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Localized expression of regulatory genes during *Ilyanassa* development

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City University of New York, 1994

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LOCALIZED EXPRESSION OF REGULATORY GENES DURING *ILYANASSA* DEVELOPMENT

by

KANG YAN

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Abstract

LOCALIZED EXPRESSION OF REGULATORY GENES DURING *ILYANASSA* DEVELOPMENT

by

Kang Yan

Advisor: Professor Jack R. Collier

We have searched for the presence and the expression of a proto-oncogene and two homeobox genes in *Ilyanassa*. Both the H-*ras*-like and the homeobox genes (*Sex comb reduced*-like and *Antennapedia*-like) are present in the *Ilyanassa* genome and expressed during *Ilyanassa* embryogenesis. Immunostaining of *Ilyanassa* embryos with an antibody against the *ras* protein showed that a *ras*-like gene was expressed during organogenesis in both normal and lobeless embryos. The *ras*-like protein was first synthesized in normal 3 day old embryos and in 5 day old lobeless embryos. The *ras*-like protein was localized in the mesodermal lineages of both normal and lobeless embryos and involved in the differentiation of the mesodermal cells. We suggest that the *ras*-like gene plays a role in the induction between the mesodermal cells and other cell types or in the proliferation of the mesodermal cells. *In situ* hybridization provides evidence for localized transcription of the *Scr*-like and the *Antp*-like homeobox genes in the ectoderm of *Ilyanassa* embryos. Their transcripts were first detected in the presumptive head primordium of postgastrula embryos. Expression of both genes is then extended to the ventral region along the anterior-posterior axis of embryos in early and middle stages of

organogenesis. This expression is also localized in the foot primordium. Finally, the transcripts of both genes are localized in the larval foot. They may play a role in the formation of the body axis, establishing positional information for the location of the foot primordium along the anterior-posterior axis of the embryo, and the final differentiation of the foot. The most important point is that the transcription of these two homeobox genes is polar lobe-dependent and the influence of the polar lobe on their transcription is different. This is the first evidence that cytoplasmic factors localized in the polar lobe affect the transcription of specific genes.

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Introduction

Cytoplasmic Localization in *Ilyanassa*

Localized cytoplasmic domains in an oocyte are an essential feature for embryonic determination in animal development. They are particularly obvious in "mosaic" embryogenesis in which cytoplasmic determinants are localized in the ooplasm, segregated differentially into blastomeres during early cleavage, and, thereby, establish the fate of specific cell lineages.

Whittaker's (1973) observation on the segregation of acetylcholinesterase and tyrosinase activities into the early blastomeres of the ascidian embryo established a relationship between the segregation of cytoplasmic factors and the appearance of tissue-specific proteins. This work was an important beginning for the molecular biology of embryonic determination. Other studies on ascidian embryogenesis have shown that cytoplasmic determinants for muscle cell specification were localized in vegetal blastomeres at the 8-cell stage (Meedel *et al.*, 1987).

The marine mud snail, *Ilyanassa obsoleta* (Say, 1882), has been studied for almost a century as an experimental organism. Embryogenesis of *Ilyanassa* is also of the mosaic type. Prior to the first cleavage, three **polar lobes** are formed. Cytoplasmic factors contained in the polar lobe are differentially segregated into blastomeres during early cleavages and these factors determine the fate of many blastomeres in *Ilyanassa* development. Therefore, *Ilyanassa* embryos are important in studying the function of localized cytoplasm in embryonic determination.

An experimental analysis of *Ilyanassa* development by Crampton in 1896 showed

that a unique feature of *Ilyanassa* development was the formation of a cytoplasmic bulge, the polar lobe, at the vegetal pole of the egg just before the first cleavage. Crampton found that removal of the polar lobe from the *Ilyanassa* egg resulted in a number of changes in the pattern of cleavage. Notable was the failure of the 4d cell or the primary mesentoblast to form, which was later shown (Clement, 1952) to contain the mesodermal and endodermal determinants of the embryo.

Clement (1952) extended Crampton's work with *Ilyanassa* and showed that the removal of the polar lobe produced a partial larva without a foot, an organized shell, a heart, an intestine, eyes and an operculum. Clement's experiments (1952, 1956, 1967, 1986a, 1986b) showed that cell fates were determined by the progressive segregation of cytoplasmic determinants into different blastomeres, and by inductive interaction between some blastomeres. The latter aspect of determination is obvious in the eye formation. The formation of eyes in *Ilyanassa* development is not only influenced by the polar lobe, but also affected by the interaction between 1a and 1c micromeres which are not derived from the polar lobe lineage, *i.e.* the D macromere. Atkinson (1971) found that although there were many structures which the lobeless embryo of *Ilyanassa* was unable to develop, the differentiation of several specific cell types occurred during the development of the lobeless embryos, for example, muscles, velum, digestive glands and stomach tissues were seen to differentiate. This suggests that the polar lobe plays a major role in the organogenesis.

Earlier efforts to recognize the influence of the polar lobe on the molecular events of *Ilyanassa* embryogenesis have shown that, the rate of DNA and RNA synthesis is less

in lobeless embryos (Collier, 1975a and 1977), polyadenylation of mRNAs is comparable in these two types of embryos (Collier, 1975b), there is no detectable difference in the electrophoretic profiles of RNAs from normal and lobeless embryos (Koser and Collier, 1976), and the patterns of protein synthesis, as determined by two dimensional electrophoresis, are similar for both classes of embryos (Collier, 1981a, 1983, 1984 and 1989). These studies were unsuccessful in finding molecular events associated with the determinants in the polar lobe because available methodologies were neither specific nor sensitive enough to assay for the expression of specific genes. With the methodology of recombinant DNA technology in hand we have returned to investigate the role of polar lobe determinants in the expression of specific genes.

Ras Proto-oncogenes in Development

Proto-oncogenes (cellular oncogenes) are normal DNA sequences in animal genomes, which can be activated to become v-oncogenes by viral transduction, mutation and rearrangement. Because they are highly conserved throughout evolution, proto-oncogenes may be involved in growth, regulation and differentiation. Many proto-oncogenes such as *c-src*, *c-erbB*, *c-ras*, *c-myc* and *c-fos* have been shown to be active during embryonic development and expressed in a specific region of an embryo or at a specific stage in development.

A major class of proto-oncogenes are the *ras* genes, which were first identified as the viral oncogene in the Harvey rat sarcoma virus (Defeo, *et al.*, 1981). Cellular *ras* genes have been found to code for a remarkably well-conserved protein localized on the cytoplasmic face of plasma membranes, designated p21. The *ras* proteins have both a

high guanine nucleotide affinity and a weak GTPase activity. They are activated by the binding of GTP and inactivated when the GTP is hydrolysed to GDP by the intrinsic GTPase. Because the *ras* proteins are structurally and functionally analogous to G protein, (GTP-binding protein,), *ras* genes are thought to be involved in transducing extracellular signaling molecules such as growth factors to effector proteins. There are two types of proteins which regulate the activation of the *ras* proteins: guanine nucleotide releasing factors (GRF) and GTPase activating proteins (GAP). GRF act as positive regulators by promoting the release of GDP from the *ras* proteins, allowing GTP to bind to the *ras* proteins. GAP act as negative regulators by stimulating the weak GTPase of the *ras* proteins to return the active *ras* proteins to the inactive GDP-bound state. Therefore, the relative activities of GRF and GAP acting on p21 at any moment determine its activation state. Generally, GRF act on the upstream of the *ras* proteins and GAP act on the downstream of the *ras* proteins. Furthermore, GAP may work both as the negative regulators of the *ras* proteins and as an effector of *ras* to provide a negative feedback to the signal transduction by *ras* (Barbacid, 1987; McCormick, 1989; Feig, 1993).

Cellular *ras* genes have been found in most organisms. They are expressed in many cell lineages of both invertebrates and vertebrates. Unlike other proto-oncogenes, c-*ras* is expressed throughout the development of the mouse embryos (Muller *et al.*, 1983; Slamon and Cline, 1984). Increasing expression of c-*ras* has been reported in actively proliferating tissues such as the regenerating rat liver (Geyette *et al.*, 1983). When H-*ras* protein was injected into the *Xenopus* oocytes, it induced maturation of the

oocytes by progressing from the prophase to the metaphase of meiosis judged by the breakdown of the germinal vesicle (Birchmeier *et al.*, 1985). In mouse 3T3 cells, more p21-GTP complexes were found in growing cells than in resting cells. The p21-GTP was increased about 2-fold when those resting cells were stimulated to initiate DNA synthesis with fetal bovine serum and p21 transduced the growth signal from the PDGF (platelet-derived growth factor) receptor (Satoh *et al.*, 1990).

However, increased levels of c-*ras* expression do not always correlate with cellular proliferation. Tanaka *et al.* (1986) found that the highest level of p21 was present in the brain, an organ consisting of nondividing cells, whereas proliferating tissues only showed limited expression. In mice, the highest level of c-*ras* expression was found in the heart (Spandidos and Dimitrov, 1985). In *Drosophila*, a correlation between the *ras* gene expression and the cell proliferation has been observed. Although the mRNAs were uniformly distributed in embryos, they were restricted to the dividing cells (e.g. imaginal discs, gonads and brain) in larvae. This picture, however, was changed in the adult flies. The strongest hybridization signals were localized in the adult ovaries, the cortex of the brain and the ganglia, which at this stage were comprised of differentiated, nondividing cells (Segal and Shilo, 1986). In *Aplysia*, a marine mollusk, *ras* proteins were most abundantly expressed in the nervous tissues and ovotestis (Swanson *et al.*, 1986). In *C. elegans*, *let-60*, coding for a *ras* protein, is a control gene in the determination of six vulval precursor cell fates during vulval induction. The determination of cell fates depends on the activation of *let-60/ras*. The activated *let-60/ras* specifies vulval cell fate and the inactivated *let-60/ras* specifies hypodermal cell fate (Han and Sternberg, 1990).

These observations support the view that c-*ras* genes play a dual role in basic cellular proliferation and in determination or differentiation of certain cell types.

Homeobox Genes in Development

The homeobox is a 180-183 bp sequence first found in some *Drosophila* homeotic genes. It is highly conserved in evolution because it exists in the genomes of both invertebrates and vertebrates such as sea urchins, nematode worms, mollusks, fish, frogs, chickens, mice and humans (Holland and Hogan, 1986; McGinnis *et al.*, 1984a). The homeobox is one part a DNA sequence coding for a protein and the protein portion encoded by the homeobox is called the homeodomain. The homeodomain, consisting of 60-61 amino acids, is also highly conserved in evolution and even more conserved than the homeobox.

Obvious features of the homeodomain include its defined size and its large content of basic amino acids (~30%) which indicates the possibility that the products of homeobox genes are DNA binding proteins. This was supported by the localization of the *Ubx* proteins in the nuclei of *Drosophila* cells (White and Wilcox, 1984). The homeodomain contains a helix-turn-helix (HLH) DNA binding motif which is highly conserved in the products of different homeobox genes and is responsible for the site-specific recognition and binding to DNA (Kissinger *et al.*, 1990). It is generally accepted that the products of homeobox genes act as sequence-specific binding proteins that regulate gene expression (*i.e.* transcription factors). Some homeobox genes autoregulate their own transcription and also regulate the transcription of other homeobox genes to establish correct spatial and temporal expression patterns (Hoey *et al.*, 1988). They may

regulate the expression of structural genes in cell differentiation.

The pattern formation of *Drosophila* is controlled by three major groups of genes, maternal effect genes, segmentation genes and homeotic genes. The homeobox has been found in more than 20 members of the three groups of regulatory genes. The most interesting is the presence of homeoboxes in homeotic genes, most of which belong to two complexes, the Antennapedia Complex (*ANT-C*) and the Bithorax Complex (*BX-C*) and are organized in clusters. They are expressed in domains along the anterior-posterior axis of the embryo. Furthermore, the physical order of the homeotic genes in the complexes corresponds to the order along the anterior-posterior axis of the segments affected by the genes (Gilbert, 1991). The expression of *ANT-C* and *BX-C* in development suggest that at least one role of these homeobox genes is to establish the anterior-posterior axis of the embryo.

Similarly, many of the homeobox genes are organized in clusters in the mouse genome. Many of them are expressed in overlapping domains along a head-to-tail axis of the embryo. The best example is the Hox-2 cluster. For example, Hox-2.6 is expressed in ectodermal derivatives such as the spinal cord, the hindbrain, the dorsal root ganglia and the Xth cranial ganglia (Graham *et al.*, 1988). Hox-2.1 is expressed in a domain extending from the hindbrain throughout the length of the spinal cord, indicating a possible role in the anterior-posterior positional specification in the neuroectoderm (Krumlauf *et al.*, 1987; Holland and Hogan, 1988a). Further study on the correlations of a gene order and an expression pattern (Graham *et al.*, 1989) showed that all members of the Hox-2 cluster were expressed in slightly different domains in the central nervous

system with respect to the anterior-posterior axis. For the genes close to the 5' end of the cluster, their expression boundaries were more posterior in the spinal cord. The expression limits of genes the which locate at the 3' end in the cluster were more anterior in the spinal cord. The position of a gene in the Hox-2 cluster is correlated with its relative expression domain in the anterior-posterior axis of a mouse embryo. Therefore, individual genes in the Hox-2 cluster are organized and expressed in the same relative order as their structural homologs in the *Drosophila* homeotic complexes, suggesting that these homeobox genes may play a similar role in the establishment of positional information in both invertebrates and vertebrates. This has been confirmed by the functional substitution of a *Hox*-gene (*Hox*-1.3) for its cognate *Drosophila* homeotic gene (*Scr*) in *Drosophila* development (Zhao *et al.*, 1993).

Interference with the function of a homeobox gene in *Xenopus* has further confirmed its role in development. The homeobox gene Xhox-1A is expressed in the gastrula stage. Deregulating its expression by injecting an excess of Xhox-1A mRNA into one blastomere at 2-cell stage disrupts the formation of the somites. The somites are disorganized or totally lost in the side of an embryo that receives the injected mRNA. The function of Xhox-1A seems to specify the proper pattern formation of the somites instead of cell differentiation (Harvey and Melton, 1988). Another example is blocking the function of the homeobox gene XIHbox 1, which is normally expressed in both the nervous system and the mesoderm of *Xenopus* embryos (Wright *et al.*, 1989). Injection of an antibody specific for the homeodomain protein into the embryos at the 1-cell stage changes the anterior spinal cord into the next anterior-most structure, the hindbrain.

