CRABP IN AMPHIOXUS: EXPRESSION, FUNCTION, AND RELATION TO VERTEBRATES

by

JOLEE MUNSON

A THESIS

Presented to the Department of General Science
and the Honors College of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Bachelor of Arts

June 2000

An Abstract of the Thesis of

Jolee Munson for the degree of Bachelor of Arts

in the Department of General Science to be taken June 2000

Title: *CRABP* IN AMPHIOXUS: EXPRESSION, FUNCTION AND RELATION TO VERTEBRATES

Approved: [redacted]

Dr. Charles Kimmel

CRABP is the gene which is believed to make the Cellular Retinoic Acid Binding Protein. In this thesis, I will show the sequence of *CRABP* in amphioxus and explain where the gene is expressed in amphioxus which are between 4 and 58 hours of development. There is expression in the posterior cerebral vesicle, rostrum, and mouth regions. By comparing the expression pattern in amphioxus to the expression patterns of the *CRABP* homologs in vertebrates (*CRABPI* and *CRABPII*), I hypothesized which structures may be homologous. I found evidence for homology between the rostral region of amphioxus and the frontonasal mesenchyme of vertebrates, the posterior cerebral vesicle of amphioxus and part of the midbrain in vertebrates, and the mouth of amphioxus and the mouth of vertebrates. This thesis will also examine possible functions of CRABP in amphioxus, including a role in regulating the retinoic acid levels in some parts of the developing embryo.

Introduction

Background

For my thesis, I studied a small, worm-like animal called amphioxus. From this research, I hope to contribute to the knowledge of the human evolution and development. How can the study of one gene in an organism that looks nothing like humans, teach us about our species?

Although humans and amphioxus do not look very similar, they are related and share many characteristics. They are both members of the Chordate phylum, which means that they have notochords, central nervous systems, and pharyngeal slits (which develop into gills slits in fish). Some of these features are only present during early development of the organism, at which time many organisms look more similar than during adulthood (Figure 1). For example, human embryos have a notochord, similar to the notochord that amphioxus has during adulthood. As the embryo develops, the notochord is replaced by the vertebral column, but even so, studying the notochord in amphioxus can give us insights into the human notochord and its development. Because some of these features are present only during development, I studied amphioxus embryos in hopes of learning more about the development of humans and also how humans have evolved.

Humans and amphioxus are related because they share a common ancestor (Figure 2). This common ancestor lived more than 500 million years ago, and has been extinct for millions of years. But its descendants, vertebrates and cephalochordates, still exist, and by studying them we can learn about the common ancestor. Speculating about the common ancestor's features will teach us more

about the features of the chordates: the basic body plan and the functions of their genes. This will help us find which features are ancient (and would be present in the common ancestor), and which have evolved since the lineage diverged, making them unique to vertebrates or amphioxus.

Homology and Gene Expression

Homologous features are characteristics shared by two or more organisms that the organisms' common ancestor had as well. For example, the wings of an ostrich are homologous to the wings of a sparrow because their common ancestor, an early bird, had wings. But, not all homologous features are as obvious as birds' wings; convergence occurs when structures that appear the same actually evolved independently in different organisms. The wings of a bat and the wings of a sparrow are not homologous because the common ancestor, an early tetrapod, had legs, not wings. The two wing structures evolved at different times in different, unrelated evolutionary lines. The convergence of these structures is supported by the fact that although both sets of wings have the same function, the internal structure is dissimilar. In order to help prevent mistaking convergent, or analogous, structures for homologous structures, we can study the genes that they express; during development, homologous structures often express homologous genes, which can be found by comparing gene sequences.

In cells that use a gene (i.e. by making proteins with it), the gene is said to be expressed. By finding and studying the expression of a gene in different organisms, we can compare the regions of expression and attempt to find

characteristics which support arguments of homology and to discover genes which each structure use during development.

The CRABP *Gene*

I studied a gene in amphioxus that is homologous to *CRABP* in hopes of learning more about the comparative anatomy of amphioxus and vertebrates, and possibly finding homologous structures. *CRABP* derives its name from the name of the protein that it is suspected to make: Cellular Retinoic Acid Binding Protein. The *CRABP* genes are interesting because studies of their function suggest that they affect the levels of retinoic acid, a substance which causes malformations when it is applied to the embryo in excess during development. In this section, I describe what these genes are thought to do during the development of vertebrates.

Retinoic acid, a form of vitamin A, is essential for the proper embryonic development of an organism (Wilson, 1953). However, excess retinoic acid, especially during development, can lead to severe deformities, notably posteriorization, an effect seen in both vertebrates and amphioxus (Holland and Holland, 1996 and Marshall, et al., 1992). Posteriorization occurs when anterior structures begin to take on posterior characteristics. An example of posteriorization is when the cells of the developing head region express "neck" genes, a more posterior structure; as a result, instead of forming a head, an extension of the neck develops (Figure 3). When excess *xCRABP* (a frog *CRABP* gene) is injected into a fertilized frog egg, the embryo develops similarly to the

way it would develop if treated with a high concentration of retinoic acid (Dekker, et al., 1994). So *xCRABP* seems play an important role in balancing levels of retinoic acid in the developing embryo. *CRABPI* and *CRABPII* are also *CRABP* genes, one or both of which have been cloned in mouse, chick, and human. In mouse, CRABPII seems to stimulate the production of retinoic acid receptors, which could bind retinoic acid and inactivate it, or facilitate the transport to its destination (Dong, et al., 1999). The function of CRABPI in mouse is less certain, but it has been suggested to hasten the break down of retinoic acid by directing it to enzymes that will degrade it (Boylan and Gudas, 1992 and Dong, et. al, 1999). Because the homologous genes in mouse have a large effect on retinoic acid levels, it is possible that CRABP affects retinoic acid levels in amphioxus as well.

