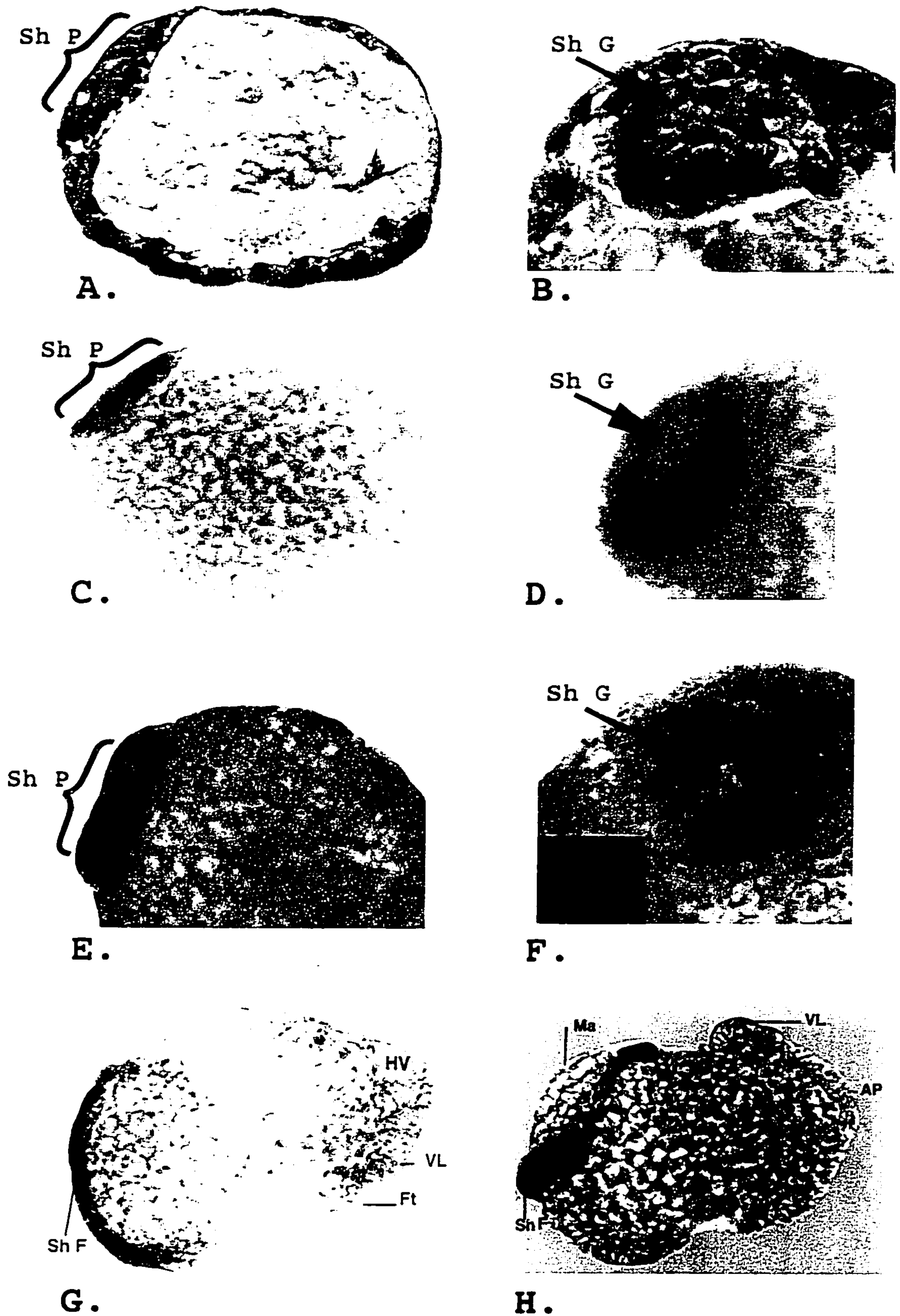

Figure 8

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Expression of *Ily*-en in Lobeless Embryos

The removal of the polar lobe at trefoil results in the ablation of external shell development. Figures 7A and 7B show a stage 3 normal and lobeless embryo, respectively. Note the radial symmetry and the absence of axiation in the lobeless embryo (Figure 7B) in contrast to the anterior-posterior and dorsal-ventral axes of the normal embryo (Figure 7A). In the dorsal-posterior region of a stage 4.5 embryo (Figure 7C) is a well-developed shell gland that is absent in the lobeless embryo (Figure 7D). While no external shell is formed, there are three irregularly placed clusters of cells in the lobeless embryo (marked by arrows in Figure 7D). These clusters of cells are randomly distributed in the lobeless embryo and they contain birefringent particles of shell-like material (McCain, 1992). In lobeless embryos these cells never form an external shell, but do form pieces of internal shell from fragments of differentiated non-evaginated shell gland (Labordus and van der Wal, 1986), that can be recognized because it is birefringent as is the external shell (McCain, 1992; Eyster, 1986). These clusters of cells stain with the 4D9 antibody (Figure 7D) which shows a nuclear localization (Figure 7E and 7F). In Figure 7F, the *engrailed* stained cells are arranged in a rosette similar to cells of the shell gland in a normal embryo (Figure 6B).

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Figure 7. Expression of *engrailed* in the lobeless embryo. (A) A 3-day old normal embryo stained with hematoxylin. (B) A 3-day old lobeless embryo stained with hematoxylin; note the radial symmetry and absence of polarity in the lobeless embryo. (C) A 4.5-day old normal embryo stained with hematoxylin as in (A). (D) A 4.5-day old lobeless embryo immunostained with the *en* antibody; arrows denote the appearance of cells (out of focus) stained by the *en* antibody. (E) An enlarged picture of the embryo in (D) showing nuclear immunostaining by the *en* antibody; these cells are in focus and are marked with arrows. (F) Photograph of another lobeless embryo showing nuclear staining by the *en* antibody of a rosette of shell gland cells.

Abbreviations: A. C., apical cell plate; Es, esophagus; Sh G, shell gland; Sto, stomadeum; A, P, D and V are the anterior, posterior, dorsal, and ventral areas, respectively of the normal embryo.