Overexpression of another homeobox gene, XIHbox 6, in *Xenopus* embryos further indicates its role in body axis formation (Cho *et al.*, 1991). XTC-MIF (*Xenopus* tissue culture mesoderm-inducing factor) can induce mesoderm in the blastula animal caps *in vitro*, which can induce the formation of a secondary head structure once transplanted into the early gastrula embryos of *Xenopus*. If the animal cap is isolated from an embryo injected with XIHbox 6 mRNA at the 2-cell stage, the secondary anterior-posterior identity is respecified. The induced secondary structure is a tail-like one instead of a head due to the posteriorizing effects of XIHbox 6. Furthermore, overexpression of the gene in animal cap tissue without the treatment of XTC-MIF directly induces the tail-like structure in the transplants. These results indicate that the above homeobox genes in the *Xenopus* embryos work similarly to the homeotic genes in *Drosophila* in the formation of a body axis.

Rationale of Research

What genes regulate *Ilyanassa* development and how are they controlled? On one hand, the *Ilyanassa* embryos are valuable for the study of cytoplasmic determination because of the polar lobe, the clear cell lineages of the micromeres and the mosaic development, but they are not suitable for a genetic analysis because of prolonged life cycle. On the other hand, many genes such as proto-oncogenes and homeobox genes are highly conserved during evolution and can be used as probes which can reveal the expression patterns and possible roles of these genes in the development of *Ilyanassa*. If developmental mechanisms are universal throughout evolution, these genes should be present in the *Ilyanassa* genome, should be expressed developmentally, and may be regulated by components of the polar lobe. The goal of my study is to search for the presence and the expression of proto-oncogenes and homeobox genes in *Ilyanassa*. This search is based on the suggestion that these highly conserved genes also have a role in the *Ilyanassa* development. This research is the first attempt to assess the role of these genes in *Ilyanassa* development.

The proto-oncogene we have chosen is the c-*ras* gene. We are interested in the expression of *ras* during *Ilyanassa* development because it may play a role in embryonic induction or cell proliferation, both of which are influenced by the polar lobe.

The homeobox genes we have chosen are *Antennapedia (Antp)* and *Sex combs reduced (Scr)*. We are interested in the expression of *Antp* and *Scr* homologs because they may play a role in axiation, a polar lobe-dependent event. When the polar lobe is removed, the *Ilyanassa* embryo develops with radial symmetry and completely lacks

bilateral symmetry.

Materials and Methods

Rearing and Staging of Embryos

Snails were collected from Plumb Beach in Brooklyn, New York and kept in the laboratory in a tank of recirculating sea water. The eggs and embryos were reared at 18^0 C in filtered sea water containing 50 μg/ml of gentamicin sulfate. The developmental stages are indicated numerically and each number is equivalent to days of development under the above conditions.

The cleavage of *Ilyanassa* is spiral in which the cleavage plane is oblique to the animal-vegetal axis of the egg. Prior to the first cleavage, three **polar lobes** are formed. The polar lobe is an enucleated cytoplasmic structure formed at the vegetal pole of the egg just before the first cleavage. The embryo with the third polar lobe is called the 'trefoil' stage. The polar lobe merges into the CD blastomere after the first cleavage. At the next division, a small polar lobe (the fourth polar lobe) enters the D blastomere. The third cleavage separates four micromeres from the A, B, C and D macromeres. They are the first quartet, 1a-1d. The fourth cleavage separates the second quartet, 2a-2d, from the macromeres. The third quartet, 3a-3d, is formed by the sixth cleavage. The eighth cleavage produces the 4d cell. The embryo now consists of 25 cells and these cleavages take about 24 hours. Bilaterality of the embryo is established when the 4d cell divides into the right and left mesentoblasts that separate the mesoblast and enteroblast cells in the next divisions. The mesoblastic derivatives produce the mesodermal bands that form the mesoderm. The enteroblastic derivatives, in conjunction with the macromeres, form the larval digestive system. Therefore, the 4d cell is responsible for forming the

mesentoblast which gives rise to both the mesodermal and endodermal organs. Because the 4d cell is the primary mesentoblast, the 25-cell stage embryo is called the mesentoblast stage (stage 1).

It takes two days for a fertilized egg to gastrulate. The gastrula (stage 2), consisting of 81 cells, is a spherical structure covered by micromeres and with a blastopore on the ventral side. Stage 3 is a 3-day old embryo, consisting of 165 cells with a visible stomodeal invagination on the ventral surface of the embryo. Stage 4 is a 4-day old embryo in an early stage of organogenesis, consisting of 239 cells. The first organ primordium, the shell gland, can be observed on the posterior-dorsal surface of this embryo. Stage 5 is a 5-day old embryo in mid-organogenesis, consisting of 469 cells. Some organ primordia such as a foot and a head vesicle have formed. A complete veliger forms after 7-8 days of development.

Polar lobes were isolated by agitation in 9 parts of Ca^{++}-Mg^{++} free sea water and 1 part of Jamarin artificial sea water (Collier, 1981b). The Ca^{++}-Mg^{++} free sea water was buffered with 0.05 M Tris at pH 8.2. The ages of lobeless embryos are defined as days of development.

Isolation of DNA and RNA

Ilyanassa genomic DNA was isolated from the testis of individual snails. Each testes was gently dispersed in 1 ml of STE buffer (0.02 M NaCl, 0.05 M Tris, pH 8.0, 0.02 M EDTA), digested with 200 μg/ml of proteinase K for 2 hrs at 53^0 C. Then, after the addition of another 200 μg/ml of proteinase K, the digestion was continued for overnight at 37^0 C. The next day the DNA extract was digested for 2 hrs after the

addition of another 200 μg/ml of proteinase K and extracted three times with phenol and twice with chloroform. All extractions were for 2 hrs with gentle agitation on a rocker platform. The aqueous phases after the extraction were precipitated with 2.5 M ammonium acetate and two volumes of ethanol and the precipitated DNA was dissolved in TE buffer (0.05 M Tris, pH 8.0, 0.02 M EDTA).

Ilyanassa total RNA was extracted in guanidinium thiocyanate as described by Sargent *et al.* (1986). Briefly, the frozen embryos were homogenized directly in 4.2 M guanidinium thiocyanate buffered with Tris (pH 8.0) containing 0.5% sarkosyl and 0.7% 2-mercaptoethanol. The homogenate was extracted twice with two volumes of phenol/chloroform (1:1) and one volume of extraction buffer (0.1 M Tris, pH 8.0, 1% SDS, 10 mM EDTA), followed by one extraction with chloroform. The extracted nucleic acids were precipitated with 2.5 M ammonium acetate and two volumes of ethanol and digested at 37° C for 1 hr with 200 μ/ml of RNase-free DNase I (Boehringer-Mannheim Biochemicals). The RNA was stored in ethanol at -80° C.

Southern Blot Analysis of DNA

Ilyanassa genomic DNA isolated from the testes was digested at 37° C overnight with restriction enzymes, including *Eco*RI, *Bam*HI, *Pst*I and *Hind*III. The digested DNA was fractionated through a 0.8% agarose gel, denatured in 0.5 M NaOH/1.5 M NaCl for 60 min, neutralized in 1 M Tris (pH 7.5)/1.5 M NaCl for 60 min, transferred to nitrocellulose in 10× SSC and baked in a vacuum oven at 80° C for 2 hr.

For hybridization using the *ras* cDNA as a probe, the reaction was performed at high stringency conditions. Briefly, the filter was prehybridized for 2 hrs in 6× SSC, 5×

Denhardt's solution, 0.5% SDS and 100 μg/ml of denatured salmon sperm DNA and hybridized overnight by addition of a radio-labeled probe (3.0 x 10^6 cpm/ml). The filter then was washed in 2× SSC for 15 min and 0.1× SSC for 5 × 20 min. All hybridization reactions and washes were at 65^0 C.

For the hybridization using *Scr* cDNA as a probe, the reaction was performed at the reduced stringency conditions (McGinnis *et al*, 1984b). The filter was prehybridized for 2 hrs in 43% formamide, 5× SSC, 5× Dendardt's solution, 50 mM Na_2HPO_4(pH 7.2) and 100 μg/ml of denatured salmon sperm DNA and hybridized for 40 hrs at 37^0 C by adding a radio-labeled probe (3.0 x 10^6 cmp/ml). The filter then was washed at 50^0 C in 2× SSC, 0.1% SDS (2 × 20 min) and 1× SSC, 0.1% SDS (3 × 20 min). These hybridized filters were dried and exposed to Kodak X-AR X-ray film with intensifying screens at -80^0 C.

For the hybridization using *Antp* cDNA as a probe, the reaction was performed at the same stringency conditions as for the *ras* except the probe DNA was labelled with digoxigenin (20 ng/ml in the hybridization).

The *ras* probe was an 0.8 kb cDNA restriction fragment of the human H-*ras* proto-oncogene cloned into pSP64 (Oncogene Science, Inc). The rDNA probe was a single repeat unit of *X. laevis* ribosomal DNA inserted into pBR322 (Bakken *et al.*, 1982). The *Scr* probe was a 0.4 kb homeobox-containing fragment from the *Drosophila sex combs reduced* gene and cloned into pAT153 (provided by Dr. M. McGinnis, Yale University). The *Antp* probe was a 2.2 kb cDNA from the *Drosophila Antennapedia* gene and cloned into pBluescript (provided by Dr. M. McGinnis, Yale University). These

DNA probes were labeled with either digoxigenin or ^{32}P-dCTP by the random primed DNA labeling method (Boehringer Mannheim), with a specific activity of 0.5-1 $\times$ 10^9 dpm/μg.

Northern Blot Analysis of RNA

20 μg of total RNA isolated from *Ilyanassa* embryos at different stages was fractionated through a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose in 20$\times$ SSC and baked in a vacuum oven at 80^0 C for 2 hr.

For the hybridization using the *ras* cDNA as a probe, the filter was prehybridized at 42^0 C for 2 hr in 50% formamide, 5$\times$ SSC, 0.1% SDS, 5$\times$ Denhardt's solution and 100 μg/ml of denatured salmon sperm DNA. The hybridization was at 42^0 C in 50% formamide, 5$\times$ SSC, 10% dextran, 0.1% SDS, 100 μg/ml of denatured salmon sperm DNA and a radio-labeled *ras* probe (3.0 $\times$ 10^6 cpm/ml) for overnight. Then the filter was washed at room temperature in 1$\times$ SSC, 0.1% SDS (2 $\times$ 15 min) and 0.25$\times$ SSC, 0.1% SDS (2 $\times$ 15 min).

For the hybridization using the *Scr* and the *Antp* cDNA as probes, the filter was prehybridized for 2 hr at 42^0 C in 50% formamide, 5$\times$ SSC, 5$\times$ Denhardt's solution, 0.1% SDS, 100 μg/ml of denatured salmon sperm DNA and hybridized in 50% formamide, 5$\times$ SSC, 5$\times$ Denhardt's solution, 10% dextran, 0.1% SDS, 100 μg/ml of denatured salmon sperm DNA and a digoxigenin-labeled *Antp* probe (20 ng/ml) overnight. The hybridized filters were washed at room temperature in 1$\times$ SSC, 0.1% SDS (15 min) and at 65^0 C in 0.2$\times$ SSC, 0.1% SDS (3 $\times$ 20 min). As a control, a 1.7 kb chicken beta-actin gene was labeled with digoxigenin and hybridized to the *Ilyanassa*

RNA. The hybrids were detected according to the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim).

For the "dot blot" RNA assays (Bresser *et al.*, 1983), RNA was isolated from *Ilyanassa* eggs and embryos by homogenization in 10 mM vanadyl nucleoside complex, 0.5% Brij-35 and 0.5% sodium deoxycholate. Proteinase K (200 μg/ml) was added directly to the homogenate and was then incubated for 1 hr at 37^0 C. The proteinase K-digested homogenate was diluted with one volume of supersaturated NaI (12.2 M) and filtered through nitrocellulose in a Scheicher and Shuell "dot-blot" apparatus. The RNA-containing filter was soaked in water for 15 min, in 70% ethanol for 15 min and in acetic anhydride for 10 min. The prehybridization, hybridization, washing and autoradiography were the same as those in the Northern blot analysis of *Antp* and *Scr*.

Immunocytochemistry

Ilyanassa embryos were fixed in freshly prepared 3.7% formaldehyde in sea water for 10 min, washed with four changes of PBT (PBS containing 0.1% Tween 20. PBS: 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.2) for 2 hr at room temperature, and incubated overnight at 4^0 C in 10% BSA (bovine serum albumin) in PNBT (PBT containing 0.5 M NaCl). After blocking with BSA, the embryos were washed with five changes of PBT for 2 hr at room temperature and incubated for 2 hr at room temperature with a 1:1 dilution of the monoclonal antibody v-H-*ras* (Ab-1) (Oncogene Science). Following incubation with the primary antibody, the embryos were washed for 2 hr with four changes of PBT and then incubated with a 1:1000 dilution of the secondary antibody conjugate (goat anti-mouse IgG-alkaline phosphatase conjugate)

at room temperature for 2 hr. After incubation with the secondary antibody, the embryos were washed for 2 hr with five changes of PBT and then stained at room temperature for 15 min in alkaline phosphatase substrate solution (0.1 M Tris, pH 9.5, 0.1 M NaCl, 0.005 M $MgCl_2$ containing nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate) and 1 mM levamisole. It was separately demonstrated that 1 mM levamisole blocked the expression of all endogenous alkaline phosphatase activities but did not affect the staining reaction dependent on the *ras* primary antibody. The stained embryos were dehydrated in absolute ethanol, cleared in xylene, and mounted in Permount.