CRABP *Expression*

The *CRABP* genes' expressions have been studied in the embryonic stages of several vertebrates, including humans, mice, frogs, and chickens. By finding the *CRABP* expression pattern in amphioxus and comparing the patterns to those of vertebrates, I have formulated a hypothesis describing which features of the common ancestor's *CRABP* gene, including function and expression domain, have been conserved. This will help explain how the *CRABP* genes have evolved since the lineage of vertebrates and cephalochordates diverged over 500 million years ago. The expression domains in amphioxus seem to correspond to some expression domains in vertebrates, and may be marking areas of homology. However, the gene is expressed in more regions in vertebrates than in amphioxus.

This disparity suggests that although there are homologous regions in these organisms, something, perhaps the function of the ancestor's CRABP or the ways in which it is used, has changed so that vertebrates use it in more regions.

Methods and Materials

cDNA Library Screening and Sequence Analysis

cDNA libraries contain fragments (copies of mRNA) of every gene that the organism is using at a specific point during development. By screening a cDNA library, we can see if amphioxus uses a gene during development.

It was important to sequence the cDNA of the gene so that it can be compared to other *CRABP* genes, and I can be more confident that I am working with a true *CRABP* homolog.

The gene is "carried" by a plasmid, a circular, double stranded DNA into which a foreign gene (one that does dot naturally occur in the plasmid) can be inserted (Figure 4). For the first sequencing reaction, I used primers that corresponded to the plasmid sequence ("primer #1" and "primer #2" in Figure 5). The primers attach to the plasmid, enabling the sequence to be obtained.

This reaction is only reliable for about the first 600 nucleotides; so a second reaction must be performed to read the complete sequence, which consists of about 1236 nucleotides.

I designed primers to read the second part of the sequence, which begins in the middle of the *CRABP* insert ("designed primer" in Figure 5). Once designed, the primers were manufactured by Keystone Labs. I then mixed them with the

CRABP plasmid and delivered them to the sequencing facility at the University of Oregon. Sequences were obtained in both directions, resulting in two complete complimentary strands of the gene. I then compared the two strands to ensure that the sequence I obtained was correct.

When the sequence was complete, I compared it with similar genes to confirm its homology to vertebrate *CRABP* genes. To accomplish this, I did what is known as the Basic Local Alignment and Search Tool (BLAST). BLAST searches a data bank of every published, and some unpublished, gene sequence, and reports back genes of similar sequences. After gathering the sequences similar to the gene I was studying in this fashion, I entered several of them into the Clustal X computer program, where I aligned the genes and made a phylogenetic tree using the Neighbor-Joining method. The phylogenetic tree provides a pattern for comparing the similar genes found in the BLAST search to amphioxus *CRABP*. Although it is only distantly related, it is important to include *Manduca*, an arthropod commonly called the tobacco worm, because it serves as the outgroup. The outgroup is the least related organism (or gene in this case) on the tree and is used to estimate an approximation of the ancestral condition. Ideally, the outgroup is as closely related to the organisms being studied as possible, but because *Manduca* is the only non-vertebrate in which CRABP has been cloned, until now. Therefore, it is the only option for an outgroup.

The alignment and tree were adjusted to account for gaps in the sequences (which may prevent the genes from aligning appropriately), and the numbers at the node of each branch reflect bootstrap, or confidence, values. As the bootstrap

number nears 1000, there is more confidence in the arrangement of the tree at that node, or branch. High bootstrap values are important because they give us more confidence in the entire tree. It is important for the tree to be correct so we know how each gene is related. For example, we need to know whether *CRABP* is more closely related to *CRABPI* or *CRABPII*, or equally related to both.

mRNA In Situ Hybridization

mRNA in situ hybridization is a procedure which labels the regions in an organism where a particular gene is expressed. This allowed me to compare the regions of *CRABP* expression in amphioxus with *CRABP* gene expression in vertebrates, and see if the expression domains could be homologous.

First, I made a probe to bind to the mRNA of the gene (a strand of nucleotides released from the nucleus when the gene is being expressed). To make the probe, I cut the plasmid containing *CRABP* using the EcoR1 enzyme, then added free nucleotides (ATPs, UTPs, GTPs, and CTPs, the building blocks of DNA) for the polymerase (the enzyme that constructs copies of the mRNA) to use during replication. The UTPs in the free nucleotides were manufactured with digoxygenin (DIG) labels. The complete probe is a mixture of many strands of cDNA which correspond to the *CRABP* mRNA and are labeled with DIG.

When I added the probe to a sample of embryos, it penetrated the cell membranes and bound to the *CRABP* mRNA within the cells (Figure 6). I used embryos which had been fixed in formaldehyde during certain, interesting stages of embryonic development and stored in methanol. After incubating the embryos

with the probe, I added a DIG* antibody that had been manufactured with alkaline phosphatase, an enzyme, attached to it. The antibody binds to the DIG on the probes, so when I added a coloration, alkaline phosphatase catalyzes the reaction between the chemicals in the solution, creating a blue precipitate as a product. Because the enzyme is only located on *CRABP* mRNA, the blue precipitate marks the areas of gene expression. I then mounted the on slides with glycerol for viewing under a compound microscope.

I performed this procedure on about ten to thirty embryos from each of the following stages: 4, 9, 10, 11, 12.5, 16, 17, 18, 18.5, 19, 20, 21, 23, 25, 28, 30, 32, 33, 34, 35, 45, and 58 hours of development. Because my sequence analysis suggested that CRABP is equally related to CRABPI and CRABPII, I compared the expression pattern in amphioxus to both the expression patterns of the homologous vertebrate genes, which often overlap.

Results

Sequence Results

Finding the sequence of *CRABP* helped me decide if it was homologous to *CRABP* genes in other animals. To obtain the sequence, I designed appropriate primers and the gene was sequenced at the University of Oregon sequencing facility (Figure 7). Using a computer program I found the known genes most similar to amphioxus *CRABP* and created a phylogenetic tree. The phylogenetic tree shown in Figure 8 displays the relationship of amphioxus *CRABP* to reported *CRABP* genes, using the aligned, conserved regions of the translated amino acid sequences. Amphioxus is located between insect (*Manduca*) and vertebrate *CRABP* genes, supporting that it is a *CRABP* homolog as well. Amphioxus *CRABP* is equally distant from both *CRABPI* and *CRABPII* on the tree; thus, I named it without a numeral designation.