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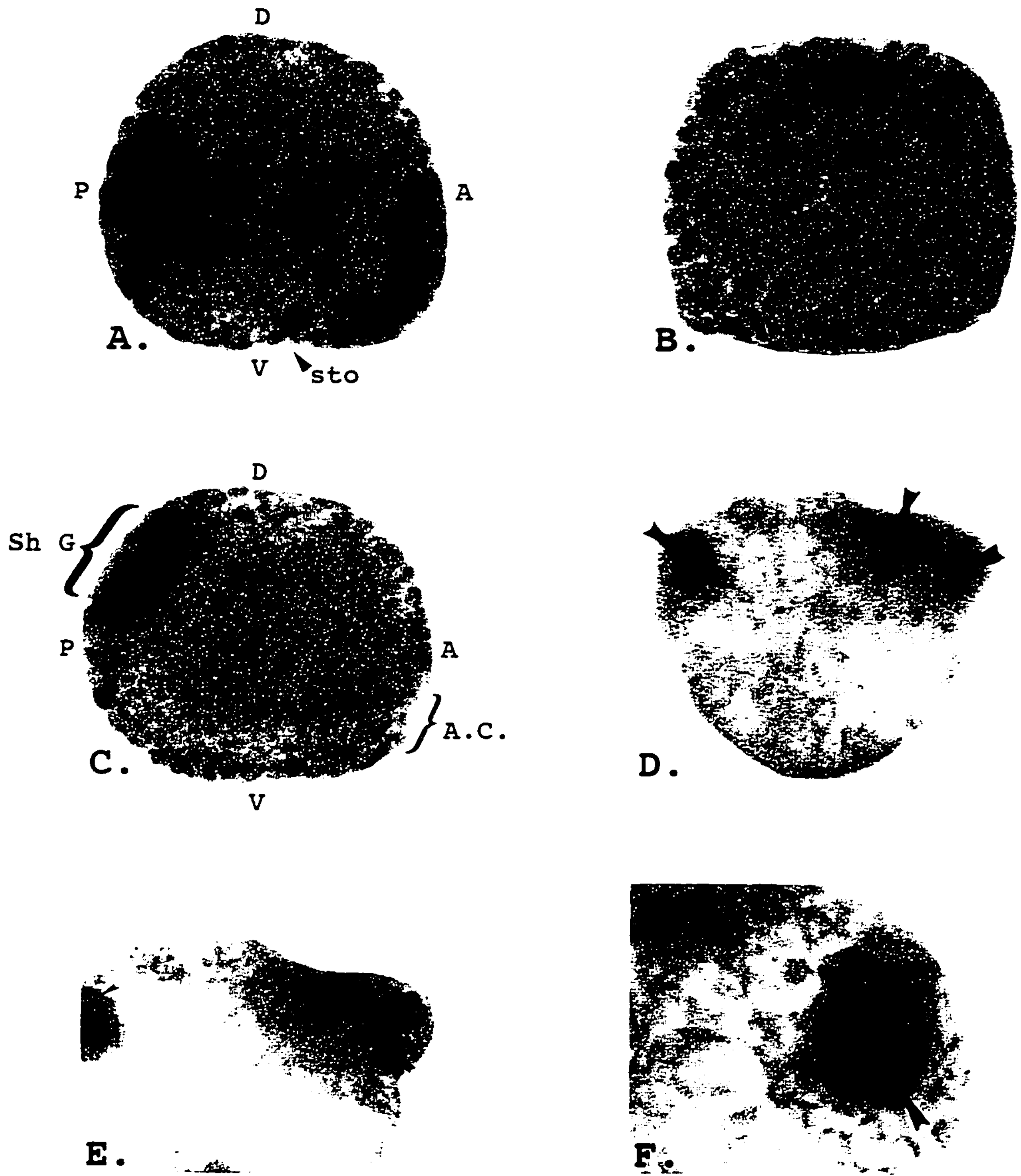

Figure 7

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Discussion

Expression of *engrailed* in *Ilyanassa*

I have shown that throughout embryogenesis and larval differentiation of *Ilyanassa* a cognate of *engrailed*, recognized by the 4D9 antibody and *in situ* hybridization, is expressed only in shell forming cells. Because I have not found any detectable expression of *Ily-en* in the 2d and 2c micromeres, which are essential for shell formation, I suggest that *Ily-en* expression is not associated with the early determination of the shell-lineage in *Ilyanassa*.

Differentiation of the Lobeless Embryo and *Ily*-en Expression

Ilyanassa embryos from which the third polar lobe is removed fail to differentiate an organized external shell. They do however have some randomly placed cells that make internal birefringent masses of shell-like material (McCain, 1992) and *Ily-en* is expressed in these randomly placed cells. Because Ily-*en* is expressed in shell gland cells in normal embryos and shell gland cells in lobeless embryos its expression is independent of the location of these cells in the embryo.

While *engrailed* may participate in establishing positional information required for polarity and regionalization in other organisms, its expression in the

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lobeless embryo of *Ilyanassa* does not alter the random placement of the fragments of internal shell. The lobeless embryo is disorganized and lacks the axial pattern of the normal embryo (compare Figures 7A, 7B, 7C, and 7D); accordingly, the failure of the lobeless embryo to properly position its shell gland cells may have arisen from a generalized disorganization of the embryo when the polar lobe was removed. Thus, we cannot conclude that *engrailed* expression is unrelated to positional information, but our results do support the conclusion that the expression of *engrailed* alone is not sufficient to provide the positional information required for the proper placement of the shell gland.

Cell Lineage of *engrailed* Expression

If the ancestral function of *engrailed* is specification of neuronal cells, as suggested by Patel *et al.* (1989), then why is it expressed in the shell gland of *Ilyanassa*?

The annelid ventral nerve cord and the molluscan shell gland arise from the lineage of the *first somatoblast* (2d) which is homologous among leeches (Whitman, 1878; Weisblat *et al.*, 1980), polychaete annelids (Wilson, 1892), and molluscs (Conklin, 1897). Rapid proliferation of the 2d lineage in both annelids and molluscs gives rise to a *posterior growing point* (Conklin, 1897; p 132) of numerous ectodermal cells; in annelids these cells will form the *ventral plate* (Wilson, 1892) from which the ventral nerve cord will be formed, in

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molluscs cells of the *posterior growing point* form the shell and part of the foot. This annelid lineage of the *first somatoblast* may account for the expression of *engrailed* in the "segmentally reiterated pattern of neuronal nuclei in the developing nervous system" of an oligochaete annelid and in the subesophageal and segmental ganglia of a leech (Patel *et al.*, 1989; Wedeen and Weisblat, 1991).

The cell lineage of *Crepidula* (Conklin, 1897) indicates the origin of the shell gland from the 2d cell or primary somatoblast; the origin of the shell gland from the 2d cell in *Ilyanassa* has also been established experimentally (Cather, 1967; Clement, 1986; Labordus and van der Wal, 1986; and McCain, 1992).

This commonality of lineages among annelids and molluscs suggests that there may be a commonality of gene expression within a lineage. Therefore, what appears to be a diverse relationship in *engrailed* expression may be readily explained by the homologous lineage of the primary somatoblast between the annelids and molluscs.

In conclusion, I suggest that cytoplasmic determinants common to the 2d lineage of annelids and molluscs are responsible for *engrailed* expression in these two phyla.

Though *Ily-en* is uniquely expressed in a polar lobe dependent structure, the shell gland, it is also expressed in lobeless embryos, therefore, I conclude that *Ily-en* expression does not require the presence of determinants contained in the polar lobe.

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It appears that evolutionarily conserved cytoplasmic determinants common to the 2d lineage of annelids and molluscs are responsible for *engrailed* expression in these two phyla, and that *engrailed* functions as an identifier of determined cells in this lineage. Furthermore, it appears that the conserved function of *engrailed* is to identify a specific set of determined cells that are to differentiate in a certain way, and, having marked these cells by its expression, it will participate in selecting a subset of genes required for the terminal differentiation of the cells it has identified. Therefore, the role of *engrailed* in development is critical. In *Drosophila engrailed* marks compartment, in *Ilyanassa* it marks the shell gland. As segmentation is crucial for making a fly, shell formation is crucial for making a snail.

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Introduction to *twist*

Establishment of mesoderm is a result of gastrulation, a process whose mechanism varies among different organisms. Whether gastrulation is achieved by invagination, involution, epiboly or induction, mesoderm differentiation is controlled by similar regulatory molecules. A principle regulatory molecule is the protein transcription factor encoded by *twist* (*twi*). *twist* is an early mesoderm-specific gene which functions in the determination and differentiation of mesoderm. The *twist* gene encodes a basic helix-loop-helix (bHLH) protein which is required for activating and maintaining the expression of further mesodermal genes, including itself.

twist was first identified in *Drosophila* (Simpson, 1983; Thisse *et al.*, 1987) and shown to be essential for mesoderm formation (Nüsslein-Volhard *et al.*, 1984). The mesodermal fate of cells in *Drosophila* is controlled by two genes, *twist* and *snail*. Upon activation by the maternal determinant *Dorsal*, the *twist* protein, found in the ventral most cells of the embryo initiates the formation of the ventral furrow, an invagination of approximately 1000 cells, leading to a layer of mesodermal tissue beneath the ectoderm. All the mesodermal derivatives of the embryo such as muscles, fat bodies and gonads are derived from these cells. *snail*, a gene that encodes a zinc-finger protein, is also activated by the *dorsal* protein and its function is the repression of

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lateral genes such as *rhomboid*, a gene essential for neuroectoderm (Jiang and Levine, 1991) In *snail* mutants, *rhomboid* is not repressed causing the ventral cells to take on a more lateral state causing a disruption of the mesoderm (Nambu *et al.*, 1990).