***In situ* Hybridization**

Ilyanassa embryos were fixed in freshly prepared 3.7% formaldehyde in fixation solution (PBS, 0.05 M EGTA) for 1 hr at room temperature and washed with three changes of PBT, two changes of methanol (embryos can be stored in methanol at -20° C for a few weeks), two changes of ethanol, one change of xylene, three changes of ethanol and five changes of PBT. The embryos were then treated with proteinase K (10 μg/ml) at room temperature for 10 min, washed with three changes of PBT, refixed in 3.7% formaldehyde for 30 min and washed with three changes of PBT. After two changes with the hybridization buffer (50% formamide, 5x SSC, 0.1% Tween 20, 50 μg/ml of heparin and 200 μg/ml of *E.coli* and pBluescript DNA), the embryos were prehybridized in the hybridization buffer at 45° C for 2 hr and hybridized by adding a digoxigenin-labeled DNA probe (10 ng/50 μl) for 18 hr at 45° C. The hybridized embryos were washed with five changes of PBT for 30 min, and, in some cases in three changes of nuclease S1 buffer (0.2 M NaCl, 0.05 M Na acetate, pH 4.5, 1 mM $ZnSO_4$,

0.5% glycerol), followed by incubation with 1000 U/ml of nuclease S1 at 37° C for 1 hr to remove the non-hybridized probe DNA sequences. After washing with three changes of PBT, embryos were incubated with an anti-digoxigenin antibody-alkaline phosphatase conjugate (1:1000) either at 4° C overnight or at room temperature for 1 hr, and then washed with five changes of PBT containing 1 mM levamisole for 100 min. Embryos were stained in alkaline phosphatase substrate solution (30-90 min), dehydrated in ethanol, cleared in xylene and mounted in Permount.

The DNA probes used were a 400 bp homeobox-containing sequence from *Drosophila Scr* cDNA and a 2.2 kb *Drosophila Antp* cDNA. The *Scr* fragment was released from the plasmid pAT153 by digestion with *Eco*RI and *Xba*I. The *Antp* sequence was released from pBluescript by digestion with *Eco*RI and *Bam*HI. After digestion, the cDNA inserts were separated from the vector DNA by electrophoresis through an agarose gel. These DNA probes were labeled by the random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (digoxigenin-dUTP), according to the Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim).

pBluescript has both T_7 RNA polymerase and T_3 RNA polymerase promoters flanking the multiple cloning site (MCS). RNA probes were produced and labeled with digoxigenin-11-uridine 5' triphosphate according to the Nonradioactive RNA Labeling and Detection Kit (Boehringer Mannheim). By using the *Antp* DNA insert as a template, which was cloned in pBluescript, both T_7 and T_3 RNA polymerase were used to produce sense and antisense RNAs. These RNA probes were stored in hybridization buffer at -20° C. Embryos hybridized with RNA probes were treated with RNase A (Sigma) at 37° C

for 30 min to remove the non-hybridized RNA probe sequences (RNase A: 20 μg/ml in 0.5 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA).

As a control, cross-hybridization was done between DNA probes by a dot blot analysis. Briefly, denatured *Antp* and *Scr* DNAs were blotted onto nitrocellulose in amounts of 0.1 ng, 0.5 ng, 1 ng and 10 ng. The DNA-bound nitrocellulose filters were then denatured in 0.5 N NaOH/1.5M NaCl (2 × 5 min), neutralized in 1 M Tris (pH 7.5)/1.5 M NaCl (2 × 5 min), dried at room temperature and baked in a vacuum oven at 80° C for 1-2 hr. The filters were prehybridized for 2 hr in 6× SSC, 5× Denhardt's solution, 0.1% SDS and 100 μg/ml of denatured salmon sperm DNA and hybridized overnight with a digoxigenin-labeled DNA probe (10 ng/ml) at 50° C (low stringency), 60° C (moderate stringency) and 65° C (high stringency), respectively. The final wash was at 50° C in 1× SSC, 0.1% SDS (low stringency), 60° C in 0.3× SSC, 0.1% SDS (moderate stringency) and 65° C in 0.1× SSC, 0.1% SDS (high stringency), respectively. The high stringency conditions were equivalent to those of *in situ* hybridization. The immunological detection was done according to the Nonradioactive Labeling and Detection Kit (Boehringer Mannheim).

Other controls included treating embryos with RNase ONE (Promega) before hybridizing to a digoxigenin-labeled probe, incubating the embryos with the anti-digoxigenin antibody conjugate without hybridization, staining the embryos in alkaline phosphatase staining solution in the presence of 1 mM levamisole, and hybridizing the embryos with the digoxigenin-labeled pBluescript DNA with amounts equivalent to 1-20% of the *Antp* probe in the hybridization reaction.

Expression of a *Ras*-like Gene in *Ilyanassa* Development

Results

Southern blot analysis of DNA

The presence of a proto-oncogene *ras* homolog in the *Ilyanassa* genome was identified by hybridizing restriction enzyme digested *Ilyanassa* genomic DNA with a human c-*ras* probe at high stringency. *Xenopus* rDNA and plasmid pSP64 were radio-labeled and used as probes for controls. The hybridization results are shown in both Fig. 1 and Fig. 2. In Fig. 1, the *Ilyanassa* genomic DNA was digested with *Eco*RI, hybridized with *ras* (lane 2) and rDNA (lane 3) probes. The arrows at lane 1 indicate the DNA repeat sequences derived from the *Eco*RI digestion. In Fig. 2, the *Ilyanassa* genomic DNA was digested with *Sst*I (lane 1) and *Bam*HI (lane 2-4), hybridized with the *ras* (lane 1-2), rDNA (lane 3) and pSP64 (lane 4) probes. These observations support the presence of DNA sequences in the *Ilyanassa* genome complementary to the *ras* probe. The stringency level (hybridization at 65° C and washing in 0.1 × SSC at 65° C) of the hybridization reactions and washes indicate a close homology between the H-*ras* sequences in human and those in *Ilyanassa*.

Dot blot analysis of RNA

Ilyanassa RNA isolated from several stages of development was bound to nitrocellulose (dot blot) and hybridized to a radio-labeled *ras* probe. Column A of Fig. 4 shows hybridization with RNA from mature, unfertilized eggs and embryos at several stages of development. In this series, the RNA from each stage was prepared from an equal number of eggs or embryos. In column A, *ras*-like transcripts were not detected

in eggs, 2-4 cell, mesentoblast or gastrula stages. The first transcripts were observed in stage 3 embryos, and by stage 5 there was a substantial increase in the number of *ras*-related transcripts, which is due in part to the increased number of cells in the stage 5 embryos.

In column B of Fig. 4, equal amounts of RNA from each stage were spotted onto nitrocellulose, and hybridized with the *ras* probe This confirms the absence of *ras*-like expression during early cleavage, mesentoblast and gastrula stages. It also confirms the onset of expression in stage 3 embryos, continued expression in stage 4 embryos, a moderate increase of expression in stage 5 embryos and a decline of expression by stage 6. Column D of Fig. 4 is a control, showing hybridization of *Ilyanassa* RNA with a chicken β-actin as a probe. The transcripts of an actin-related gene is present in all stages during *Ilyanassa* embryogenesis.

Column C of Fig. 4 shows hybridization between the *ras* probe and RNA from *Ilyanassa* embryos lacking the cytoplasm contained in the polar lobe, i.e. lobeless embryos. Because of the difficulty of obtaining large numbers of the lobeless embryos, only two stages, stage 5 and stage 6, were assayed for expression of the *ras*-like gene. In this series of experiments, the number of the embryos was adjusted so that each stage assayed contained the same amount of RNA. The results demonstrate that in lobeless embryos the expression of the *ras*-like gene is considerably less than that in wild-type embryos reared under the same conditions and for the same time. There is also a significant, but lower than normal, increase in *ras*-like expression after another day of development.

Northern blot analysis of RNA

The expression of a *ras*-like gene in *Ilyanassa* embryos was confirmed by hybridizing size-fractionated *Ilyanassa* embryo RNA with a *ras* probe. A single *ras*-like transcript (about 1.9 kb) was observed in both stage 4 (Fig.3, lane 1) and stage 5 (Fig. 3, lane 2) embryos. These embryos are in early and late stages of organogenesis, respectively.

Localization of a *ras*-like protein in embryos

We have used a cross-reacting anti-*ras* antibody to determine the spatial pattern of expression of a *ras*-like protein in embryos at different stages of development (Yan and Collier, 1993). The *Ilyanassa* embryos have an endogenous alkaline phosphatase activity that parallels the localization of *ras*-like protein. Therefore, the staining for the *ras* antibody was in the presence of 1 mM levamisole which was shown to completely block the endogenous alkaline phosphatase activity (Fig. 5a). We have also used secondary antibodies conjugated to horseradish peroxidase to confirm the localization of *ras*-like protein (data not shown).

Separate controls with normal rat IgG or with secondary antibodies alone (Fig. 5b and 5c, respectively) show that the staining is not caused by normal rat serum or by secondary antibodies conjugated to alkaline phosphatase. These controls indicate that the immunostaining reaction with the *ras* antibody is antibody specific.

We have immunostained *Ilyanassa* embryos during early and late stages of development. We did not detect any *ras*-like protein in unfertilized eggs (Fig. 5d), early cleavage stage (4- to 12-cell stage; Fig.5e), mesentoblast stage embryos (25-30 cells; Fig.

5f) and gastrula stage embryos (Fig. 5g). Staining time for these early embryos was threefold longer than for other stages. While we can not rule out the presence of *ras*-like proteins in the early development, they were not detectable within these limits of sensitivity.

The *ras*-like protein was detected in all later stages of development, beginning with stage 3 embryos. Stage 3 embryos have 165 cells with a visible stomodeal invagination at the ventral part of the embryo (Fig. 5h). The mesodermal cells in stage 3 embryos (marked with small arrowheads in Fig. 5h) are stained with the anti-*ras* antibody (Fig. 5i).

Stage 4.5 embryos, consisting of 239 cells, are at a stage of early organogenesis during which the first organ primordium, the shell gland, can be observed in the dorsal-posterior ectoderm of the embryo. Fig. 5j is a frontal section of a stage 4.5 embryo illustrating the mesodermal band (mb) and larval mesoderm (lm). The mesoderm bands are formed from the left- and right- primary mesoblasts which are the lineage products of the 4d cell. The larval mesoderm is derived from the 2a, 2b and 2c micromeres of the second quartet. Expression of the *ras*-like protein in stage 4.5 embryos (Fig. 5k and 5l, dorsal and side view, respectively) is observed in the mesoderm. In Fig. 5k, the larval mesoderm and mesodermal bands are marked with lm and bm, respectively. The exact location of the *ras*-like protein is indicated by the *ras* antibody immunostaining of the mesodermal cells (mb; lm) in a section of a stage 4 embryo shown in Fig. 5m.

Stage 5 embryos, which are in an intermediate stage of organogenesis, have 469 cells, a well-developed shell gland (shg), a small foot (f) and a head vesicle (hv). Fig.

5n is a side view of a stage 5 embryo immunostained with the *ras* antibody. As in the earlier stages, *ras*-like expression is observed in mesodermal cells. Fig. 5o is a section of a stage 5 embryo showing immunostaining of the mesodermal cells (m); the non-stained endodermal and ectodermal cells are marked with en and ect, respectively.

Lobeless embryos are the products of the eggs delobed just before the completion of the first cleavage. The lobeless embryo fails to form the 4d cell, which is the primary mesentoblast (Clement, 1952) and, as a consequence, the mesodermal bands are absent in lobeless embryos. Fig. 6a and 6b show lobeless embryos that have been immunostained with the *ras* antibody after 3 and 4 days of development, respectively. The 3-day lobeless embryo has 162 cells compared to the 165 cells in the normal embryo of the same stage; the 4-day lobeless embryo has 219 cells compared to 239 cells in the normal embryo. The *ras*-like protein is not detected in lobeless embryos at either of these stages, even though its presence is evident in comparably aged normal embryos (Fig. 5i, 5k and 5l). The difference in cell numbers between normal and lobeless embryos at these stages is insignificant. The known absence of the mesodermal bands in the lobeless embryos is meaningful. The absence of immunostaining for the *ras*-like protein in the region of the mesodermal bands is not surprising, since it was previously observed that the development of these bands depends on the presence of the polar lobe determinants. However, the absence of staining of the larval mesoderm at this stage is unexpected.

Fig. 6c and 6d show the 5- and 6-day old lobeless embryos, respectively, that have been immunostained with the antibody to the *ras*-like protein. The 5-day old lobeless embryo consists of 304 cells in contrast to 469 cells of a normal embryo at the

same stage; the 6-day old lobeless embryo has 433 cells in comparison to 936 cells in the normal embryo at the same age (Collier, 1975). This is the first stage when lobeless embryos have significantly fewer cells than normal embryos. The lobeless embryos at these stages stain intensely for the *ras* protein in the apical region of the embryo, which is in the area of the velar lobe, but neither stage stains in the posterior protrusion of the lobeless embryos.

Discussion

The presence and expression of a *ras*-like proto-oncogene in *Ilyanassa* were confirmed by Southern and Northern blot analysis. The Southern hybridization was done at high stringency which indicates high homology between the human c-*ras* and an *Ilyanassa ras*-like gene. The *Ilyanassa ras*-like gene was found, by Northern blot, to be expressed as a single transcript in stage 4 and 5 embryos.

A more extensive analysis of developmental regulation of the *Ilyanassa ras*-like gene was investigated by a dot blot assay. The *ras*-like transcript was first detected in stage 3 embryos. The expression continued throughout the early and middle stages of development and reached a maximal level in stage 5 embryos when the embryos are under the mid-organogenesis and a few organ primordia (shell gland, foot and head vesicle) have been formed. In contrast to normal embryos, expression of a *ras*-like gene was delayed and first began after 5 days in lobeless embryos, with a moderate increase after 6 days of development.

Consistent with the appearance of the *ras*-like transcript, the *ras*-like protein was found in 3 day old embryos and continuously detected in later stages of development. As

shown in Fig. 5, the *ras*-like protein is localized in both the mesodermal bands and the larval mesoderm in the anterior region of normal embryos. The localization of the *ras*-like protein in these two types of mesoderm is best shown in Fig. 5k which is a dorsal view of a stage 4 embryo. Sections of normal embryos immunostained with the anti-*ras* antibody clearly show that the *ras*-like protein is exclusively localized in the mesoderm, including both the mesodermal bands and the larval mesoderm (Figs. 5m and 5o).

A *ras*-like protein in lobeless embryos was not detected until the fifth day of development, which also corresponds to the first appearance of a *ras*-like mRNA in these embryos. This localization of *ras*-like gene is limited to the apical part of the embryo (Figs. 6c and 6d). The delayed appearance of the *ras*-like mRNA and protein in lobeless embryos is correlated with a slower rate of development of these embryos, as shown by a significant decrease in the rate of DNA synthesis after five days of development (Collier, 1975).