CRABP *Expression*

The expression pattern shows which cells are using *CRABP* (making the CRABP protein). Knowing this, I can compare it to expression patterns of *CRABP* in vertebrates, and hypothesize which structures are homologous.

The embryos that were preserved after developing for 12.5 hours, were the youngest to express *CRABP*. This early expression pattern is in the rostrum of the embryo (Figures 9, 10, and 11). Each embryo I studied between 12.5 and 58 hours of development (the latest stage that I studied) showed labeling in this region. It is difficult to tell which cells are expressing *CRABP* because the

expression domain seems to be located between the ectoderm and endoderm layers of the embryo, an unusual result of mRNA in situ hybridization. The expression may be labeling a layer of mesenchymal cells, which would be very small and may not have been noticed before. If there is not a mesenchymal layer, the endoderm is probably expressing *CRABP* because the majority of embryos show little or no expression in the ectoderm, while the endoderm is slightly darker.

There is also bilateral labeling in a few cells posterior to the neuropore, located in the posterior part of the cerebral vesicle (an expansion of the neural tube) (Figures 9, 10, and 11). The expression begins by 16 hours of development and continues with little change through 58 hours. However, by 58 hours, the domain seems to be moving ventrally (towards the floors of the neural tube) (Figure 11), which may be a result of a structure called the lamellar body forming above it and displacing it downwards (Thurston Lacalli, personal communications).

By 30 hours, expression begins in the region that will develop into the mouth. The expression begins as a diffuse cluster of cells, and when the mouth opens, expression forms a circle, several cells wide, about one cell from the opening (Figures 10 and 11). This circle of expression is present through 58 hours of development, the latest stage examined.

Discussion

Sequence Analysis

Using the amphioxus CRABP sequence I obtained, I made a phylogenetic tree of the representative members of the *CRABP* gene family. The tree shows how closely related the genes are to each other and provides a model for comparing vertebrate and cephalochordate *CRABP* genes. It also reveals the two subgroups of the *CRABP* gene family in vertebrates (Figure 8). Two *CRABP* genes have been cloned in mouse embryos (Ruberte, et al., 1992); they are represented on the phylogenetic tree as "Mouse *CRABPI*" and "Mouse *CRABPII*," respectively. Genes that are this similar in one organism are probably paralogs, structures or genes within the same organism that evolved by the duplication of a single ancestral gene. The grouping of vertebrate *CRABP* genes on the tree suggest that a *CRABP* duplication event occurred fairly early in the vertebrate line (as opposed to occurring only in the mouse line). This would account for the two groups of *CRABP* homologs within the vertebrate section of the tree. This also suggests that the duplication happened after the cephalochordate and vertebrate lines split; the common ancestor probably had only one copy of *CRABP*. A similar idea has been proposed by several biologists using data from other genes. Amores, et al. suggested that the entire vertebrate genome was duplicated early in the vertebrate line (1998). This could account for the duplicate copies of *CRABP* and several other vertebrate genes, like *Hox* genes, that do not always have duplicates in non-vertebrates.

The duplication theories raise the question of whether amphioxus may have more than one *CRABP* homolog. Previous comparisons of amphioxus to vertebrates show that genes which have been duplicated in vertebrates usually seem to have only one copy in amphioxus. When a duplicated gene is found in amphioxus, they appear to be due to an independent, single gene duplication event in the cephalochordate line (reviewed in Jackman, et al., 2000). The fact that only one copy of the gene was found in the cDNA library may indicate that there are no duplicates of *CRABP* in amphioxus. However, screening *CRABP* genes in several different amphioxus cDNA libraries could potentially isolate other *CRABP* genes. Ultimately, this question cannot be answered until the entire amphioxus genome is sequenced.

The phylogenetic tree including amphioxus can be used to help classify newly discovered vertebrate genes. *xCRABP*, which isolated in the frog (*Xenopus*), was found to be a member of the *CRABP* family, as expected (Dekker, et al., 1994). However, the gene is much larger than the average lengths of *CRABPI* and *CRABPII*, and the expression pattern is unlike other patterns seen in vertebrates. For these reasons, researchers thought that they may have cloned a novel *CRABP* that does not seem to fit into either the *CRABPI* or the *CRABPII* subdivision. Placing *xCRABP* on the phylogenetic tree including vertebrates and amphioxus, shows that *xCRABP* (labeled Frog *xCRABP* in Figure 6), like the other *Xenopus CRABP* gene cloned, probably evolved after the original duplication of *CRABP*, and appears to be more closely related to *CRABPII*.

Homologous Structures

mRNA in situ hybridization of amphioxus *CRABP* in 12.5 to 58 hour embryos revealed some cells in the developing rostrum, which appeared to label the area between the ectoderm and the endoderm (Figures 9, 10, 11). There is expression in a similar region of vertebrates; the frontonasal mesenchyme is labeled by both *CRABPI* and *CRABPII* mRNA of frog, chick, and mouse (Decker, et al., 1994; Maden, et al., 1991; Ruberte, et al., 1992). A mesenchyme is a layer of loosely organized cells which forms by the disaggregation of ectodermal or mesodermal tissues. Amphioxus is thought to have little or no mesenchyme, but this cryptic layout of cells near the rostrum which express *CRABP* may be a small layer of mesenchymal cells. If this is true, it is possible that the cells are homologous to the cells in the frontonasal mesenchyme in vertebrates, which develops into the connective tissue of the nose.