The initial activation of *twist* involves *Dorsal* binding to regions of activation, which contain *Dorsal*-binding sites, upstream of the promoter (Pan *et al.*, 1991; Ip *et al.*, 1991). *twist* then maintains its own expression and that of *snail*, as well as activating expression of other mesoderm-specific genes. In *twist* mutants, *snail* expression is limited to a narrow band, but this pattern disappears in the cellular blastoderm demonstrating the need for *twist* to maintain *snail* expression. Embryos mutant in both *twist* and *snail* form no mesoderm and die at the end of embryogenesis, demonstrative of their essential role in mesodermal differentiation and morphogenesis (Leptin, 1991).

In *Drosophila* the level of *twist* expression determines the differentiation of mesoderm into various mesodermal tissues. High levels of *twist* cause the formation of somatic muscle, where as low levels of *twist* permit the formation of other mesodermal derivatives, such as visceral muscle fibers and heart (Baylies and Bate, 1996). By modulating *twist* expression *Drosophila* has a mechanism to produce different derivatives from mesoderm. *twist* homologs, *Xtwist* (Xenopus), *Mtwist* (mouse), and bHLH-EC2 (human), share a similar expression pattern, first present in the determination of

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mesoderm and then in the subdivision of the mesodermal derivatives. These observations show that there is an analogy in function of the *twist* family of proteins among chordates and arthropods in patterns of expression leading to the formation of mesodermal derivatives. These results suggest that *twist* is an ancient regulatory gene whose function as a mesodermal selector gene has been retained throughout evolution.

Because entomesoderm formation is polar lobe dependent in *Ilyanassa*, and because the teloblastic mechanism of mesoderm formation in *Ilyanassa* is radically different from *Drosophila* and other organisms I have undertaken a study of whether the regulatory gene *twist* has been conserved in *Ilyanassa* and if it has retained its role as a mesoderm-specific gene.

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Results

Sequence of *twist*

Using degenerate primers and PCR amplification I have sequenced a 171 nucleotide fragment of *Ilyanassa* DNA (Figure 8A). This DNA fragment has a 77% identity to the bHLH *twist* gene of *Drosophila.* The conceptual translation (Figure 8B) of this fragment has an 82% identity to the bHLH protein encoded by the *Drosophila twist* gene. From this close identity to *Drosophila twist,* I conclude that there is in *Ilyanassa* a homolog of *twist;* I designate this homolog as *Ily-twist.*

Regional identities of this fragment of *Ily-twi* to corresponding regions of *Drosophila, Mus* and *Xenopus twist* are shown in Figure 8C. There is 100 percent identity in the DNA binding site and the loop region of all four *twist* genes. The closest identity of *Ily-twi,* in both Helix I and Helix II is to *Mus twist.*

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Figure 8. *twist* sequence homology. (A) DNA sequence homology between *Ilyanassa*, *Drosophila*, *Mus* and *Xenopus*. *Ilyanassa* has a 77% homology with *Drosophila*, a 78% homology with *Mus* and a 74% homology with *Xenopus*. (B) Deduced amino acid homology. Identical residues are highlighted in pink, conservative substitutions are highlighted in yellow. (C) Percent identity in each region.

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A. *twist* DNA Homology

```
Consensus   CARMGGGTSA TGGCYAAYGT VMGGGAGMGB CAGMGSACBC AGWSSCTSAA   50
Ilyanassa   CAAAGGGTGA TGGCTAATGT ACGGGAGAGG CAGAGGAGGC AGAGCCTGAA
Drosophila  CAGCGGGTCA TGGCCAATGT GAGGGAGCGC CAGCGCACCC AGAGCCTCAA
Mus         CAGCGGGTCA TGGCTAACGT GCGGGAGCGC CAGCGCACGC AGTCGCTGAA
Xenopus     CAGAGGGTCA TGGCCAATGT CAGGGAGCGT CAGAGGACTC AGTCCCTCAA

Consensus   CGAVGCSTWC DMSBMSYTGM RVMAGATCAT CCCCACSCTS CCCWSSGACA  100
Ilyanassa   CGACGGGTAC GCCCAGCTGA GGCAGATCAT CCCCACGCTG CCCTCGGACA
Drosophila  CGACGCCTTC AAGTCCCTGC AGCAGATCAT CCCCACGCTG CCCAGCGACA
Mus         CGAGGCGTTC GCCGCCCTGC GCAAGATCAT CCCCACGCTG CCCTCGGACA
Xenopus     CGAAGCCTTC TCGTCCTTGA GAAAGATCAT CCCCACCCTC CCCTCGGACA

Consensus   ARCTSAGYAA RATYCAYACS CTSAARYTGG CSDCVMGVTA CATCGACKKY  150
Ilyanassa   AGCTCAGCAA GATCCAGACG CTGAAGTTGG CCACGCGCTA CATCGACGGT
Drosophila  AGCTCAGCAA GATCCAGACC CTCAAACTGG CCACAAGGTA CATCGACTTC
Mus         AGCTGAGCAA GATTCAGACC CTCAAACTGG CGGCCAGGTA CATCGACTTC
Xenopus     AACTGAGTAA AATCCAAACG CTCAAACTGG CCTCCAGATA CATCGACTTT

Consensus   CTSTRCCRSR TSYTVMRSWS S                                 174
Ilyanassa   CTGTACCAGG TGCTGAGGAC G
Drosophila  CTGTGCCGCA TGCTCAGCTC G
Mus         CTGTACCAGG TCCTGCAGAG C
Xenopus     CTCTGCCAGG TCTTACAGAG C
```

B. Deduced Amino Acid Homology

```
                     10        20        30        40        50
            123456789012345678901234567890123456789012345678901234567
Consensus   QRVMQNVRERQRTQSLN.AF..LR.IIPTLPSDKLSKIQTLKLA.RYIDFL.QVL.S
Ilyanassa   QRVMQNVRERQRTQSLNDAYAQLRQIIPTLPSDKLSKIQTLKLATRYIDGEYQVLRT
Drosophila  QRVMQNVRERQRTQSLNDAFKSLQQIIPTLPSDKLSKIQTLKLATRYIDFLCRMLSS
Mus         QRVMQNVRERQRTQSLNEAFAALRKIIPTLPSDKLSKIQTLKLAARYIDFLYQVLQS
Xenopus     QRVMQNVRERQRTQSLNEAFSSLRKIIPTLPSDKLSKIQTLKLASRYIDFLCQVLQS
            <-----DNA BINDING SITE-><----HELIX I----><------LOOP-----><---HELIX II--->
```

DNA BINDING SITE — HELIX I — LOOP — HELIX II

C. Regional Homologies to *Ilyanassa*

	DNA Binding Site	Helix I	Loop	Helix II	Overall Homology
Drosophila	100%	66%	100%	54%	82%
Mus	100%	73%	100%	69%	86%
Xenopus	100%	66%	100%	62%	82%

Figure 8

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Ily-twi Expression

Because the *Ilyanassa* mesodermal lineage is established during early cleavage, I looked for *twist* expression during early stages of development. *In situ* hybridization and immunostaining were used to follow the localization of *twist* mRNA and the *twist* antigen, respectfully. A 171 nucleotide digoxigenin-labeled DNA probe of *Ily-twi* was used for *in situ* hybridization and a polyclonal antibody against *Drosophila twist* was used for immunostaining. Controls for *in situ* hybridization were (1) hybridization in the absence of labeled probe and (2) RNase digestion of embryos prior to the hybridization reaction (Figures 9F and 9G). In both cases there was no hybridization signal after incubation with anti-digoxigenin antibody and staining. Immunostaining controls were reactions in (1) the absence of all antibodies and (2) in the absence of the primary antibody. Immunostaining did not occur in either of these controls. These controls support the reliability of the *in situ* hybridization and immunostaining reactions.