There is no evidence for the presence of *ras*-like protein in any other regions of lobeless embryos because the mesodermal bands are not formed in lobeless embryos (Clement, 1952). The presence of the *ras*-like protein in the apical region of the lobeless embryos is consistent with the localization of this protein in larval mesoderm which forms in the head region of normal embryos and in the apical region of lobeless embryos. The radial mesodermal lineage, from the second quartet of micromeres 2a, 2b and 2c (Lillie, 1895; Conklin, 1897), produces the so-called 'larval mesoderm' that accounts for the contractile elements in lobeless embryos (Clement, 1952). This radial mesodermal lineage accounts for all of the mesoderm anterior to the blastopore in

Ilyanassa embryos and is therefore the probable source of the cells containing the *ras* protein in lobeless embryos.

Clement's studies (1952, 1956, 1967, 1986a, 1986b) on the early development of *Ilyanassa* included deletion experiments in which the cell in question was killed by puncture with a glass needle. By analyzing the deficiencies of larvae that developed from deleted embryos, the normal roles or embryonic values of the micromeres in the development of *Ilyanassa* were determined. Clement found that the cell fates were determined at early stages by the gradual segregation of cytoplasmic determinants. Inductive events, for example, the formation of the eyes and the shell gland, also occur during the early cleavages of the *Ilyanassa* embryos. The absence of detectable expression of the *ras* gene during the early cleavage stages indicates that this proto-oncogene is not involved in the determinative events which occur during the early development of *Ilyanassa*. Because the mesodermal lineages are determined at the stage when the 4d cell gives rise to the mesoblast and when the *ras*-like transcript and protein can not be detected, the *ras*-like gene does not appear to be involved in the determination of the mesoderm, although later in development the *ras*-like protein is exclusively localized in the mesoderm band and the larval mesoderm and is involved in functional differentiation of the mesoderm.

The polar lobe of *Ilyanassa* appears to be involved in both induction and cell proliferation. Its inductive role in the formation of eyes and shell gland was shown by Clement. From Atkinson's cytological study of lobeless embryos (1971), in which he found the differentiation of many cell types, Collier (1983) suggested that the failure of

lobeless embryos to differentiate might result from the inability of stem cells to proliferate. In other words, certain cell types, such as digestive gland cells, differentiate, but fail to form a normal organ because the stem cells had not proliferated prior to differentiation. We undertook a study of the *ras* gene during *Ilyanassa* development because we thought it might be involved in these putative functions of the polar lobe. Although the *ras*-like protein does not appear to be involved in the determination of the mesodermal lineages in *Ilyanassa*, its basic roles in both differentiation and proliferation in other organisms lead us to think that the *ras*-like protein may have a role in the organization and distribution of the mesodermal cells during organogenesis of *Ilyanassa*. This role may be the transmission of signals between the mesodermal cells and other cell types. At an early stage of organogenesis in *Ilyanassa* development, the mesodermal cells contribute to the organogenesis of several organs such as foot, heart, the musculature, velum and intestine. The mesodermally derived components of these organs are consistently stained by the *ras* antibody. Another possible role of the *ras*-like protein could be to stimulate the proliferation of presumptive mesodermal stem cells required for the further development of the veliger larva.

In summary, our results demonstrate that a *ras*-like gene is present in the *Ilyanassa* genome and expressed developmentally. The expression of the *ras*-like gene is detected in normal embryos at stage 3 and delayed in lobeless embryos at 5 days old. Immunostaining of *Ilyanassa* embryos with an antibody against the *ras* protein shows that the *ras*-like protein is localized in the mesodermal lineages of both normal and lobeless embryos and involved in the mesoderm differentiation. We suggest that the *ras*-like gene

plays a role in the induction between the mesodermal cells and other cell types or in the proliferation of the mesodermal cells.

Transcription of Homeobox genes in *Ilyanassa* development

Results

Southern blot analysis of DNA

The presence of homeobox genes in the *Ilyanassa* genome was detected by hybridizing restriction enzyme digested *Ilyanassa* genomic DNA with a probe from the *Drosophila* homeobox gene *Scr*. Restriction enzyme digested *Drosophila* genomic DNA, pBR322 (data not show) and *Xenopus* rDNA were used as controls. The hybridization results are shown in Fig. 7 where *Drosophila* genomic DNA (lanes 1-2) was digested with *Eco*RI (lane 1) and *Hind*III (lane 2) and hybridized with a *Scr* probe. *Ilyanassa* genomic DNA (lanes 3-6) were digested with *Eco*RI (lanes 3 and 5) and *Hind*III (lanes 4 and 6), hybridized with the *Scr* (lanes 3-4) and rDNA (lanes 5-6) probes. The hybridization was at reduced stringency. Multiple fragments hybridizing to the *Scr* homeobox were observed in *Ilyanassa* genomic DNA (lanes 3-4). These observations show that the homeobox-containing sequences complementary to the *Drosophila Scr* probe are present in the *Ilyanassa* genome.

Southern hybridization using an *Antp* cDNA probe confirmed the presence of a *Drosophila Antp*-like gene in the *Ilyanassa* genome. As shown in Fig. 8, the *Eco*RI (lanes 1 and 3) and *Bam*HI (lane 2) digested *Ilyanassa* DNA was hybridized to a digoxigenin-labelled *Antp* probe (lanes 1-2) and to a digoxigenin-labelled pBluescript DNA (lane 3) at high stringency conditions. A major band and a minor band were found as the results of association between the restriction fragments of *Ilyanassa* genomic DNA and the *Antp* probe, indicating the presence of an *Antp*-like gene in the *Ilyanassa*

genome.

Northern blot analysis of RNA

The transcription of a homeobox-containing gene was determined by hybridizing size-fractionated *Ilyanassa* embryo RNA with digoxigenin-labeled *Scr* and *Antp* probes. As a control, embryo RNAs were hybridized with a β-actin probe. In Fig. 9, *Ilyanassa* total RNA was extracted from the mesentoblast stage (lane 1), gastrula (lane 2), stage 3 (lane 3), stage 4 (lane 4) and stage 5 (lane 5) embryos, and hybridized with *Scr* (Fig. 9A), *Antp* (Fig. 9B) and β-actin (Fig. 9C) probes. The transcripts (about 2.2 kb) from a *Scr*-like gene were detected in these embryos at three developmental stages (stages 3-5). The *Ilyanassa* DNA sequence complementary to the *Antp* probe was also expressed in *Ilyanassa* development. In Fig. 9B, the digoxigenin-labelled *Antp* probe was hybridized to *Ilyanassa* RNA. A 3.5 kb single transcript was found in stage 3-5 embryos. The absence of hybridization signals with both *Scr* (Fig. 9A) and *Antp* (Fig. 9B) probes from early cleavage (lane 1) and mesentoblast embryos (lane 2) show that the transcription of sequences complementary to these probes were not detectable during early development. The hybridization signals from RNA of older embryos (lanes 3, 4, and 5) indicate the transcription of sequences complementary to these probes during later stages of development. Fig. 9C shows a control where a digoxigenin-labelled actin DNA was used as a probe which hybridized to *Ilyanassa* RNA from all stages of development.

Localization of the transcripts of homeobox genes

We determined the localization of homeobox gene transcripts in *Ilyanassa*

embryos by *in situ* hybridization with digoxigenin-labeled DNA probes. We used a 2.2 kb cDNA probe from *Drosophila Antp* cDNA and a 400 bp fragment of a *Scr* cDNA probe. Hybridized probes were detected by immunostaining *Ilyanassa* embryos with an anti-digoxigenin antibody alkaline phosphatase conjugate. We also used an anti-digoxigenin antibody conjugated to horseradish peroxidase to confirm localization of homeobox gene transcripts in the embryos (data not show).

We hybridized *Ilyanassa* embryos at different stages of development. Fig. 10 summarizes the results of hybridization from unfertilized eggs to stage 3 embryos. We did not detect the presence of the transcripts in eggs, mesentoblast stage embryos or gastrula stage embryos with either the *Antp* (Fig. 10a, 10b and 10c) or *Scr* (Figs. 10g, 10h and 10i) probes. Transcripts complementary to the *Antp* and *Scr* probes were, however, found in stage 3 embryos (Figs. 10e and 10k, respectively) where they were localized in the ectoderm of the anterior regions of embryos. Because we have not yet shown that these transcripts are encoded by homologs of *Antp* and *Scr* in the *Ilyanassa* genome, we define the genes encoding these transcripts as *Antp*-like gene and *Scr*-like gene. Figs. 10d and 10i illustrate stage 3 embryos treated with RNase ONE to digest embryonic RNA before hybridization. The absence of signals in RNase-treated embryos shows that the hybridization in Figs. 10e and 10k is RNA-dependent.

The transcription of the *Antp*-like gene is dynamic in older embryos. Stage 4 embryos are in an early stage of organogenesis, and, as mentioned above, the first organ primordium, the shell gland, is formed at this stage. The *Antp*-like transcripts were found in the ventral region of ectoderm along the anterior-posterior axis of the embryo. Fig.

11a is a lateral view of a stage 4 embryo, showing the expression pattern in the ventral ectoderm. The development of *Ilyanassa* continues and a visible foot and a head vesicle are formed in stage 5 embryos. The expression pattern of *Antp*-like gene in stage 5 embryos is similar to that in stage 4 embryos. Fig. 11b is a lateral view of a stage 5 embryo, showing that *Antp*-like transcripts were localized in the ventral region, including the foot primordium.

The transcription of the *Scr*-like gene is also dynamic in *Ilyanassa* development. The transcription pattern of the *Scr*-like gene in older embryos, summarized in Fig. 12, is similar to that of the *Anpt*-like transcripts. The *Scr*-like transcripts are also localized in the ventral ectoderm of both stage 4 embryos (Fig. 12a, a lateral view) and stage 5 embryos (Fig. 12b, a lateral view).

It takes 7-8 days for a fertilized egg to develop into a young veliger which has many well developed organs or structures, including a shell gland, a foot, eyes, a velum, an operculum, muscles, cilia, a digestive system and so on. The *Antp*-like transcripts were exclusively localized in the foot. Fig. 13a is a lateral view of a veliger showing expression in the foot as indicated by an arrow. Fig. 13b is a dorsal view of a young veliger, showing that the whole ectoderm of the foot is stained.

The transcription of the *Scr*-like gene was also observed in late stages of *Ilyanassa* development. Fig. 14a is a lateral view of a young veliger, showing expression in the foot. Fig. 14b is a ventral view of a young veliger, showing peripheral staining in the ectoderm of the foot. These observations show that the expression of the *Antp*-like and *Scr*-like genes are not only dynamic during embryogenesis, but also overlapping in

Ilyanassa embryos.

Our most important observation was that the *Antp* probe did not hybridize with 3 day old lobeless embryos (Fig. 10f). Therefore, the synthesis of mRNA complementary to this probe appears to be polar lobe-dependent. While the *Scr*-like transcripts were present in 3 day old lobeless embryos (Fig. 10l), they were distributed in a radial pattern and not localized in a specific region as in normal stage 3 embryos (Fig. 10k). This is consistent with the absence of bilateral symmetry in the lobeless embryo and its failure to form an anterior-posterior axis.

This observation was extended to older lobeless embryos. Like the 3 day old lobeless embryos, the *Antp* probe failed to hybridize to 4 day, 5 day and 6 day old lobeless embryos (Figs. 11c, 11d and 11e, respectively), while normal embryos at comparable stages showed high levels of expression. Unlike the 3 day old lobeless embryos, we did not find any transcripts of the *Scr*-like gene in these older lobeless embryos. Figs. 12c, 12d and 12e are 4, 5 and 6 day old lobeless embryos, respectively that were hybridized with a *Scr* probe. In 7 day old (Fig. 13c and Fig. 14c) and 8 day old (Fig. 13d and Fig. 14d) lobeless embryos, we could not detect any transcripts of either the *Antp*-like gene (Figs. 13c-d) or the *Scr*-like gene (Figs. 14c-d). Failure of the two probes to hybridize to older lobeless embryos again suggests that the transcription of the *Antp*-like and *Scr*-like genes are influenced by the polar lobe and that the failure of the hybridization is not caused by a delay in RNA synthesis.

The transcription patterns of the *Antp*-like gene in *Ilyanassa* embryos were further confirmed by *in situ* hybridization of embryos with digoxigenin-labeled *Antp* RNA

probes, which showed the same pattern of hybridization seen with DNA probes. Fig. 15 shows the results from *in situ* hybridization with the RNA probes. We found that the antisense RNA probe hybridized to embryos (Figs. 15a-b), while the sense RNA probe did not hybridize (Figs. 15c-d). Fig. 15a is a stage 3 embryo with hybridization in the anterior ectoderm (indicated by an arrow). Fig. 15b is a stage 5 embryo showing hybridization in the ventral region of the embryo. Figs. 15c and 15d show a stage 3 and a stage 5 embryos, respectively, hybridized with a sense RNA probe. The hybridization results with the antisense RNA probe are the same as those with the DNA probes.

In order to confirm that the staining was caused by hybridizing a digoxigenin-labeled probe to its complementary mRNA in *Ilyanassa* embryos, embryos were treated with RNase ONE prior to hybridization (Fig. 16a). The staining was negative, indicating that the hybridization reaction was RNA-dependent. Another control was to test if the staining was caused by non-specific binding of the anti-digoxigenin antibody conjugate. Embryos were incubated with the antibody without hybridizing to a probe. Without a probe, the antibody conjugate was unable to bind to the embryos (Fig. 16b). Because the antibody is conjugated with an alkaline phosphatase and there is an endogenous alkaline phosphatase activity in *Ilyanassa* embryos, we used 1 mM levamisole in the staining reaction. Fig. 16c shows that 1 mM levamisole completely suppresses the activity of the endogenous alkaline phosphatase in the *Ilyanassa* embryos.

The *Antp* was cloned in the plasmid pBluescript and the *Scr* was cloned in the plasmid pAT153. DNA inserts were released from vectors by digestion with restriction enzymes and purified by agarose gel electrophoresis and electroelution. It is necessary

to know whether the hybridization with the *Antp* and the *Scr* probes was caused by potentially contaminating vector sequences. If there is potential contamination by vector sequences, the contamination should be very minor and the amount of the contaminating vector sequences should not be more than 1% of the total DNA probe. Even so, we hybridized embryos with different amounts of digoxigenin-labeled pBluescript DNA which were equivalent to 1%, 5%, 10% and 20% of the *Antp* or *Scr* probe used in the hybridization, respectively. While embryos hybridized with normal amounts of the *Antp* and *Scr* probes were well stained, embryos hybridized with the pBluescript probe were unstained. Fig. 16d shows the hybridization of a stage 5 embryo with digoxigenin-labeled vector (pBluescript). The amount of pBluescript probe was equivalent to 20% of the *Antp* probe in this case. This experiment confirms that the staining of the embryos hybridized with the *Antp* and *Scr* probes was not caused by contamination with vector sequences.