If the mesenchymal layer is not present in amphioxus, and the labeling is in the ectoderm of the rostrum, there still may be an argument for homology between this area and the frontonasal mesenchyme. In vertebrates, the mesenchyme in this region is derived from neural crest cells which migrate from the midbrain (Ruberte, et al., 1992). The posterior cerebral vesicle, which has been hypothesized to be homologous to part of the midbrain (Lacalli, et al.,1994), is near the rostrum of the embryo during early development, before the embryo elongates into more of an oval-like shape (data not shown). The cells of the cerebral vesicle, and the ectoderm of the rostrum could have developed from the same, or adjacent groups of cells. If so, it is conceivable that the anterior ectoderm is homologous to the

midbrain neural crest of vertebrates, which develops into the frontonasal mesenchyme. However, this hypothesis is only plausible if the CRABP expression domain is indeed in the ectoderm, and the posterior cerebral vesicle is sufficiently close to the rostrum during early development, both of which are still uncertain.

The mRNA in situ hybridizations also marked a few cells in the posterior cerebral vesicle (Figures 9, 10, and 11). Because the embryos and larvae that I examined were very young, it is difficult to distinguish what these cells might become. They may become the most anterior pair of dorsal bipolar cells (neurons recognizable in the 12 day amphioxus larvae) (Thurston Lacalli, personal communications). The fate of these cells could be supported if older embryos showed *CRABP* expression in pairs of more posterior neural cells, showing that all dorsal bipolar cells express *CRABP* during development, and we are seeing only the beginning of the dorsal bipolar cell development in the 18 to 58 hour embryos. Creating a fate map of amphioxus, which follows cells through development to see what they become, would be the ideal way to determine the late identity of these early *CRABP*-expressing cells. If the labeled cells do become the dorsal bipolar cells, they may be homologous to the optic tectum in the midbrain of vertebrate embryos (Lacalli, et al., 1994). *CRABP* is expressed in the midbrain of mice, frogs, and chick embryos (Decker, et al., 1994; Maden, et al., 1991; Ruberte et al., 1992). Considering this, it would make sense that a homologous structure in the area homologous to the midbrain of vertebrates would express *CRABP* as well.

CRABP expression begins in the ectoderm near the mouth before the mouth opens, beginning at about 30 hours (Figure 11). By 34 hours, the mouth is

beginning to open, and there is one non-*CRABP*-expressing cell layer between the circle of *CRABP* expression and the opening (Figures 10 and 11). This pattern continues as the mouth grows through 58 hours. The homology of the amphioxus mouth to the vertebrate mouth is unclear (Willey, 1894). An alternate theory is that the mouth of amphioxus is homologous to gill slits in vertebrates, which develop between the branchial arches. However, the early gill slits in amphioxus, which develop by 58 hours, do not appear to express *CRABP*, an observation which leads me to examine the hypothesis that the mouths of amphioxus and vertebrates are homologous. The mouth of vertebrates does not express *CRABP*; however, the maxillary arch, a mesenchymal tissue derived from ectoderm, which forms a section of the upper jaw (maxillary bone) (Hopper and Hart 1985), is marked (Maden, 1991) . The tongue, another mesenchymal tissue derived from neural crest tissues (ectodermal) and mesodermal tissue, is also marked in mouse (Ruberte, 1992). The expression in the ectoderm surrounding the mouth of amphioxus could share homology with either the tongue, the maxillary arch, or both structures in vertebrates.

Function of CRABP

The expression patterns of *CRABP* may be compatible with the hypothesis that the function of CRABP in amphioxus is to regulate the concentration of retinoic acid within certain tissues. Excess retinoic acid applied to both developing vertebrate and amphioxus embryos causes posteriorization (Holland and Holland 1996 and Marshall, et. al, 1992). CRABP may protect the anterior region from posteriorization by effectively lowering the concentration of retinoic acid, while still ensuring that enough is present for proper development. Also, amphioxus treated with retinoic acid develop unusually small mouths, and, if the treatment is strong enough, fail to develop a mouth. CRABP may help regulate the concentration of free retinoic acid in the mouth region, protecting it from the teratogen and allowing the mouth to develop normally. When excess retinoic acid is added, the effect of CRABP is overwhelmed and the mouth does not form properly. CRABP's regulating effect on retinoic acid may help the posterior cerebral vesicle develop as well, as embryos treated with retinoic acid show a shortened cerebral vesicle (Holland and Holland 1996). CRABP's function may be to help maintain an appropriate concentration for this region. An interesting point, which lends credence to the hypothesis that the posterior cerebral vesicle is homologous to part of the midbrain in vertebrates, is that the midbrain fails to develop in frogs and chicks that are treated with retinoic acid (Durston, 1989 and Sundin and Eichele, 1992). Both structures are highly sensitive to retinoic acid and express *CRABP*, similarities which support the hypothesis that they are homologous.

Like amphioxus *CRABP*, *CRABPI* and *CRABPII* in vertebrates are both expressed in regions that are highly susceptible to the effects of retinoic acid. This similarity suggests that the role of CRABP may have remained stable throughout the evolution of amphioxus and vertebrates. This characteristic is important to consider when proposing homology between expressing tissues because a conserved function may increase the likelihood that the regions in which the gene is expressed have been conserved also.

CRABP may help form a gradient of retinoic acid within the embryo, like the gradient of retinoids present in the *Xenopus laevis* (frog) embryo (Chen, et al., 1994). The concentration gradient in frog is thought to be responsible for neural transformation and providing positional cues during development. In amphioxus, this gradient could play a role in the regional organization of the neural tube. Although *CRABP* mRNA (which is labeled by the in situs) does not appear to be in a gradient, the protein may be, with areas of high CRABP concentration possibly corresponding to areas of low concentrations of retinoic acid. CRABP may also work with other proteins to establish a gradient of retinoic acid, aiding the embryo in proper development.

However, it is unlikely that the function of CRABP has been entirely conserved. First, the extended area of expression in vertebrates may signify that the gene has been recruited to new areas of expression, or that the gene's expression domain could have been reduced in amphioxus. This change in the number of expression domains may be a sign of the gene's function changing, or the body plans changing enough that the original gene is needed, or not needed, in

different areas. Second, CRABPI and CRABPII appear to have different functions (See *Introduction*). They both involve retinoic acid, but affect it in different ways. The function of amphioxus CRABP may resemble either CRABPI or CRABPII, or it may perform a different function that may or may not involve retinoic acid.