In situ hybridization revealed the presence of *twist* mRNA in the germinal vesicle (Figure 9A), suggesting a maternal origin of *twist* mRNA in *Ilyanassa*. Localization of the *twist* mRNA continues in the animal hemisphere throughout polar lobe formation and trefoil stage (Figure 9B). To further determine the time and place of *twist* expression, I immunostained whole-mount embryos with a polyclonal *twist* antibody. There was, as expected, no staining in the

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germinal vesicle (Figure 9C), but by the time the first polar lobe was formed the animal pole of the egg was immunostained, this immunostaining of *Ily-twi* persisted through trefoil stage (Figures 9D and 9E). These results show that while *twist* mRNA is maternally localized its translation begins in the zygote.

In the normal embryo *Ily-twi* mRNA continues to be translated during early cleavage. Examples of immunostained 2, 4 and 8-cell embryos are illustrated in Figures 10A, 10C and 10E. Note in Figure 10E that the first quartet of micromeres (1a, 1b, 1c and 1d) are intensively stained peripherally while the central area of each cell is less intensively stained. By focusing-through these cells it is seen that this area is the cell nucleus which is not immunostained for the *Ily-twi* antigen. Thus the *twist* antigen remains in the cytoplasm and has not accumulated in the cell nucleus at this stage.

The *Ily-twi* antigen does not accumulate in the nucleus until about the 28-cell stage when it is first seen in cells of the mesodermal lineage, namely, Me^1 and Me^2, and the right and left mesoblasts as shown in Figure 11. The appearance of staining in these cells at the final step in the delineation of the entomesoderm lineage, thus, the accumulation of *Ily-twi* in the nuclei, is precisely correlated with entomesoderm formation.

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Expression of *Ily-twi* in Lobeless Embryos

As previously discussed, the removal of the polar lobe at trefoil stage results in the absence of entomesoderm. Therefore, I studied the affect of polar lobe removal on *twist* expression. The removal of the polar lobe caused an immediate halt in the translation of *twist* mRNA as indicated by the failure of 2, 4 and 8-cell lobeless embryos to immunostain with the *twist* antibody (Figures 10B, 10D and 10F).

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Figure 9. *twist* expression. (A) *in situ* hybridization of the germinal vesicle (GV) with an *Ily-twi* DNA probe. Note staining in GV only. (B) *in situ* hybridization of second polar lobe stage. Staining is limited to the animal pole. (C) Germinal vesicle immunostained with a polyclonal antibody for *twist*, showing no staining in the germinal vesicle. (D) Second polar lobe stage immunostained with the *twist* antibody, shows similar staining as compared to *in situ* hybridization. (E) Trefoil stage immunostained with the *twist* antibody. (F) Ribonuclease treated germinal vesicle, then hybridized with the *Ily-twi* probe. Note the lack of staining in the GV with Figure 9A. (G) Ribonuclease treated second polar lobe stage prior to *in situ* hybridization results in the loss of staining.

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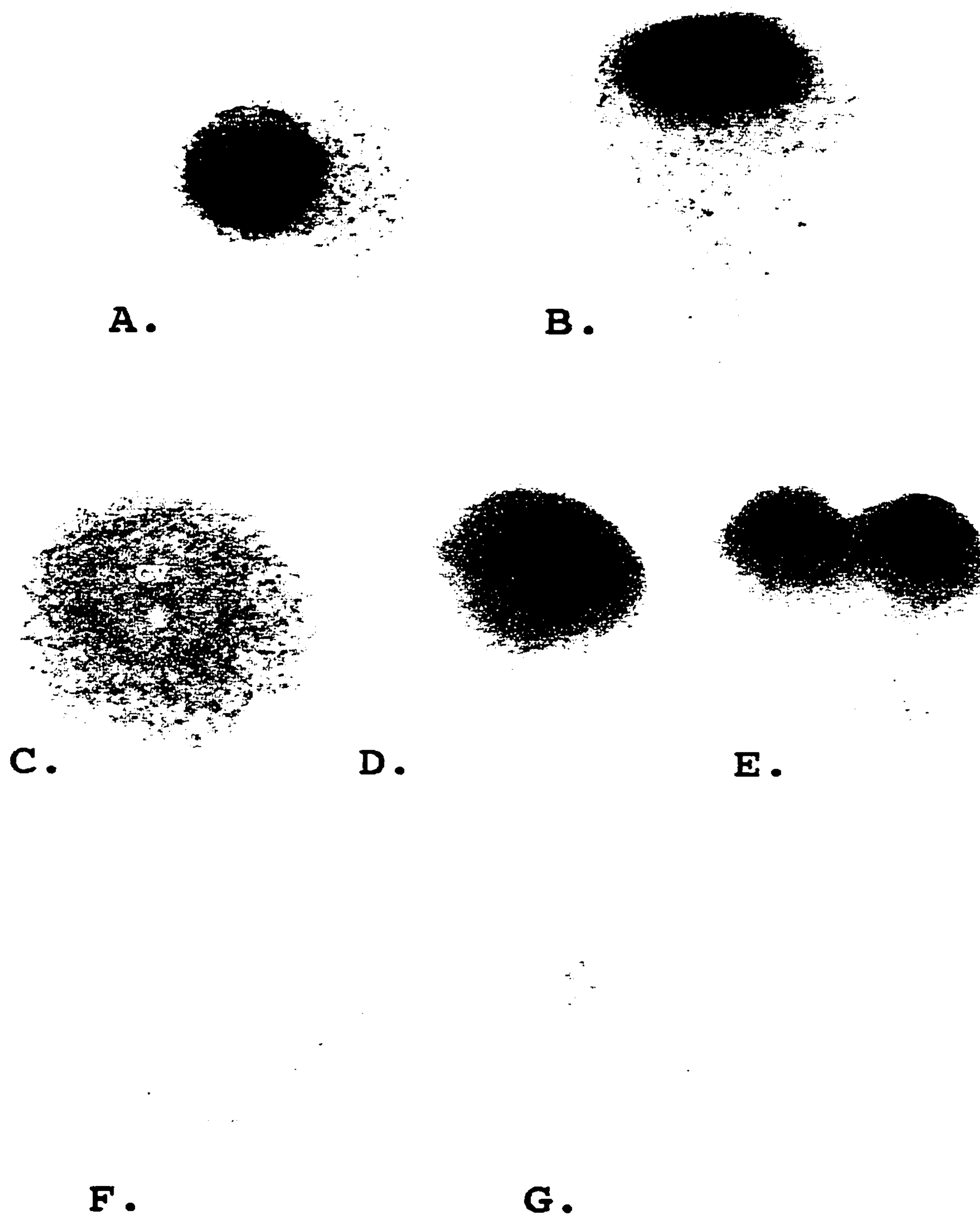

Figure 9

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Figure 10. Expression of *twist* in normal and lobeless embryos. (A) A 2-cell embryo immunostained with the twist antibody. Note staining in the animal pole. (B) A 2-cell lobeless embryo immunostained with the *twist* antibody showing no staining. (C) A 4-cell embryo immunostained with the *twist* antibody. Note no staining in the nuclei. (D) A 4-cell lobeless embryo showing no localization when immunostained with the *twist* antibody. (E) An 8-cell embryo with its first set of micromeres immunostained with *twist* antibody (staining in both the macromeres and micromeres in the cytoplasm). (F) An 8-cell lobeless embryo showing no localization when immunostained with *twist* antibody.