One question about our experiments is whether the *Scr* and *Antp* probes hybridized with the same *Ilyanassa* transcript, *i. e.* are we using two probes to detect the transcripts of a single gene? The hybridization patterns in normal embryos for the both probes look similar and both the *Scr* and *Antp* probes have a homeobox sequence which shares about 82% homology in DNA sequence. Under low stringency hybridization conditions, the *Antp* homeobox sequence could hybridize to the *Scr* probe (Kuroiwa *et al.*, 1985). Dot blot experiments were done to answer this question (Figs. 17A and 17B). In the dot blot analysis, we assayed cross-hybridization between *Antp* and *Scr* probes at different levels of stringency in which a labelled *Antp* probe hybridized with *Scr* DNA and a labelled *Scr* probe hybridized with *Antp* DNA. The concentration of unlabelled

DNA on each blot ranged from 0.1 ng to 10 ng. The hybridization reactions and washes were at low (50° C in 1× SSC), moderate (60° C in 0.3× SSC) and high (65° C in 0.1× SSC) stringency levels. Although cross-hybridization between *Antp* and *Scr* probes occurred when hybridization was at low and moderate stringencies, it did not occur at high stringency conditions which were equivalent to the stringency level used in *in situ* hybridization. Therefore, the staining patterns of the embryos appear to be caused by a unique association between a probe and its complementary mRNA in *Ilyanassa* embryos.

Discussion

The role of homeobox genes in animal development have been extensively investigated in *Drosophila* and in many other organisms, including man. Based on the similarities of homeobox and homeodomain sequences, the expression patterns in both central nervous system (CNS) and mesoderm along the anterior-posterior body axis of an embryo, the correlation between the physical order of genes and their expression patterns and the morphological effects derived from homeotic mutations, homeotic genes are thought to play a role in establishing the anterior-posterior body axis in *Drosophila* development. Genes with strikingly similar homeoboxes, and presumably similar developmental functions, have been identified in many other organisms.

Homeobox genes have also been found in non-segmented worms. At least four *Caenorhabditis elegans* genes have a homeobox related to the *Drosophila Antp* gene. Surprisingly, they are also organized in a cluster with a similar order to *Drosophila* homeotic genes (Bürglin *et al.*, 1991; Kenyon and Wang, 1991). They may be involved

in pattern formation of *C. elegans*. Among them, *mab*-5 is expressed in the posterior body region, controlling epidermal, neuronal and mesoderm cell differentiation (Costa *et al.*, 1988). In *mab*-5 mutants, most of the cells normally expressing *mab*-5 show homeotic transformations and change to their adjacent anterior homologs. Therefore, the anterior-posterior axis activity of homeobox genes also appears to be conserved in non-segmented organisms.

Like *C. elegans*, *Ilyanassa* is a non-segmented organism. The universal conservation of homeobox sequences may reveal a universal mechanism in development in both invertebrates and vertebrates and in both segmented and non-segmented organisms. One effect of removing the polar lobe from *Ilyanassa* embryos is that the lobeless embryos develop without an anterior-posterior body axis, and have instead radial symmetry. This implies the presence of factors (morphogens) in the polar lobe that specify the anterior-posterior axis of *Ilyanassa* embryos at an early stage of development. One reason why we undertook the study of homeobox genes during *Ilyanassa* development was because we thought they may play a role in the formation of the anterior-posterior axis of *Ilyanassa* embryos.

Our search for homeobox genes in the *Ilyanassa* genome was initially done by Southern hybridization. At reduced stringency, a *Drosophila Scr* probe hybridized to multiple fragments of *Ilyanassa* genomic DNA digested with various restriction enzymes, suggesting the presence of more than one homeobox gene. It was reported by Holland (1986) that rDNA repeats could cross-hybridize to a homeobox sequence under reduced stringency conditions. Our control with the *Xenopus* rDNA as a probe shows that the *Scr*

probe did not hybridize with the *Ilyanassa* rDNA sequences. These observations confirm that putative homologs of homeobox genes are present in the *Ilyanassa* genome. Similarly, an *Antp* cDNA probe hybridized at high stringency to at least two restriction fragments (a major band and a minor band) of *Ilyanassa* genomic DNA. These *Ilyanassa* DNA sequences complementary to the *Antp* and *Scr* probes were shown by Northern hybridization and *in situ* hybridization to be expressed in *Ilyanassa* development, starting in stage 3 embryos.

In situ hybridization provides further evidence for the existence, expression and tissue-specificity of homeobox genes in *Ilyanassa* development. By using digoxigenin-labeled *Drosophila Scr* and *Antp* DNA probes, we found evidence for spatially and temporally regulated *Antp*-like and *Scr*-like genes. We did not detect any transcripts from these genes in embryos until the third day of development at which time *Ilyanassa* embryos have a visible stomodeal invagination (Figs. 10d-e; 10i-k). With this invagination, the anterior-posterior axis of the embryo is clearly discernible. The smaller part of the embryo is the anterior region and the larger part of the body belongs to the posterior region. The transcripts of the *Antp*-like and *Scr*-like genes are localized in the anterior region of embryos (Figs. 10e and 10k, respectively). In mouse development, homeobox genes are initially expressed at the early presomite gastrula stage during which the anterior-posterior body axis becomes apparent and the metamerism of the mesoderm is established (Holland and Hogan, 1988b). Both *Antp* and *Scr* genes are expressed in *Drosophila* embryos, with a high level expression in the anterior part of the embryo (McGinnis and Krumlauf, 1992). Their homologs, the *Hox* genes, are also expressed in

mouse embryos, with the most anterior limit of expression in the central nervous system (Graham *et al.*, 1989). The correlation between the morphological formation of the body axis and localization of both the *Antp*-like and *Scr*-like transcripts in anterior regions suggest that the *Antp*-like and *Scr*-like genes play a similar role in establishing the anterior-posterior axis in *Ilyanassa* embryos.

The expression of the *Scr*-like and *Antp*-like genes is overlapping and dynamic in *Ilyanassa* development. In young embryos, their transcripts are restricted to the head primordium. Later, localization of the transcripts extends along the ventral surface and to posterior regions of the embryo. Similar changes in the expression patterns of homeotic genes have been found during the development of *Drosophila* embryos. The *Scr* protein is first detected in parasegment 2 and later extends to parasegment 3 when the germ band elongates. At this time, the most posterior limit of the *Scr* expression is in the anterior compartment of the first thoracic segment (T1a). When the germ band is retracting, the *Scr* protein can be seen in the posterior cells of the T1 segment (Carroll *et al.*, 1988). In the early and middle stages of *Ilyanassa* organogenesis, the expression of the *Scr*-like and *Antp*-like genes extends to the ventral ectoderm along the anterior-posterior axis of the embryo in an overlapping pattern. This localization is similar to the expression pattern of homeotic genes in *Drosophila* embryos (McGinnis and Krumlauf, 1992) and Hox genes in the CNS of mouse embryos (Graham *et al.*, 1989).

Similarity of the homeobox gene expression among *Drosophila*, mouse and *Ilyanassa* embryos is shown in Fig. 18. The upper part and middle part of this diagram show the expression domains of homeotic genes in the *Drosophila* embryo and *Hox* genes

in the mouse embryo along the anterior-posterior axis, respectively (McGinnis and Krumlauf, 1992. Diagram courtesy of M. McGinnis and *Cell* Press). The lower part of this diagram shows the expression of the *Antp*-like and *Scr*-like genes in *Ilyanassa* embryos. Their expression is overlapping and dynamic. The transcription (shown by dotted regions) of the *Antp*-like and *Scr*-like genes is first localized in the presumptive head primordium of a stage 3 embryo (left) and is then extended to the ventral region along the anterior-posterior axis of a stage 5 embryo (right). Although we can not distinguish the expression boundaries of the *Scr*-like gene from those of the *Antp*-like genes in *Ilyanassa* embryos, we suggest that their expression patterns are consistent with a role in forming the anterior-posterior body axis in *Ilyanassa* embryos, and in establishing the positional information required for the development of the foot primordium.

In mouse development, the expression of a homeobox gene is not always consistent with a role in specification of the body axis. Particularly, the expression at later stages of development may be partly tissue- or cell-specific, rather than regionally specific. For example, although the Hox-2.1 transcripts are regionally localized in the spinal cord in an anterior-posterior gradient extending from the hindbrain, they are also found in fetal tissues (lung, kidney, gut, spleen and liver) and in mature granulocytes in adult mice, suggesting a function of Hox-2.1 in cell determination or organogenesis in the mouse (Krumlauf *et al.*, 1987; Holland and Hogan, 1988a). When an *Ilyanassa* embryo develops to the seventh day under the conditions mentioned in text, a young veliger is formed, with visible organs such as eyes, a foot, a head vesicle, a shell gland,

a velum and an operculum. We found that the transcripts of both the *Antp*-like and *Scr*-like genes were exclusively localized in the ectoderm along the edge of the foot. This hybridization is shown in Figs. 13a and 13b (for *Antp*-like) and in Figs. 14a and 14b (for *Scr*-like). These observations on the transcription of the *Antp*-like and *Scr*-like genes during later development of *Ilyanassa* (young veliger) indicate that these genes are closely linked to the differentiation of a specific structure (the foot) and are therefore not in accord with the role of HOM-C genes in *Drosophila*, which are generally thought to function in coarse position specification and are not closely linked to the differentiation of a specific structure (McGinnis and Krumlauf, 1992; Kenyon and Wang, 1991). In the early and middle stages of organogenesis of *Ilyanassa* embryos, the transcripts of both the *Scr*-like and *Antp*-like genes are found in the foot primordium, and in later stages these genes are transcribed in the differentiation of the foot. Combining these observations, we believe a possible role for the *Antp*-like and *Scr*-like genes is to define the leading edge and growth pattern in the formation of the larval foot. Thus, these homeobox genes show regional specific expression in early stages and tissue or organ specific expression in later stages of *Ilyanassa* development.

Our Northern blot analysis and *in situ* hybridization to *Ilyanassa* embryos with *Drosophila Antp* and *Scr* cDNA probes shows that sequences complementary to these probes are transcribed during *Ilyanassa* development. Because the hybridization signals in normal embryos are overlapping for both the probes and both of the probes have a homeobox sharing 82% DNA sequence homology (Kuroiwa *et al.*, 1985), there is a possibility that the two probes detected a single *Ilyanassa* transcript. The different size

of *Ilyanassa* RNA shown in our Northern blot analysis (2.2 kb for the *Scr* probe and 3.5 kb for the *Antp* probe) and the high stringency condition in the hybridization suggest that we have detected different transcripts complementary to the two different probes. This point is further supported by our observation that the *Antp* and *Scr* probes do not cross-hybridize at high stringency conditions. Finally, the hybridization patterns of these two probes with lobeless embryos are distinctly different. These observations suggest that the *Antp* and *Scr* probes are detecting different transcripts in *Ilyanassa* embryos.

Localized cytoplasmic determinants are important in specifying cell fates presumably by regulating gene expression. If the polar lobe is removed, the larva developed from the lobeless embryo will not develop a normal anterior-posterior body axis. Although we do not know how the maternal factors in the polar lobe play a role in the formation of the body axis during *Ilyanassa* development, they may do so by regulating the expression of homeobox genes.

Failure of the *Antp* probe to hybridize with lobeless embryos is a striking demonstration of polar lobe-dependent gene transcription. The failure to detect hybridization in several older stages of lobeless embryos shows that the absence of hybridization in lobeless embryos is not caused by different rates of development of these two classes of embryos. The hybridization obtained with an antisense riboprobe and the corresponding failure of a sense riboprobe to hybridize suggest that our hybridization reaction has detected a specific mRNA. That the *Scr* probe hybridized to 3 day old lobeless embryos whereas the *Antp* probe did not supports that these probes, while overlapping in regional localization in the embryos, detect different mRNAs.

The *Scr* probe did hybridize with 3 day old lobeless embryos, but not at later stages as in the normal embryos. The hybridization in the lobeless embryos was in a radial pattern rather than along an anterior-posterior axis as in normal embryos. The absence of an anterior-posterior axis in lobeless embryos suggests that their aberrant hybridization pattern may have occurred because they lacked the positional information required for axiation, and therefore, hybridization of the *Scr* probe was aberrant.

Is the influence of the polar lobe on gene transcription direct or indirect? If it is a direct influence, one would expect the polar lobe to contain a specific factor (s) required for the transcription of a specific gene. An indirect influence might arise from some abnormal event caused by the absence of the polar lobe, such as the absence of the mesentoblast cell (4d), that secondarily alters the expression of a gene. These two possibilities are not mutually exclusive and it will be important to determine how the polar lobe exerts its influence on gene expression in both the present results and the expression of other genes may be shown to be aberrant in lobeless embryos.

In summary, our results show that two homeobox genes, the *Antp*-like and *Scr*-like genes, are present in the *Ilyanassa* genome and expressed during *Ilyanassa* embryogenesis. Their expression is overlapping and dynamic during embryonic development. The transcripts of these homeobox genes are first localized in the presumptive head primordium in early stage embryos (postgastrula) of the development. During organogenesis of older embryos (stage 5) the localization of the transcripts extends ventrally along the anterior-posterior axis of the embryo. The transcripts of the *Antp*-like and *Scr*-like genes are finally localized in the ectoderm of the foot in young

veliger larva. These expression patterns are consistent with the expression domains of the corresponding homeotic genes in *Drosophila* embryos and the *Hox* genes in mouse embryos.

The transcription of the *Antp*-like and *Scr*-like genes in the stage 3 embryo suggests that these homeobox-like genes have a role in establishing the anterior-posterior axis of the *Ilyanassa* embryo. This point is further emphasized by the failure of the *Antp*-like gene to be expressed in the radially symmetrical lobeless embryo and by the aberrant expression of the *Scr*-like gene in the lobeless embryo. The transcription of the *Antp*-like and *Scr*-like genes in the foot primordium of the stage 5 embryo suggests a role in establishing the position of the foot primordium along anterior-posterior axis of the embryo. That the transcripts of these homeobox-like genes are finally localized in the ectoderm of the foot in the young veliger larva indicates a possible role of these genes in the final differentiation of the foot. That their expression is localized along the periphery of the foot suggests that they are involved in positional information which is required for establishing the growth pattern of the foot.