Future research

There is still some uncertainty in which tissue layer expresses *CRABP* in the rostrum of amphioxus. Making serial sections of embryos that have been labeled through mRNA in situ hybridization would allow researchers to look more closely at this area, possibly determining which tissue layer is labeled, and if there is a small mesenchymal layer in this region. This would help ascertain what this area is homologous to in vertebrates, and if it is related to the neural crest cells of the midbrain.

It would also be helpful to find the expression pattern of *CRABPI* and *CRABPII* in fish, which have gill slits through adulthood, so that the later stages of gill slit development could be checked for the expression of *CRABP* homologs. I believe that expression would not be found in this area, as the early gill slits have not been found to express *CRABP* in any vertebrates that have been examined. However, if they are labeled, this would make an argument for homology between the mouth of amphioxus and the gill slits of vertebrates.

A huge barrier to the progress of research on amphioxus is that the organisms cannot, as of yet, be bred in a laboratory. This limits researchers to doing experiments during the natural breeding season of amphioxus, which must

be done at the site of amphioxus collection, away from their laboratories. The organisms can be preserved for future experiments, like mRNA in situ hybridization. But, if a method to raise amphioxus in the lab was found, a wide range of experiments which are currently done on other organisms, could be performed on amphioxus. For example, the effect of retinoic acid on *CRABP* expression could be studied, which may help determine how *CRABP* is affected by retinoic acid in amphioxus.

Researchers could also discover how an amphioxus develops without *CRABP*, a procedure commonly done in several organisms. Mice that are deficient in either *CRABPI*, *CRABPII*, or both, are essentially normal. They have normal life spans, reproductive capabilities, responses to retinoic acid treatments, and anatomy (aside from limb outgrowth on some forelimbs, which is also an effect of treatment with retinoic acid) (Lampron, 1995). This may suggest that the function of *CRABPI* and *CRABPII* is only important under extreme conditions, like starvation. Amphioxus may show a similar result, or lack of result, to a *CRABP* deficiency, suggesting that the function of CRABP has been highly conserved.

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Figure Legends

Figure 1
Organisms which seem very diverse as adults may actually share many characteristics, especially during development. These illustrations of vertebrate embryos display the similarities of their body plans. (Drawings by Charles Kimmel)

Figure 2
Phylogenetic trees are helpful in displaying the relationship of organisms. This tree shows the relationship of amphioxus to some familiar animals. Note the asterisk, which marks where the common ancestor of amphioxus and vertebrates would be placed on the tree.

Figure 3
Retinoic acid levels must be balanced for an embryo to develop properly. A) a normal *Xenopus* larva B) a *Xenopus* larva treated with excess retinoic acid during development and showing signs of posteriorization. (Figure from Durston, et al., 1989)

Figure 4
The *CRABP* gene cDNA was inserted into a double-stranded plasmid to make working with the unknown sequence possible. (Figure from Watson, et al., 1992)

Figure 5
Primers were used to obtain the sequence of the gene within the plasmid. The binding sites of the primers on the plasmid (Primer binding site #1 and #2) and on the inserted gene (Designed Primer binding sites) are depicted here. Each circle represents a strand of the double stranded plasmid. Both strands were sequenced to ensure that the correct sequence was obtained.

Figure 6
mRNA in situ hybridization uses a blue precipitate to labels\ the cells in the embryo which are expressing a particular gene. This illustration shows the steps performed during this procedure. (Figure adapted from Boehringer Mannheim's 1997 Biochemical Catalog)

Figure 7
The sequence of the amphioxus *CRABP* cDNA. This sequence was compared to other CRABP sequences and placed in a phylogenetic tree.

Figure 8
The phylogenetic tree relating amphioxus *CRABP* to selected *CRABP* genes from other animals. Note the two subgroups of vertebrate *CRABP*. The branches are

labeled with the name of the gene as it was published, and the common name of the animal from which it was cloned.

Figure 9
Photographs of amphioxus embryos displaying the *CRABP* expression pattern. The anterior of the embryo is pointed left. A) a side view of a 16 hour embryo showing the anterior expression domain. B) a dorsal view of a 16 hour embryo showing the anterior expression domain. C) a side view of a 21 hour embryo showing cerebral vesicle and rostral expression. D) a dorsal view of a 20 hour embryo showing the bilateral cerebral vesicle expression and the rostral expression. E) a 32 hour larva showing diffuse labeling in the region where the mouth will form, and still exhibiting the expression domains in the cerebral vesicle and the rostrum. F) a 34 hour embryo showing a circle of expression around the developing mouth. Labeling in the cerebral vesicle and the rostrum is also present. G) A 45 hour embryo, which displays the ventral movement of the cerebral vesicle expression when compared to earlier stages. The rostral expression is faint in this specimen, but all three expression domains are still present (the mouth is out of the plane of focus).

Figure 10
An Illustration of a 20 hour amphioxus embryo showing *CRABP* mRNA labels in the posterior cerebral vesicle and the rostrum. Abbreviations are as follows: ra, rostrum expression; cv, cerebral vesicle; np, neuropore; cve, cerebral vesicle expression; nt, neural tube; not, notochord.

Figure 11
An illustration of a 36 hour amphioxus larva showing *CRABP* mRNA labels in the posterior cerebral vesicle, the rostrum, and in a circle around the mouth. Abbreviations are as follows: ra, rostrum expression; cv, cerebral vesicle; np, neuropore; cve, cerebral vesicle expression; nt, neural tube; not, notochord; m, mouth; me, mouth expression.