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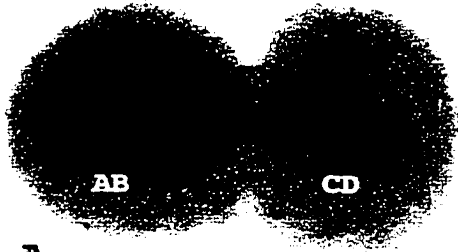
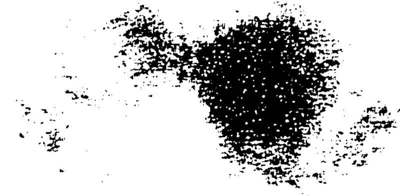
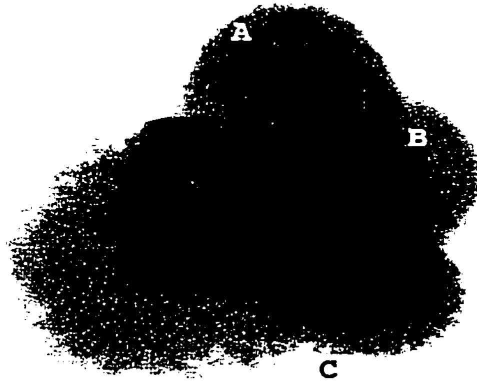


A.

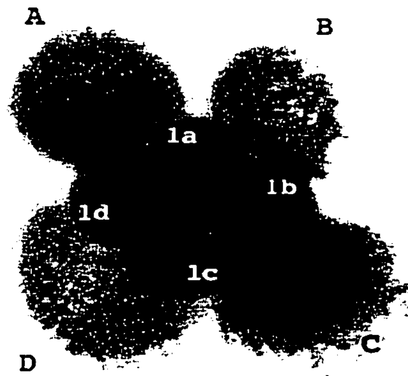


B.



C.

D.



E.

F.

Figure 10

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Figure 11. Localization of *twist* in the mesodermal lineage of *Ilyanassa.* Immunostained with *twist* antibody showing localization of *Twist* in the nuclei of the endodermal lineage (Me^1 and Me^2) and the primary mesoblast cells (m^1 and m^2) of *Ilyanassa.*

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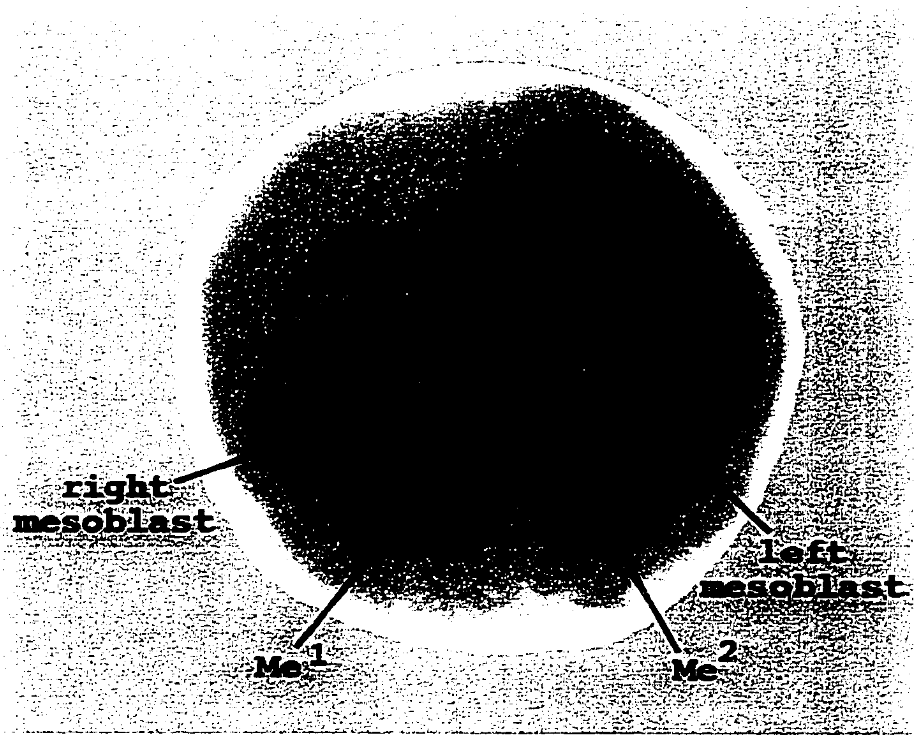

Figure 11

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Discussion

Expression of *twist* in *Ilyanassa*

The results of whole mount *in situ* hybridization of *Ilyanassa* embryos with an *Ily-twi* DNA probe revealed the presence of *Ily-twi* maternal messenger RNA (mmRNA) in the germinal vesicle of the *Ilyanassa* oocyte (Figure 9A) and in the animal pole cytoplasm of the fertilized egg through the second polar lobe stage (Figure 9B). The *Ily-twi* mRNA localized in the germinal vesicle indicates that *Ily-twi* is a maternal gene whose transcripts have been retained in the germinal vesicle. After fertilization, when the germinal vesicle breaks down, the *Ily-twi* mRNA is released into the egg cytoplasm where it remains localized in the animal hemisphere of the egg.

From *in situ* hybridization of later cleavage stages the presence of *Ily-twi* mRNA is not evident, however, immunostaining with a *twist* antibody shows that the *Ily-twi* protein is rapidly translated and accumulated in all of the micromeres (Figure 10E; data not shown for later quartets of micromeres). *Ily-twis*t protein is accumulated only in the cytoplasm of the micromeres. The first nuclear appearance of this protein is, as described above, in the mesodermal lineage, Me^1, Me^2 and the right and left mesoblasts (Figure 11). This precise correlation between the nuclear localization of the *Ily-twi* protein and the final step of the establishment of the entomesodermal lineage suggests that

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Ily-twi plays a determinative role in entomesoderm differentiation.

Thusfar, it is not known whether *twist* is active in determining ectomesoderm derived from the micromere 3a, 3b and 3c.

The suggested role of *Ily-twi* in entomesoderm determination extends the role of *twist* to another animal group, the molluscs, which has yet another mechanism for gastrulation and mesoderm formation. Homologs of *twist* showing remarkable homology and similar functions in mesoderm formation have been found in arthropods (Thisse *et al.*, 1988), annelids (Soto *et al.*, 1997), molluscs (Moshel and Collier, 1997) and chordates (Wolf *et al.*, 1991; Hopwood *et al.*, 1989; Quertermous *et al.*, 1994). Thus, it appears that the *twist* gene has been adopted early in animal evolution as a designator of mesoderm, and that this function has been conserved even though the structural modes of gastrulation and mesoderm differentiation have varied from one phylum to another. This suggests that structural features of development are less conserved than the molecular genetics of development; indeed, it raises the question that these two aspects of development may be subjected to different evolutionary selection pressures.

Aside from the above evolutionary implications this study has brought to light what appears to be a non-transcriptional and non-translational mode of gene regulation involved in the embryology of territory-specification. The

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ubiquitous dispersal of the *twist* protein among the micromeres with a final segregation by localization into the cell nuclei of the entomesodermal lineage is yet another, but not unique, mechanism for regulating gene expression in the establishment of specific cell types. Thus, there must be selective cues for the transport of transcription factors into specific cell nuclei, whereupon, these transcription factors can further regulate the expression of other genes.

This mode of specifying the entomesodermal lineage by *Ilyanassa* is similar to the segregation of the *dorsal* protein, also produced by a mmRNA, into the nuclei of the ventral cells of the *Drosophila* embryo (Roth *et al.*, 1989; Rushlow *et al.*, 1989). Similarly, the activity of the transcription activator *Oxt* is regulated by its specific accumulation in the cell nuclei of the sea urchin embryo (Klein *et al.*, 1995).

Expression of *twist* in the lobeless embryo

As shown in Figures 10B, 10D and 10F the *twist* protein is not present in the lobeless embryo (compare with *twist* protein in normal embryos in Figures 10A, 10C and 10E), even though mmRNA was in the germinal vesicle of the oocyte before removal of the polar lobe. Note from Figures 9D and 9E that the *twist* protein is localized in the animal hemisphere of the egg and is absent in the polar lobe (Figure 9E). Figure 9E is a trefoil stage, which has synthesized *twist* protein, and the isolatable polar lobe is still attached to the egg.