Most importantly we wish to emphasize that our observations have shown that the transcription of the *Antp*-like and *Scr*-like homeobox genes are polar lobe-dependent, and that this is the first evidence that cytoplasmic factors in the polar lobe affect the transcription of specific genes. Lacking sequence data for the *Antp*-like and *Scr*-like genes in *Ilyanassa* we can not determine the extent of their homology to the corresponding *Drosophila* genes, but setting aside this question for the moment, we think that finding two genes whose transcripts are polar lobe-dependent is a significant step

toward understanding the way localized cytoplasmic factors influence development.

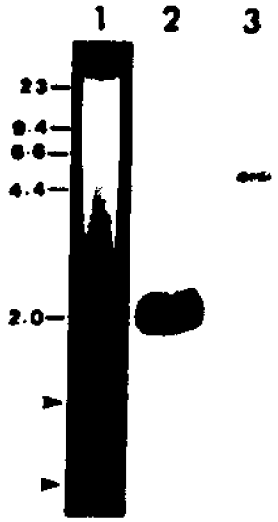

Fig. 1

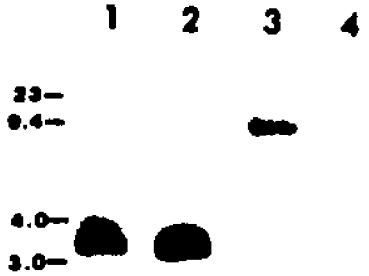

Fig. 2

Fig. 1. **Southern blot analysis of a *ras*-like gene in the *Ilyanassa* genome.** *Ilyanassa* DNA was digested with *Eco*RI, fractionated through a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with a *ras* probe (lane 2) and a rDNA probe (lane 3) at high stringency. A ~2.0 kb DNA fragment bound to the *ras* probe and a ~5.0 kb fragment bound to the rDNA probe. The arrows at lane 1 indicate repetitive sequences produced by *Eco*RI digestion.

Fig. 2. **Hybridization of *Ilyanassa* DNA with a *ras* cDNA probe.** *Ilyanassa* genomic DNA was digested with *Sst*I (lane 1) and *Bam*HI (lane 2-4), fractionated through a 0.8% agarose gel, transferred to a filter, and hybridized with a *ras* cDNA probe (lane 1-2), a rDNA probe (lane 3) and a pSP64 probe (lane 4) at high stringency. The *ras* probe hybridized to a 3.5 kb DNA fragment (lane 1-2).

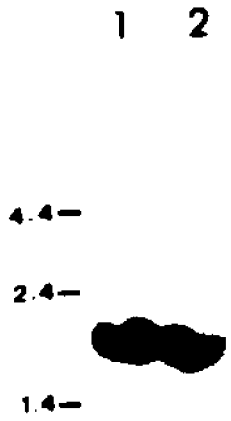

Fig. 3. **Transcription of a *ras*-like gene in *Ilyanassa* embryos**. Total RNA from stage 4 (lane 1) and stage 5 (lane 2) embryos was fractionated through a 1.0% agarose gel containing formaldehyde, transferred to nitrocellulose, hybridized to a radio-labeled *ras* cDNA probe at high stringency. A ~1.9 kb mRNA was complementary to the *ras* probe.

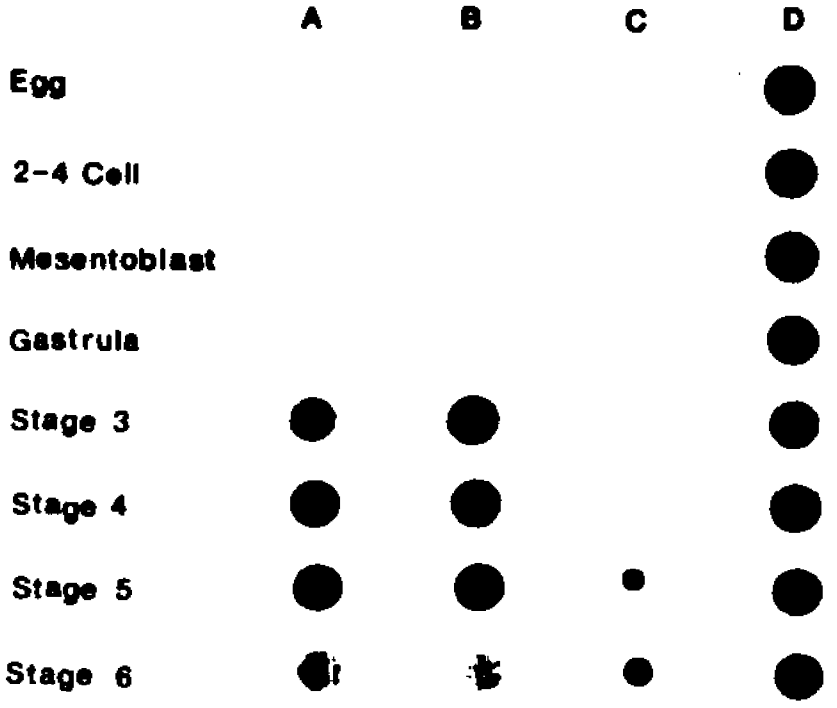

Fig. 4. **Developmental expression of a *ras*-like gene in *Ilyanassa* embryos.** RNA from different stages of *Ilyanassa* embryos was blotted onto nitrocellulose, and hybridized to a *ras* cDNA probe (Column A, B, and C). The stages are as labeled. Column A, a 'dot blot' analysis of RNA from equal numbers of the normal embryos; Column B, analysis of equal amount of RNA from normal embryos; Column C, analysis of RNA from lobeless embryos with an amount of RNA used for normal embryos in lane B. Column D, hybridization of *Ilyanassa* RNA from all stages with an actin probe. The *ras* transcript was detected in normal embryos at stage 3 and in lobeless embryos at the age of 5 days old.

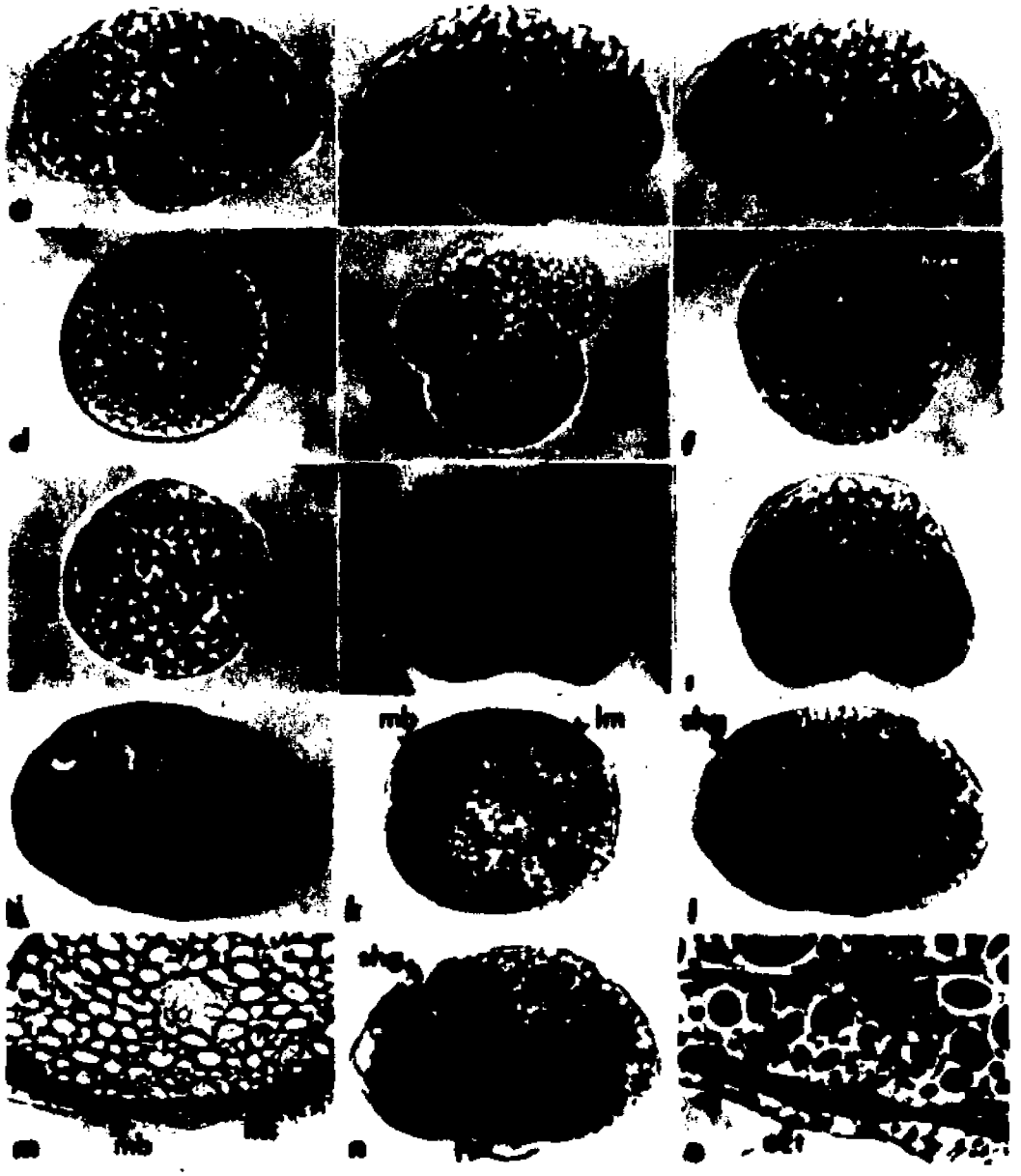

Fig. 5. **Immunostaining of embryos with an antibody to the *ras* encoded protein.** Stage 5 embryos stained for alkaline phosphatase in the presence of 1 mM levamisole (a), immunostained with normal rat IgG as the primary antibody (b) and with the secondary antibody-alkaline phosphatase conjugate (c) alone. Early stages of development immunostained with an antibody to the *ras* protein, mature unfertilized egg (d); four-cell stage (e); mesentoblast stage (f); gastrula (g); paraffin section of a stage 3 embryo (h); whole mount of stage 3 embryo immunostained with the *ras* antibody (i); paraffin section of a stage 4.5 embryo (j); dorsal (k) and ventral (l) view, respectively, of stage 4.5 embryos immunostained with the *ras* antibody; section of immunostained stage 4.5 embryo (m); whole mount of immunostained stage 5 embryo (n); and section of immunostained stage 5 embryo (o). The *ras* protein is detected in the mesoderm of stage 3-5 embryos. Abbreviations: f, foot; gv, germinal vesicle; ect, ectoderm; en, endoderm; m, mesoderm; lm, larval mesoderm; mb, mesoderm band; shg, shell gland.

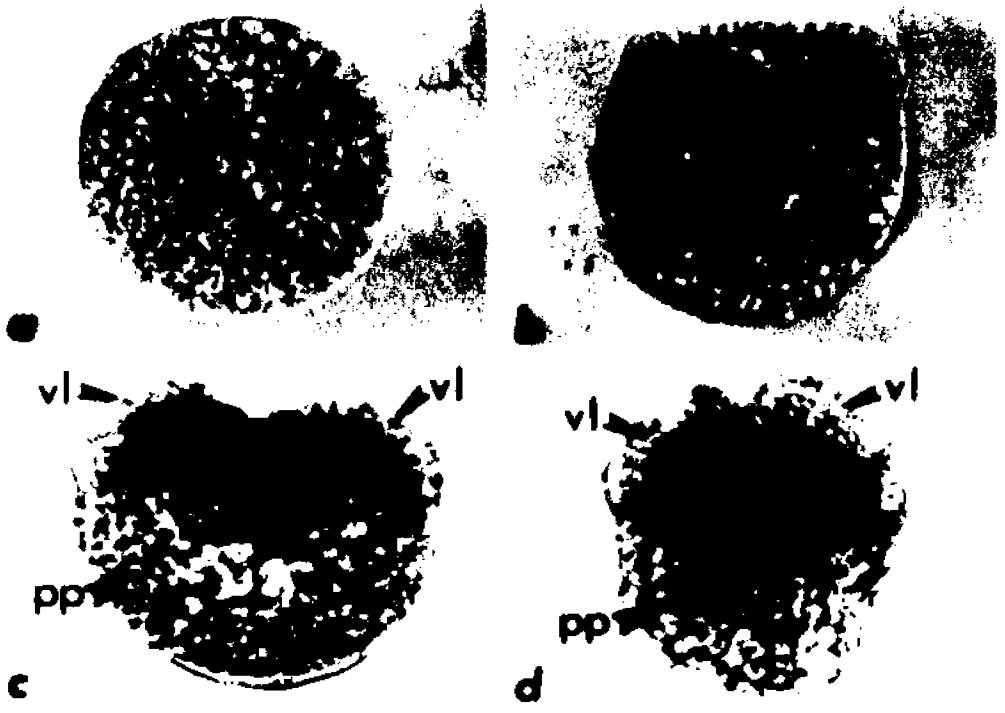

Fig. 6. **Immunostaining of lobeless embryos with an antibody to the *ras* encoded protein**. Immunostained lobeless embryos after 3 (a) and 4 (b) days of development, and similarly stained lobeless embryos after 5 (c) and 6 (d) days of development. The *ras* protein is detected in the lobeless embryos at the age of 5 days old and 6 days old. Abbreviations: pp, posterior protrusion; vl, velar lobe.