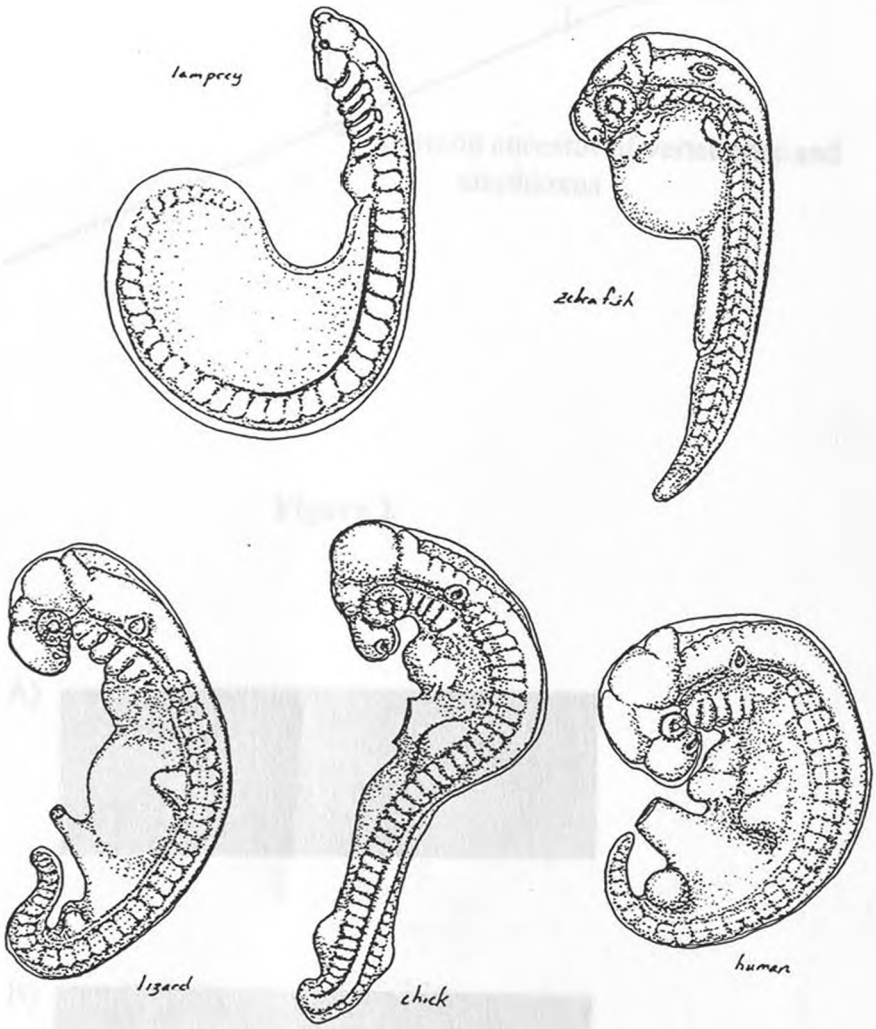


Figure 1

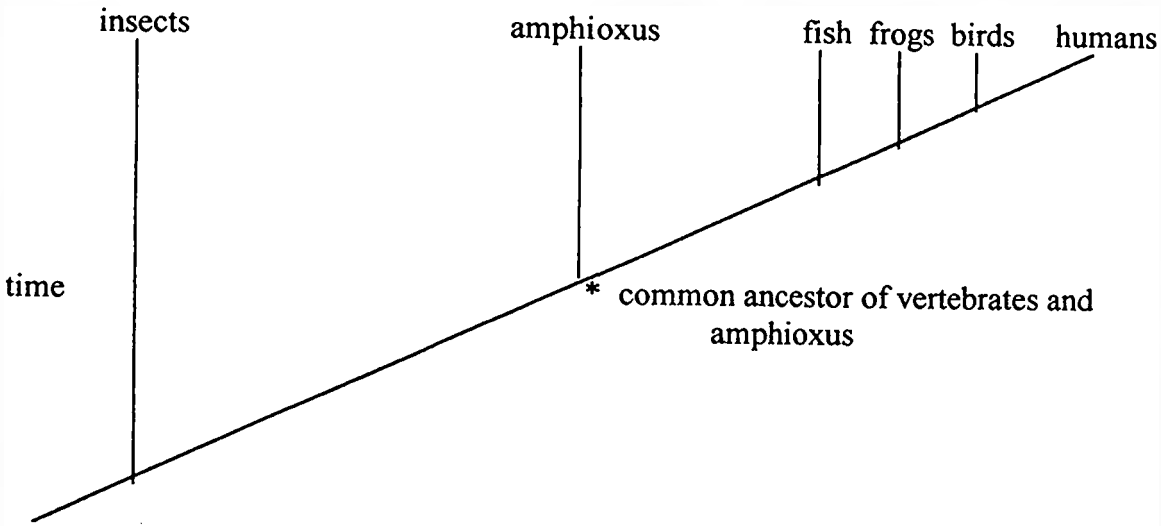


Figure 2

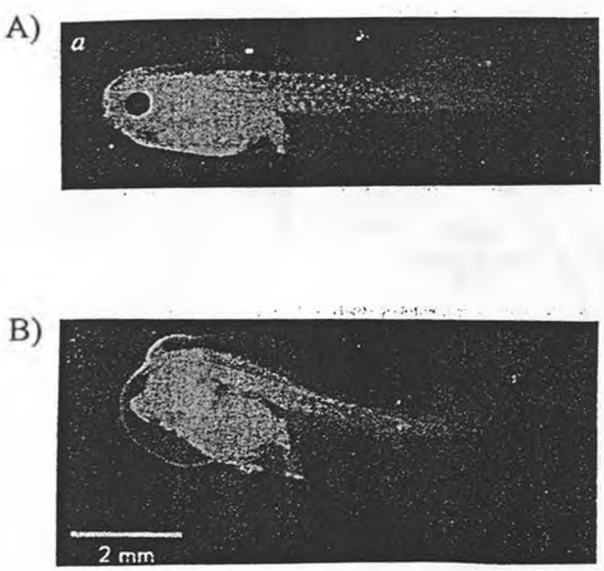
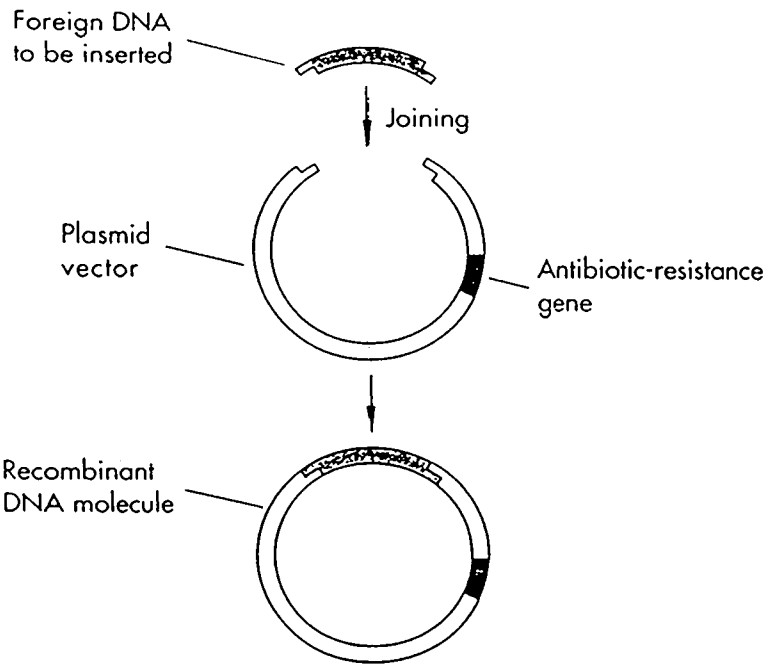