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Although *Twist* is present in the trefoil stage it is absent moments later in the 2-cell lobeless embryo; presumably it has degraded.

On the basis of the present observations the absence of the polar lobe prevents the translation of *twist* mRNA. The lobeless egg does not show a general reduction in its ability to synthesize proteins as Collier and McCarthy (1981) and Collier (1981) have shown by two-dimensional electrophoresis of radioactive proteins. Both normal and lobeless embryos synthesize the same set of proteins. The failure of the lobeless embryo to synthesize the *twist* protein may be caused by a specific polar lobe factor(s) that for this protein or a special class of proteins, are not detectable by the electrophoresis of radioactive proteins.

This study has shown that there are factors in the polar lobe that are required for synthesis and accumulation of the transcriptional factor encoded by *Ily-twi*. This influence of the polar lobe at the translation level is a mechanism for the regulation of the expression of maternally transcribed genes, to what extent the polar lobe influences the expression of zygotic genes remains to be determined.

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REFERENCES

Atkinson, J. W. (1971). Organogenesis in normal and lobeless embryos of the marine prosobranch gastropod *Ilyanassa obsoleta*. *J. Morph.* 133, 339-352.

Atkinson, J. W. (1986). An atlas of light micrographs of normal and lobeless larvae of the marine gastropod *Ilyanassa obsoleta*. *Intern. J. Invert. Reprod. Dev.* **9**, 169-178.

Baylies, M. K. and Bate, M. (1996). *twist*: A myogenic switch in *Drosophila*. *Science* **272**, 1481-1484.

Bower, D. L. (1986). *engrailed* gene expression in *Drosophila* imaginal discs. *EMBO J.* **5**, 2649-2656.

Brandhorst, B. P. and Newrock, K. M. (1981). Post-translational regulation of protein synthesis in *Ilyanassa* embryos and isolated polar lobes. *Dev. Biol.* **83**, 250-254.

Carroll, S. B. (1995). Homeotic genes and evolution of arthropods and chordates. *Nature* **376**, 479-485.

Cather, J. N. (1967). Cellular interactions in the development of the shell gland of the gastropod *Ilyanassa*. *J. Exp. Zool.* **166**, 205-224.

Clement, A. C. (1952). Experimental studies on germinal localization in *Ilyanassa*. I. The role of the polar lobe in determination of the cleavage pattern and its influence in later development. *J. Exp. Zool.* **121**, 593-626.

Clement, A. C. (1956). Experimental studies on germinal localization in *Ilyanassa*. II. The development of isolated blastomeres. *J. Exp. Zool.* **132**, 427-445.

Clement, A. C. (1962). Development of *Ilyanassa* following removal of the D macromere at successive cleavage stages. *J. Exp. Zool.* **149**, 193-216.

Clement, A. C. (1967). The embryonic value of the micromeres in *Ilyanassa obsoleta*, as determined by deletion experiments. I. The first quartet cells. *J. Exp. Zool.* **166**, 77-88.

Clement, A. C. (1971). *Ilyanassa*. In "Experimental Embryology of Marine and Fresh-water Invertebrates," (G. Reverberi, Ed.), North-Holland Pub. Co., Amersterdam-London. pp. 188-214.

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Clement, A. C. (1986). The embryonic value of the micromeres in *Ilyanassa obsoleta*, as determined by deletion experiments. I. The second quartet cells. *Intern. J. Invert. Reprod. Dev.* **9**, 139-153.

Clement, A. C. (1986). The embryonic value of the micromeres in *Ilyanassa obsoleta*, as determined by deletion experiments. I. The third quartet cells and the mesentoblast cell, 4d. *Intern. J. Invert. Reprod. Dev.* **9**, 155-168.

Coleman, K. G., Poole, S. J., Weir, M. P., Soeller, W. C. and Kornberg , T. (1987). The *invected* gene of *Drosophila*: sequence analysis and expression studies reveal a close kinship to the *engrailed* gene. *Genes & Dev.* **1**, 19-28.

Collier, J. R. (1975). Polyadenylation of nascent RNA during the embryogenesis of *Ilyanassa obsoleta*. *Exptl. Cell Res.* **95**, 263-268.

Collier, J. R. (1976). Nucleic acid chemistry of the *Ilyanassa* embryo. *Amer. Zool.* **16**, 483-500.

Collier, J. R. (1977). Rates of RNA synthesis in the normal and lobeless embryo of *Ilyanassa obsoleta*. *Exptl. Cell Res.* **106**, 390-394.

Collier, J. R. (1981). *Methods of obtaining and handling eggs and embryos of the marine mud snail Ilyanassa obsoleta.* In "Marine Invertebrates," Report of the Committee on Marine Invertebrates, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington, D.C. pp. 217-232.

Collier, J. R. (1981). Protein synthesis in the polar lobe and lobeless egg of *Ilyanassa obsoleta*. *Biol. Bull.* **160**, 366-375.

Collier, J. R. and McCarthy, M. E. (1981). Regulation of peptide synthesis during early embryogenesis of *Ilyanassa obsoleta*. *Differentiation* **19**, 31-46.

Collier, J. R. (1983). *The biochemistry of molluscan development.* In "The Mollusca Development," (N. H. Verdonk, J. A. M. Van Den Biggelaar, and A. S. Topma, Eds.), Academic Press, NY. pp. 253-297.

Condron, B. G., Patel, N. H. and Zinn, K. (1994). *engrailed* controls glial/neuronal fate decisions at the midline of the central nervous system. *Neuron* **13**, 541-554.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Conklin, E. G. (1897). The embryology of *Crepidula,* contribution to the cell lineage and early development of some marine gastropods. *J. Morph.* **13**, 1-226.

Conklin, E. G. (1905). Mosaic development in ascidian eggs. *J. Exp. Zool.* **2**, 146-223.

Crampton, H. E. (1896). Experimental studies on gasteropod development. *Roux' Arch. Entw.-mech.* **3**, 1-19.

Darnell, D. K., Kornberg, T. and Ordahl, C. P. (1986). *ChickEn*: a chick genomic clone with homology to the *Drosophila engrailed* homeo box. *J. Cell Biol.* **103**, 311a.

Davidson E. H., Haslett, G. W., Finney, R. J., Allfrey V. G. and Mirsky, A. E. (1965). Evidence for prelocalization of cytoplasmic factors affecting gene activation in early embryogenesis. *Proc. Natl. Acad. Sci.* **54**, 696-704.

Davidson, E. H. (1986). "*Gene activity in early development,*" (3rd ed.) Academic Press, New York.

DiNardo, S., Kuner, J. M., Theis, J. and O'Farrell, P. H. (1985). Development of embryonic pattern in *D. melanogaster* as revealed by accumulation of the nuclear *engrailed* protein. *Cell* **43**, 59-69.

Degnan, B. M., Degnan, S. M., Giusti, A. and Morse, D. E. (1995). A *hox/hom* homeobox gene in sponges. *Gene* **155**, 175-177.

Dolecki, G. J. and Humphreys, T. (1988) An *engrailed* class homeobox gene in sea urchins. *Gene* **64**, 21-31.

Donohoo, P., and Kafatos, F. C. (1973). Differences in the proteins synthesized by the progeny of the first two blastomeres of *Ilyanassa,* a "mosaic" embryo. *Dev. Biol.* **32**, 224-229.

Ecker, R. (1929). The recessive mutant *engrailed* in *Drosophila melanogaster. Hereditas* **12**, 217-222.

Ettensohn, C. A. and McClay, D. R. (1988). Cell lineage conversion in the sea urchin embryo. *Dev. Biol.* **125**, 396-409.

Eyster, L. S. (1986). Shell inorganic composition and onset of shell mineralization during bivalve and gastropod embryogenesis. *Biol. Bull.* **170**, 211-231.

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Fjose, A., McGinnis, W. J, and Gehring, J. (1985). Isolation of a homeobox-containing gene from the *engrailed* region of *Drosophila* and the spatial distribution of its transcripts. *Nature* **313**, 284-289.