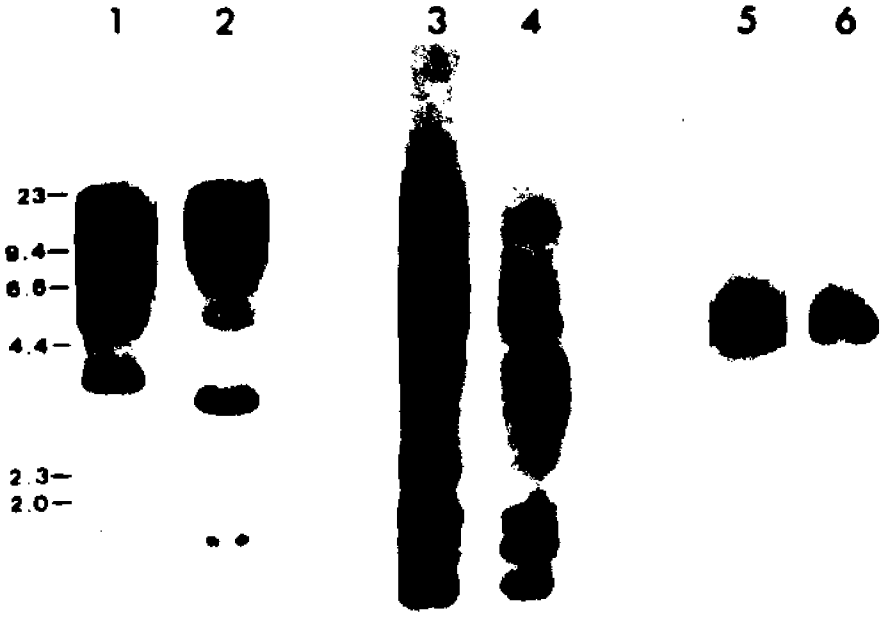

Fig. 7. **Southern blot analysis of homeobox genes in the *Ilyanassa* genome.** *Ilyanassa* genomic DNA was digested with *Eco*RI (lane 3) and *Hind*III (lane 4), fractionated through a 0.8% agarose gel, transferred to a filter, and hybridized to a *Scr* probe at reduced stringency. The *Scr* probe bound to multiple restriction fragments of the *Ilyanassa* DNA. Controls are the *Eco*RI (lane 1, 5) and *Hind*III (lane 2, 6) digested *Drosophila* DNA (lane 1-2) hybridized with the *Scr* probe and *Ilyanassa* DNA (lane 5-6) hybridized with rDNA probe.

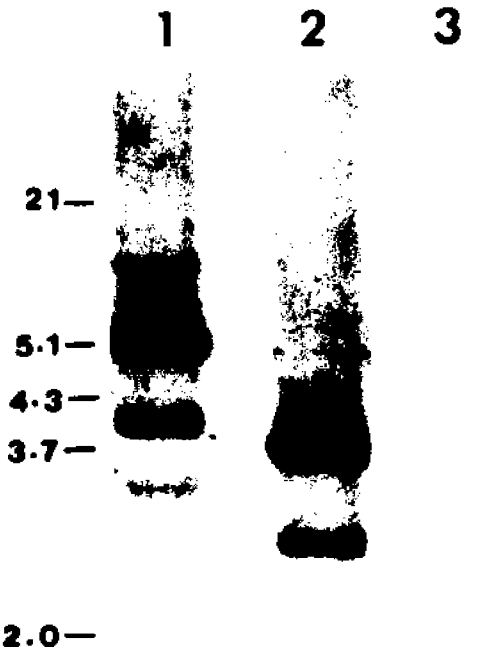

Fig. 8. **Hybridization of *Ilyanassa* DNA with a *Drosophila Antp* cDNA probe.** *Ilyanassa* genomic DNA was digested with *Eco*RI (lanes 1 and 3) and *Bam*HI (lane 2), fractionated through a 0.8% agarose gel, transferred to nitrocellulose and hybridized to an *Antp* probe (lanes 1-2) and pBluescript DNA (lane 3, control) at high stringency.

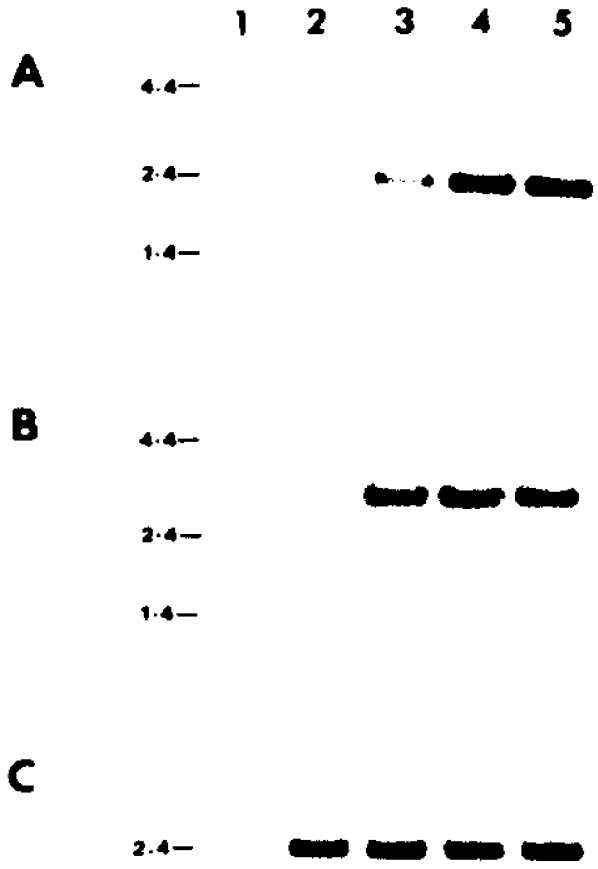

Fig. 9. **Transcription of *Scr*-like and *Antp*-like genes in *Ilyanassa* development.** Hybridization of *Ilyanassa* RNA from mesentoblast (lane 1), gastrula (lane 2), stage 3 (lane 3), stage 4 embryos (lane 4) and stage 5 embryos (lane 5) with the *Scr* probe (A) and the *Antp* probe (B). A control (C) is the same RNA hybridized with an actin DNA probe.

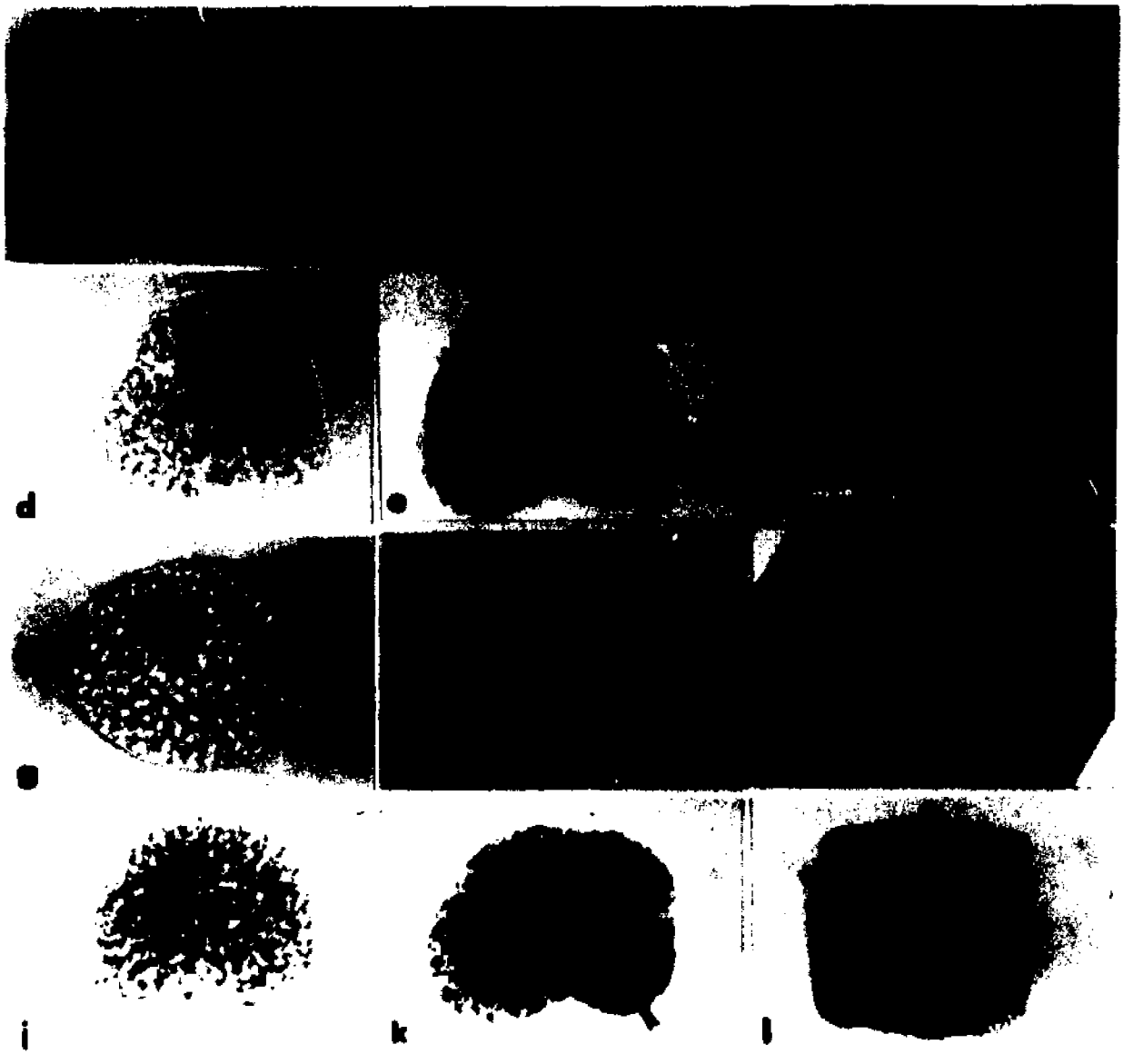

Fig. 10. *In situ* hybridization of early stage embryos with *Antp* (a-f) and *Scr* (g-l) probes. As probes, the *Antp* and *Scr* cDNAs were labeled with digoxigenin. The hybridization was detected by immunostaining with an anti-digoxigenin antibody conjugated with alkaline phosphatase. Mature unfertilized eggs (a, g); mesentoblast stage (b, h); gastrula (c, i); stage 3 embryos (e, k); 3 day old lobeless embryos (f, l). Controls are RNase ONE-treated stage 3 embryos (d, i) hybridized with the probes. Hybridization regions are indicated by arrows. The transcripts of *Antp* (e) and *Scr* (k) are first detected in the normal stage 3 embryos and localized in the anterior part of the embryos. No *Antp*-like transcripts in lobeless embryos (f). The *Scr* transcripts are also found in 3 days old lobeless embryos in an aberrant pattern (l). Abbreviation: gv, germinal vesicle.

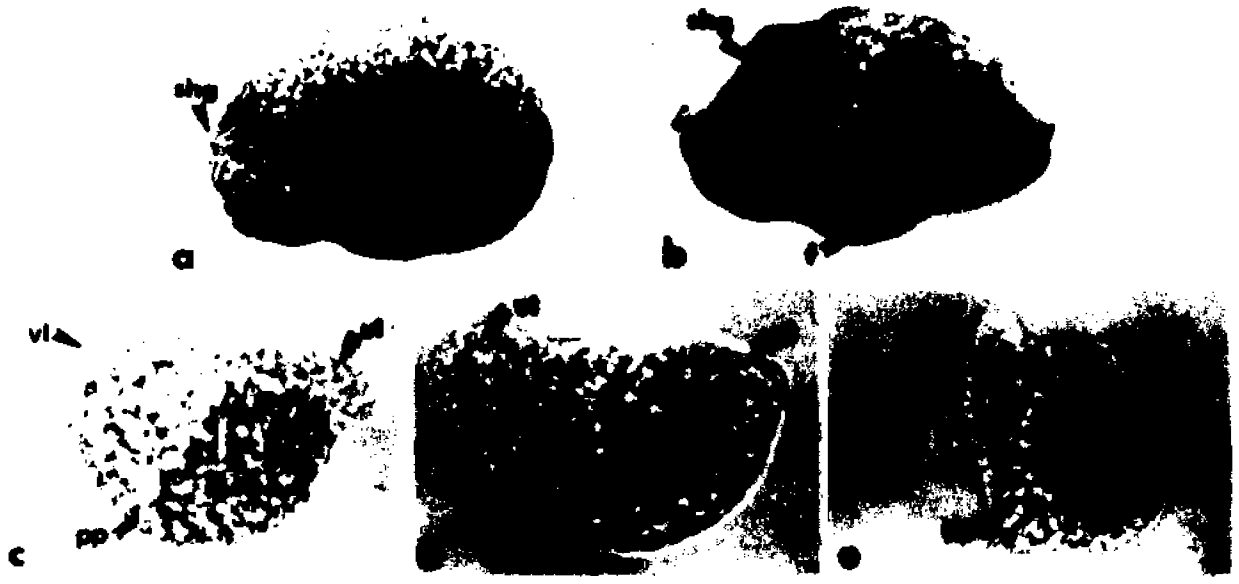

Fig. 11. *In situ* hybridization of older embryos with an *Antp* probe. A lateral view of a stage 4 embryo (a); a lateral view of a stage 5 embryo (b); a 4 days old lobeless embryo (c); a 5 days old lobeless embryos (d) and a 6 days old lobeless embryo (i). The *Antp*-like transcripts are localized in the ventral ectoderm in both stage 4 and stage 5 embryos. No hybridization signal in lobeless embryos. Abbreviations: shg, shell gland; f, foot; hv, head vesicle; pp, posterior protrusion; vl, velar lobe.

Fig. 12. ***In situ*** **hybridization of older embryos with a *Scr* probe.** A lateral view of a stage 4 embryo (a); a lateral view of a stage 5 embryo (b); a 4 days old lobeless embryo (c); a 5 days old lobeless embryo (d) and a 6 days old lobeless embryo (e). The *Scr*-like transcripts are localized in the ventral ectoderm in both stage 4 and stage 5 embryos. No hybridization signal in lobeless embryos. Abbreviations: shg, shell gland; f, foot; hv, head vesicle; pp, posterior protrusion; vl, velar lobe.