Figure 3



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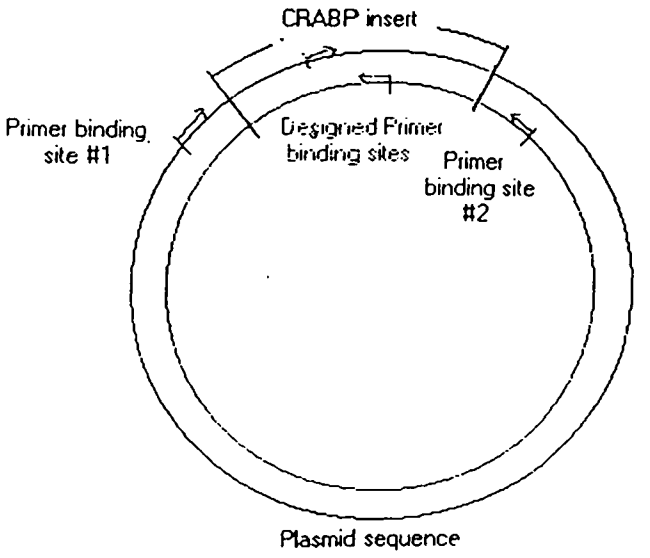


Figure 5

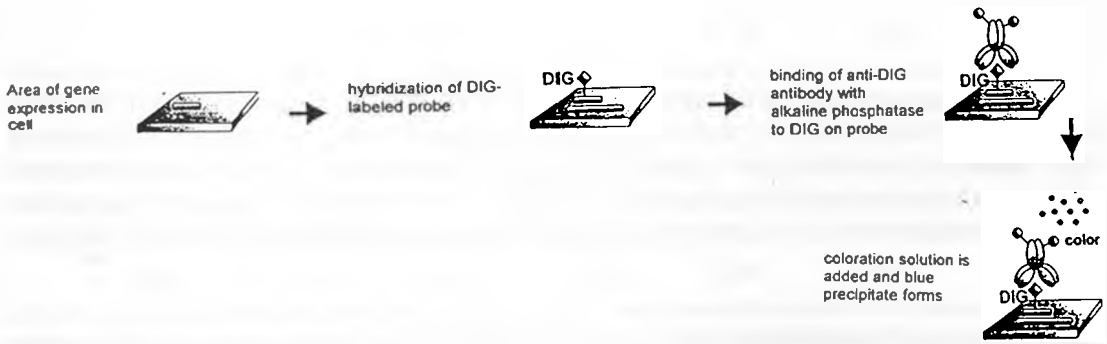
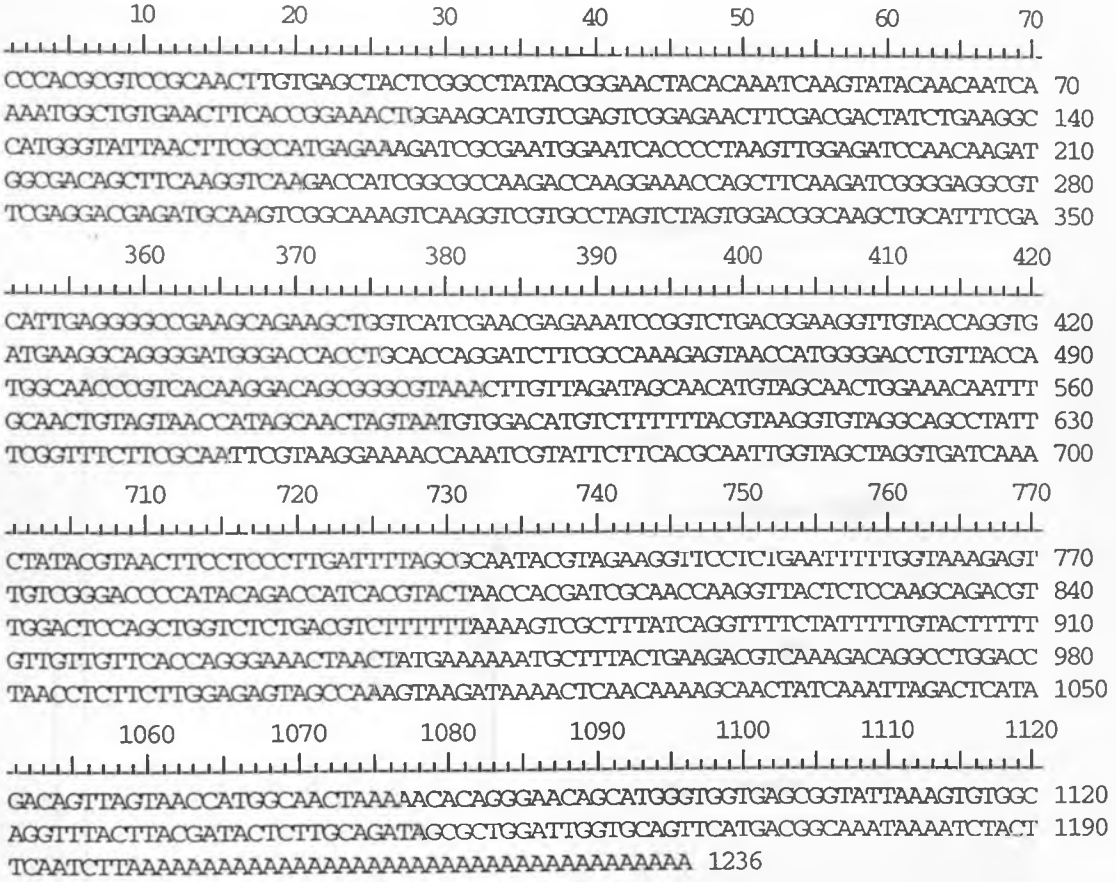