Fjose, A., Eiken, H. G., Njolstad, P. R., Molven, A. and Hordvik, I. (1988). A zebrafish *engrailed*-like homeobox sequence expressed during embryogenesis. *FEBS Lett.* **181**, 173-182.

Friedman, G. C. and O'Leary, D. D. M. (1996). Retroviral misexpression of *engrailed* genes in the chick optic tectum perturbs the topographic targeting of retinal axons. *J. Neurosci.* **16**, 5498-5509.

Greenwald, I. (1989). Cell-cell interactions that specify certain cell fates in *C. elegans* development. *Trends Genet.* **5**, 237-241.

Gilbert, S. F. (1994). "*Developmental Biology*," 4th ed. Sinauer, Sunderland, Massachusetts.

Gimlich, R. L. and Gerhart, J. C. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Dev. Biol.* **104**, 117-130.

Henry, J. J., Amemiya, S., Wray, G. A. and Raff, R. A. (1989). Early inductive interactions are involved in restricting cell fates of mesomeres in sea urchin embryos. *Dev. Biol.* **136**, 140-153.

Henry, J. J. and Grainger, R. M. (1990). Early tissue interactions leading to embryonic lens formation in *Xenopus laevis*. *Dev. Biol.* **141**, 149-163.

Hidalgo, A. (1996). The roles of *engrailed*. *Trends Genet.* **12**, 1-4.

Holland, P. W. H. and Hogan, B. L. M. (1986). Phylogenetic distribution of *Antennapedia*-like homeoboxes. *Nature* **321**, 251-253.

Holland, L. Z., Kene, M., Williams, N. A. and Holland, N. D. (1997). Sequence and embryonic expression of the amphioxus *engrailed* gene *(AmphiEn)*: the metameric pattern of transcription resembles that of its segment-polarity homolog in *Drosophila*. *Development* **124**, 1723-1732.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and neural crest. *Cell* **59**, 893-903.

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Hyman, L. H. (1951). "*The Invertebrates: Platyhelminthes and Rhynchocela.*" McGraw Hill, New York.

Ip, T. Y., Kraut, R., Levine, M. and Rushlow, C. (1991). The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell* **64**, 439-446.

Jiang, J. and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the *dorsal* gradient morphogen. *Cell* **72**, 741-752.

Joyner, A., Kornberg, T., Coleman, K. G., Cox, D. and Martin, G. R. (1985). Expression during embryogenesis of a mouse gene with sequence homology to the *Drosophila engrailed* gene. *Cell* **43**, 29-37.

Klein, W. H., Mao, C. A., Gan, K. M., Chuang, C. K. and Wikramanayake, A. H. (1995). *Manipulating cell fate in the sea urchin embryo.* In "Invertebrate Reproduction and Development," 7th ICIR Proceedings (in press).

Kornberg, T. (1981). *engrailed*: a gene controlling compartment and segment formation in *Drosophila*. *Proc. Natl. Acad. Sci.* **78**, 1095-1099.

Kornberg, T., Siden, I., O'Farrell, P. and Simon, M. (1985). The *engrailed* locus of *Drosophila*: In situ localization of transcripts reveals compartment-specific expression. *Cell* **40**, 45-53.

Kornberg, T. B. and Tabata, T. (1993). Segmentation of the *Drosophila* embryo. *Curr. Opin. Genet. Dev.* **3**, 585-593.

Labordus, V. and van der Wal, U. P. (1986). The determination of shell field cells during the first hour in the sixth cleavage cycle of eggs of *Ilyanassa obsoleta*. *J. Exp. Zool.* **239**, 65-75.

Lawrence, P. A. and Johnston, P. (1994). On the role of the *engrailed* gene in internal organs of *Drosophila*. *EMBO J.* **3**, 2839-2844.

Lawrence, P. A. and Morata, G. (1976). Compartments in the wing of *Drosophila*: a study of the *engrailed* gene. *Dev. Biol.* **50**, 321-337.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell* **85**, 951-961.

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Leptin, M. and Grunewald, B. (1990). Cell shape changes during gastrulation in *Drosophila*. *Development* **110**, 73-84.

Leptin, M. (1991). Mesoderm determination by *twist* and *snail*. *Genes & Dev.* **5**, 1568-1572.

Logan, C., Willard, H. F., Rommens, J. M., and Joyner, A. L. (1989). Chromosomal localization of the human homeo-box containing genes En-1 and En-2. *Genomics* **4**, 206-209.

Logan, C., Hanks, M. C., Noble-Topham, S., Nallainathan, D., Provart, N. J. and Joyner, A. L. (1992). Cloning and sequence comparison of the mouse, human and chicken *engrailed* genes reveal potential functional domains and regulatory regions. *Dev. Genet.* **13**, 345-358.

Loomis, C. A., Harris, E., Michaud, J., Wurst, W., Hanks, M. and Joyner, A. L. (1996). The mouse *Engrailed-1* gene and ventral limb patterning. *Nature* **382**, 360-363.

McCain, E. R. (1992). Cell interactions influence the pattern of biomineralization in *Ilyanassa obsoleta* (Mollusca) embryo. *Dev. Dynamics* **195**, 188-200.

Morata G., and Lawrence, P. A. (1975). Control of compartment development by the *engrailed* gene in *Drosophila*. *Nature* **255**, 614-617.

Moshel, S. M. and Collier, J. R. (1997). The expression of the mesodermal gene *twist* in the marine mud snail *Ilyanassa obsoleta* and the identification of a homolog. *Dev. Biol.* **186**, 273.

Nambu, J. R., Franks, R. G., Hong, S. and Crews, S. (1990). The single-minded gene of *Drosophila* is required for the expression of genes important for the development of CNS midline cells. *Cell* **63**, 63-75.

Newrock, K. M. and Brandhorst, B. P. (1981). Post-transcriptional regulation of protein synthesis in *Ilyanassa* embryos and isolated polar lobes. *Dev. Biol.* **83**, 250-254.

Nieuwkoop, P. D. (1969). The formation of the mesoderm in urodele amphibians. I. Induction by the endoderm. *Roux' Arch. Entw.-mech.* **162**, 341-373.

Nieuwkoop, P. D. (1973). The "organisation center" of the amphibian embryo: its origin, spatial organisation and morphogenetic action. *Adv. Morphogen.* **10**, 1-39.

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Nüsslein-Volhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Dev. Biol.* **193**, 267-282

Pan, D., Huang, J. and Courey, A. J. (1991). Functional analysis of the *Drosophila twist* promoter reveals a *dorsal*-binding ventral activator region. *Genes & Dev.* **5**, 1892-1901.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

Poole, S. J., Kauvar L. M., Drees, B. and Kornberg, T. (1985). The *engrailed* locus of *Drosophila*: Structural analysis of an embryonic transcript. *Cell* **40**, 37-43.

Quertermous, E. E., Hidai, H., Blanar, M. A. and Quertermous, T. (1994). Cloning and characterization of a basic helix loop helix protein expressed in early mesoderm and developing somites. *Proc. Natl. Acad. Sci. USA* **91**, 7066-7070.

Render, J. (1991). Fate Maps of the first quartet micromeres in the gastropod *Ilyanassa obsoleta*. *Development* **113**, 495-501.

Rétaux, S., McNeill, L. and Harris, W. A. (1996). *Engrailed,* retinotectal targeting, and axonal patterning in the midbrain during *Xenopus* development: an antissense study. *Neuron* **16**, 63-75.

Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsal ventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-1202.

Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in *Drosophila. Cell* **5**, 1165-1177.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Scott, M. P., Tamkun, J. W. and Hartzell III, G. W. (1989). The structure and function of the homeodomain. *Biochimica et Biophysica Acta* **989**, 25-48.

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Simpson, P. (1983). Maternal-zygotic gene interactions during the formation of the dorsoventral pattern in *Drosophila* embryos. *Genetics* **105**, 615-632.