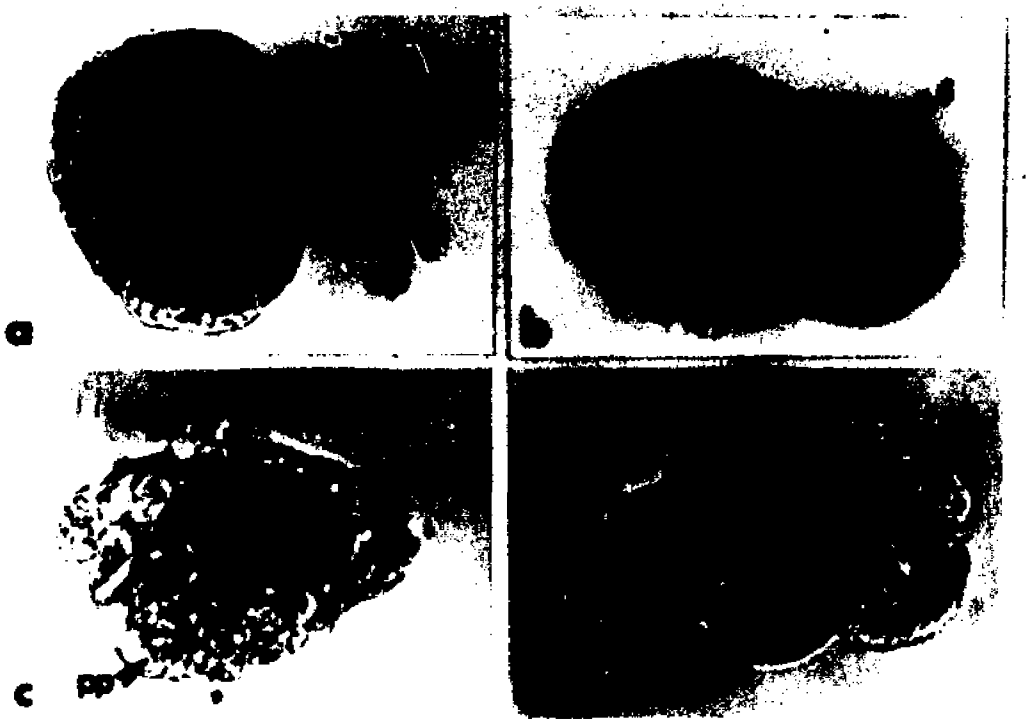

Fig. 13. ***In situ* hybridization of young veligers with an *Antp* probe.** A lateral view of a young veliger (a); a dorsal view of a young veliger (b); a 7 days old lobeless embryo (c) and a 8 days old lobeless embryo (d). The *Antp*-like transcripts are exclusively localized in the edge of the foot. No hybridization signal in lobeless embryos. Abbreviations: f, foot; op, operculum; e, eyes; pp, posterior protrusion; vl, velar lobe.

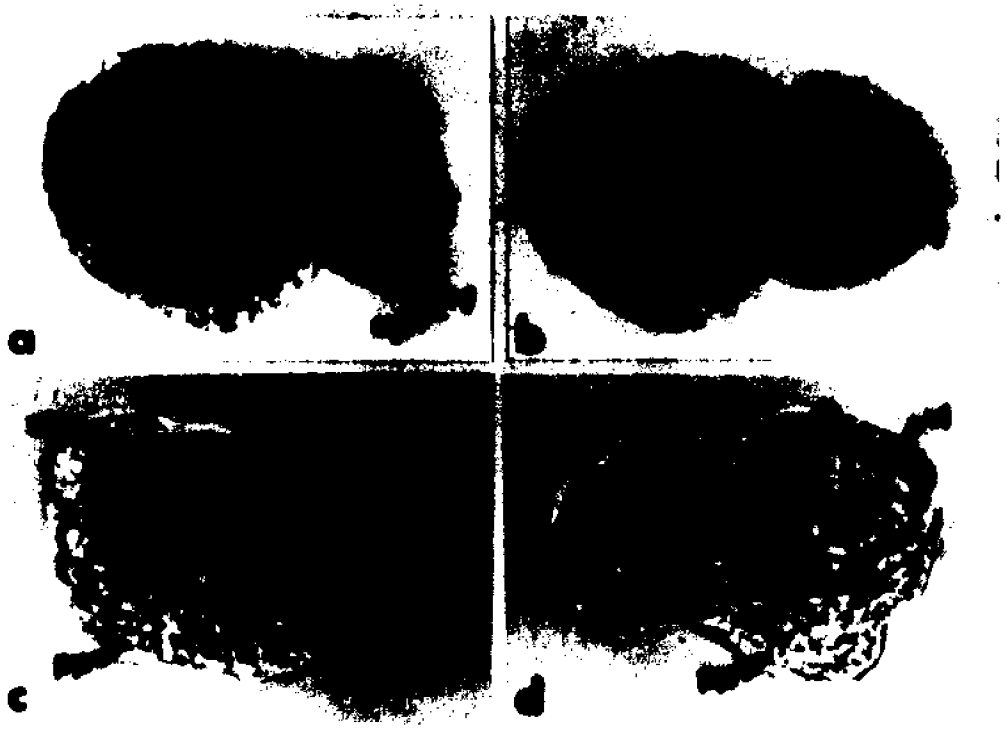

Fig. 14. *In situ* hybridization of young veligers with a *Scr* probe. A lateral view of a young veliger (a); a ventral view of a young veliger (b); a 7 days old lobeless embryo (c) and a 8 days old lobeless embryo (d). The *Scr*-like transcripts are exclusively localized in the ectoderm of the foot. No hybridization signal in lobeless embryos. Abbreviations: f, foot; op, operculum; e,eyes; pp, posterior protrusion; vl, velar lobe.

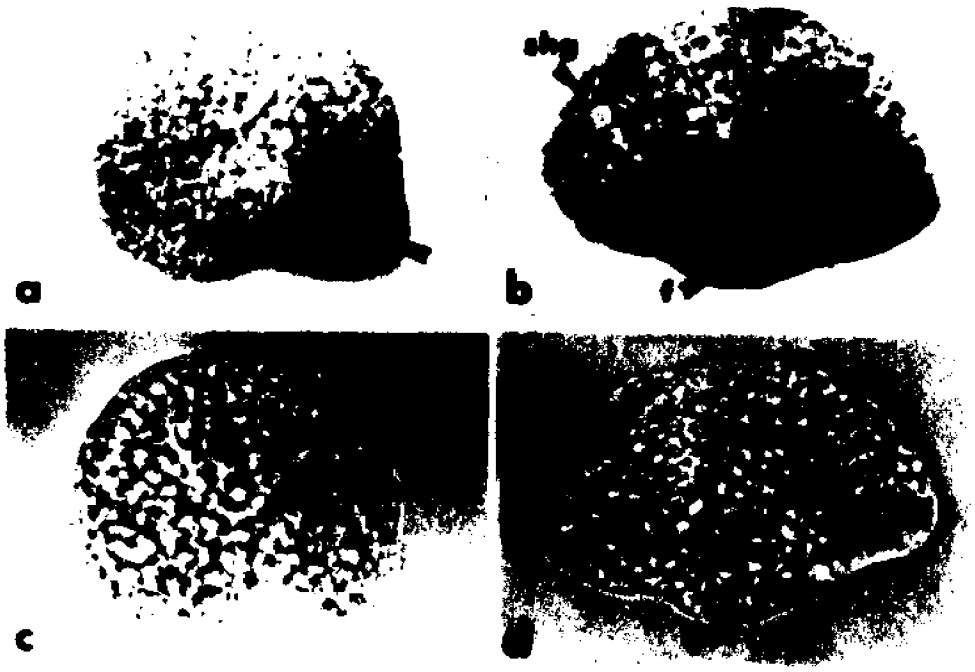

Fig. 15. ***In situ* hybridization of embryos with an *Antp* RNA probe.** The embryos are hybridized with an antisense RNA probe (a-b) and a sense RNA probe (c-d) of the *Antp*. A lateral view of a stage 3 embryo with the hybridization in the anterior part (a); a lateral view of a stage 5 embryo with the hybridization in the ventral region, including the foot (b); lateral views of a stage 3 (c) and a stage 5 (d) embryo to show negative staining. Abbreviations: shg, shell gland; f, foot; hv, head vesicle.

Fig. 16. Controls for *in situ* hybridization. Stage 5 embryos (a-d) treated with RNase ONE before hybridizing with the *Antp* probe to show that the hybridization is RNA-dependent (a); incubated with the anti-digoxigenin antibody without a probe to demonstrate the absence of non-specific binding of the antibody to the embryos (b); stained in the alkaline phosphatase staining solution in the presence of 1 mM levamisole to show that the endogenous alkaline phosphatase in the *Ilyanassa* embryos is completely suppressed (c); and hybridized with digoxigenin-labeled pBluescript DNA with the amount equivalent to 20% of the *Antp* probe to confirm that the staining patterns in Fig. 10-14 are caused by the *Antp* and *Scr* probes rather than potentially contaminating vector sequences.

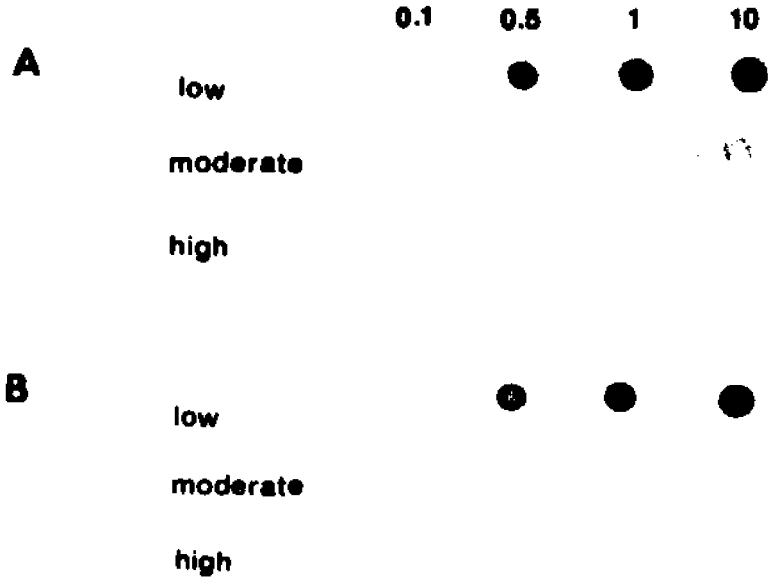

Fig. 17. **Cross-hybridization between DNA probes**. Dot blots were prepared with the *Antp* and *Scr* cDNAs. The number on the top represents the amount of unlabelled DNA (ng) bound to nitrocellulose. A: blots bound with *Antp* DNA were hybridized with the *Scr* probe at low, moderate and high stringencies mentioned in text. B: blots bound with *Scr* DNA were hybridized with the *Antp* probe at low, moderate and high stringencies. Cross-hybridization between these probes did not occur at high stringency conditions which are equivalent to those of *in situ* hybridization.

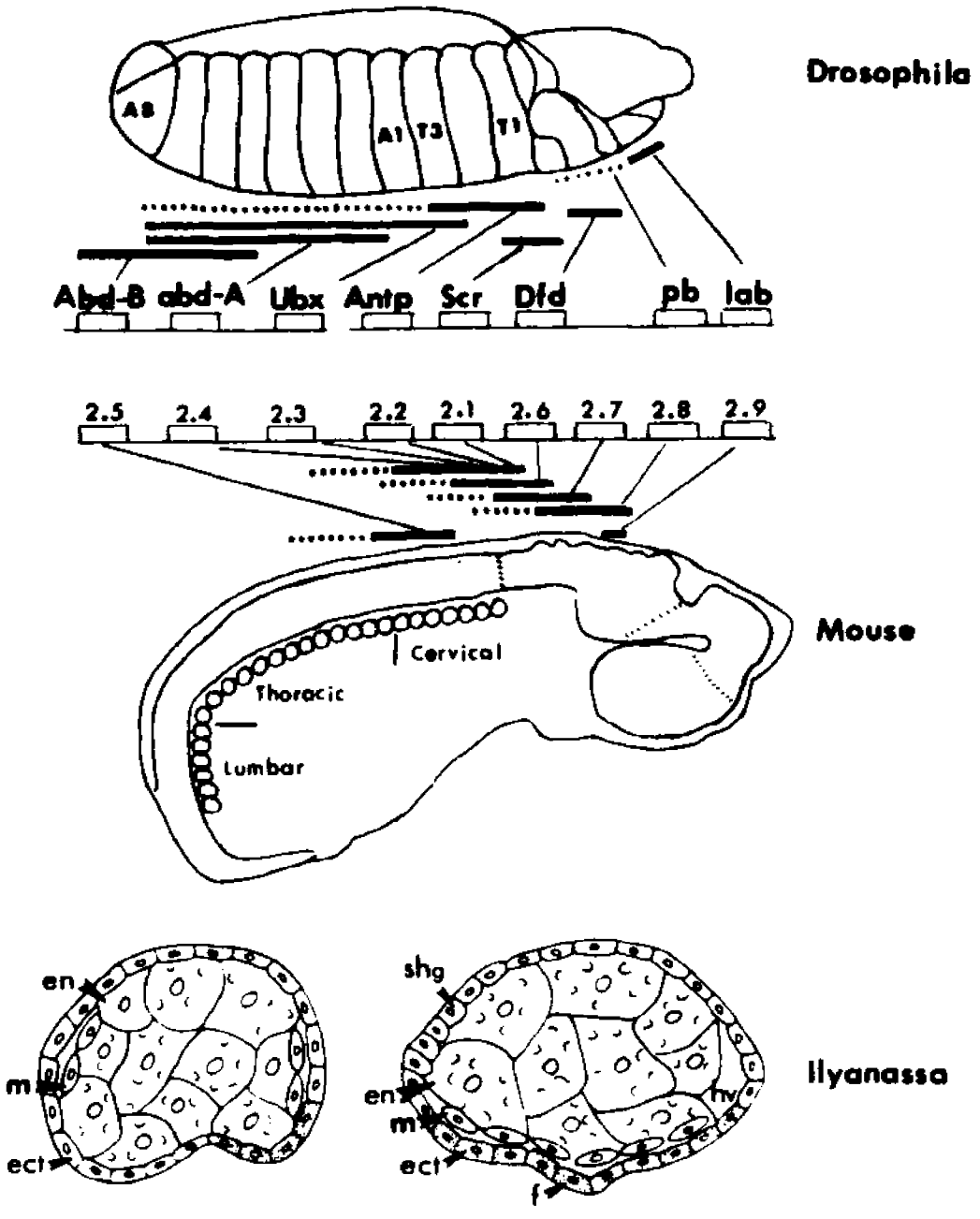

Fig. 18. **Summary of the expression patterns of homeobox genes.** The expression pattern of homeobox genes is similar among *Drosophila*, mouse and *Ilyanassa* embryos. The upper and middle: expression domains of homeotic genes in a *Drosophila* embryo and *Hox* genes in a mouse embryo, respectively (McGinnis and Krumlauf, 1992. Diagram courtesy of M. McGinnis and *Cell* Press). The lower: expression domain (dotted region) of homeobox genes in a stage 3 (left) and a stage 5 (right) embryos in *Ilyanassa* development. Abbreviations: T1-T3, thoracic segments 1-3; A1-A8, abdominal segments 1-8; f, foot; hv, head vesicle; shg, shell glang; en, endoderm; m, mesoderm; ect, ectoderm.

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