Soto, J. G., Nelson, B. H. and Weisblat, D. A. (1997). A leech homolog of *twist*: evidence for its inheritance as a maternal mRNA. *Gene* **199**, 31-37.

Spemann, H. and Mangold, H. (1924). Induction of embryonic primordia by implantation of organizers from different species. *Roux' Arch. Entw.-mech.* **100**, 599-638.

St Johnston, D., and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-219.

Steward, R. (1989). Cytoplasmic and nuclear *dorsal* protein. *Cell* **59**, 1179-1188.

Storey, K., Crossley, J. M., De Robertis, E., Norris, W. E. and Stern, C. D. (1992). Neural induction and regionalisation in the chick embryo. *Development* **114**, 729-741.

Tautz, D. (1996). Selector genes, polymorphisms and evolution. *Science* **271**, 160-161.

Thisse, B., Stoetzel, C., Messal, M. E. and Perrin-Schmitt, F. (1987). Genes of the *Drosophila* maternal *dorsal* group control the specific expression of the zygotic gene *twist* in the presumptive mesodermal cells. *Genes & Dev.* **1**, 709-715.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C. and Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J.* **7**, 2175-2183.

Turner, F. R. and Mahowald, A. P. (1977). Scanning electron microscopy of *Drosophila melanogaster* embryogenesis. *Dev. Biol.* **57**, 403-416.

Valentine, J. W., Erwin, D. H. and Jablonski, D. (1996). Developmental Evolution of Metazoan Bodyplans: The Fossil Evidence. *Dev. Biol.* **173**, 373-381.

Waddington, C. H. (1933). Induction by the primitive streak and its derivatives in the chick. *J. Exp. Biol.* **10**, 38-46.

Wedeen, C. J. and Weisblat, D, A. (1991). Segmental expression of an *engrailed*-class gene during early development and neurogenesis in an annelid. *Development* **113**, 805-814.

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Weisblat, D. A., Harper, G., Stent, G. S. and Sawyer, R. T. (1980). Embryonic cell lineage in the nervous system of the glossiphoniid leech *Helobdella triserialis*. *Dev. Biol.* **76**, 58-78.

Weisblat, D. A., Price, D. J. and Wedeen, C. J. (1988). Segmentation in leech development. *Development* **104**(Suppl.), 161- 168.

Whitman, C. O. (1878). The embryology of *Clepsine*. *Quart. J. Microsop. Sci.* **18**, 215-315.

Whittaker, J. R. (1980). Acetylcholinesterase development in extra cells caused by changing the distribution of myoplasm in ascidian embryos. *J. Embryol. exp. Morph.* **55**, 343-354.

Willmer, P. (1990). "*Invertebrate Relationships: Patterns in Animal Evolution.*" Cambridge Univ. Press, Cambridge.

Wilson, E. B. (1904). Experimental studies on germinal localization. I. The germ-regions in the egg *Dentalium*. *J. Exp. Zool.* **1**, 1-72.

Wray, C. G., Jacobs, D. K., Kostriken, R., Vogler, A. P., Baker, R. and DeSalle, R. (1995). Homologous of the *engrailed* gene from five molluscan classes. *FEBS Lett.* **365**, 71-74.

Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P. and Perrin-Schmitt, F. (1991). The *M-twist* gene of *Mus* is expressed in subsets of mesodermal cells and is closely related to the *Xenopus X-twi* and *Drosophila twist* genes. *Dev. Biol.* **143**, 363-373.

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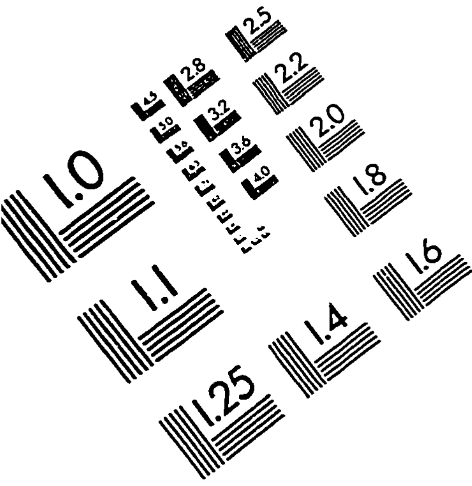
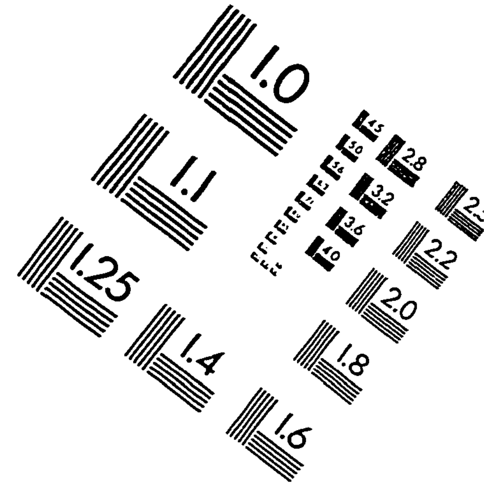
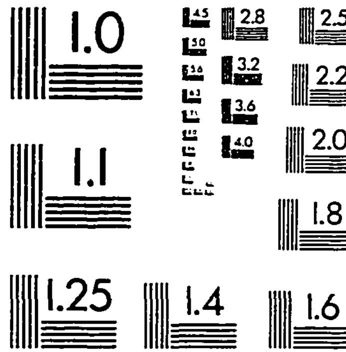
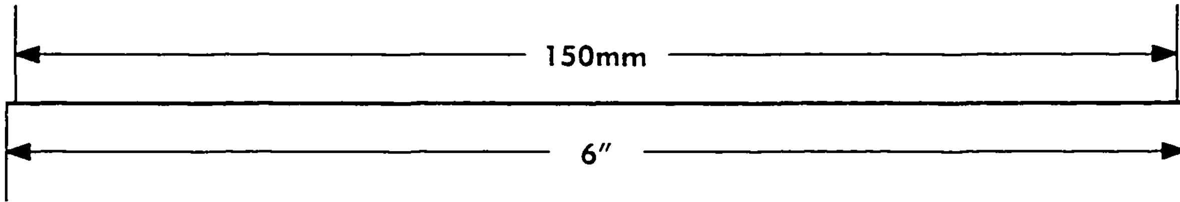

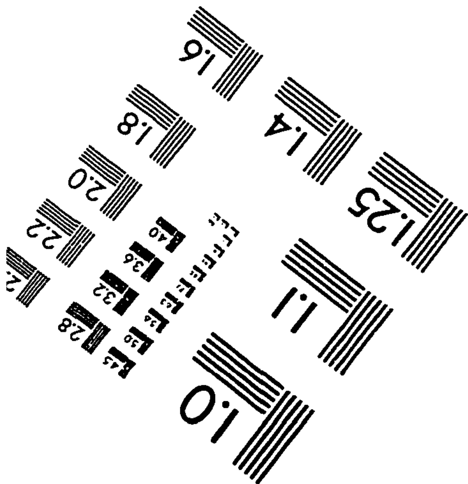

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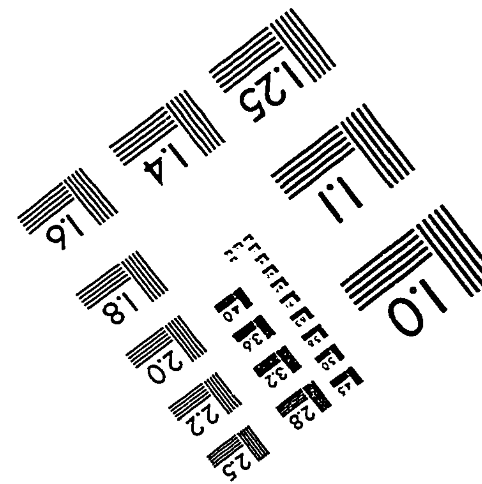

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