Area of gene expression in cell
hybridization of DIG-labeled probe
DIG
binding of anti-DIG antibody with alkaline phosphatase to DIG on probe
DIG
color
coloration solution is added and blue precipitate forms
DIG

Figure 6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360 370 380 390 400 410 420
CATTGAGGGGCCGAAGCAGAAGCTGGTCATCGAACGAGAAATCCGGTCTGACGGAAGGTTGTACCAGGTG 420
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710 720 730 740 750 760 770
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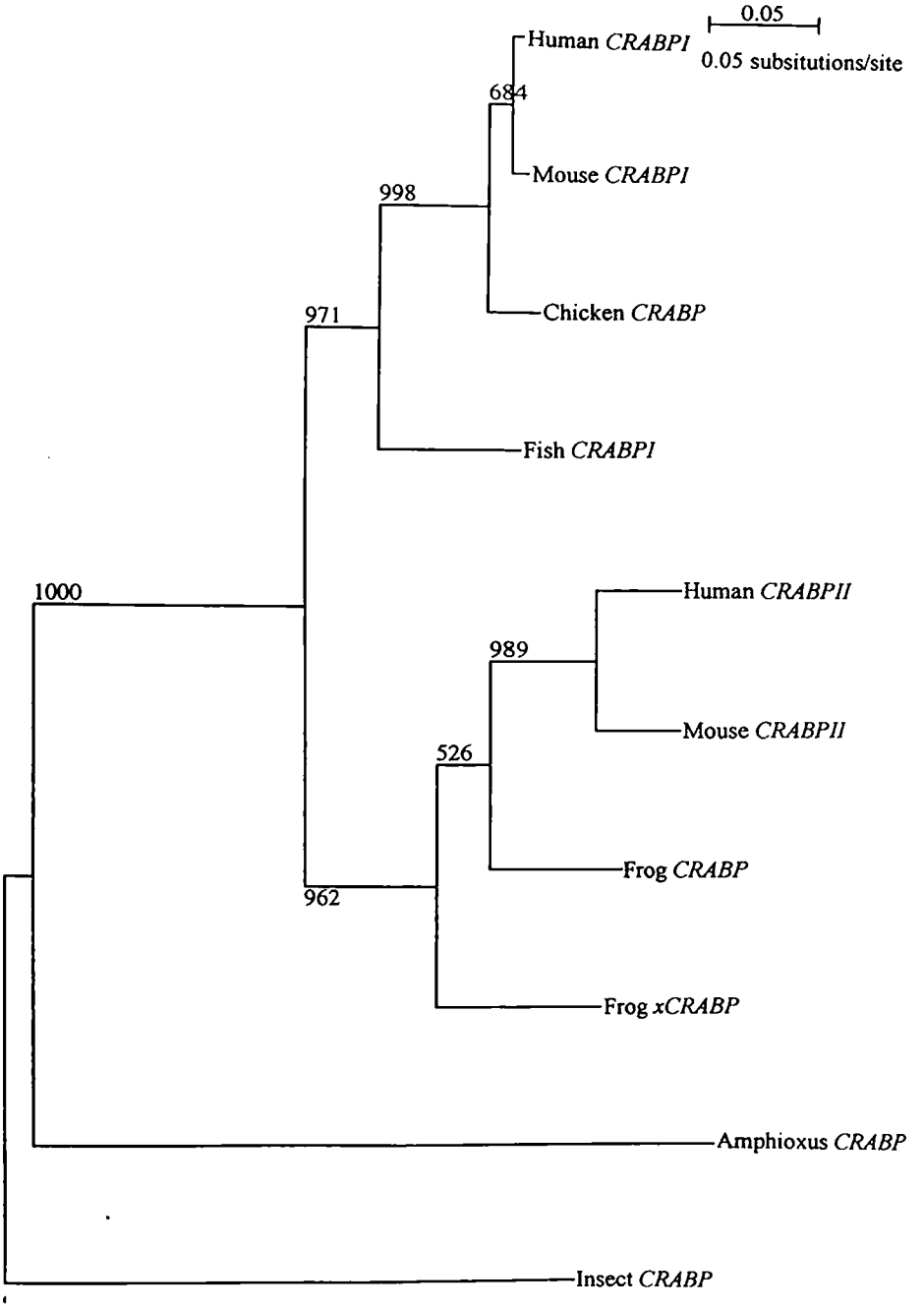
Figure 7


0.05
0.05 subsitutions/site
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Mouse CRABPI
998
Chicken CRABP
971
Fish CRABPI
1000
Human CRABPII
989
Mouse CRABPII
526
Frog CRABP
962
Frog xCRABP
Amphioxus CRABP
Insect CRABP

Figure 8

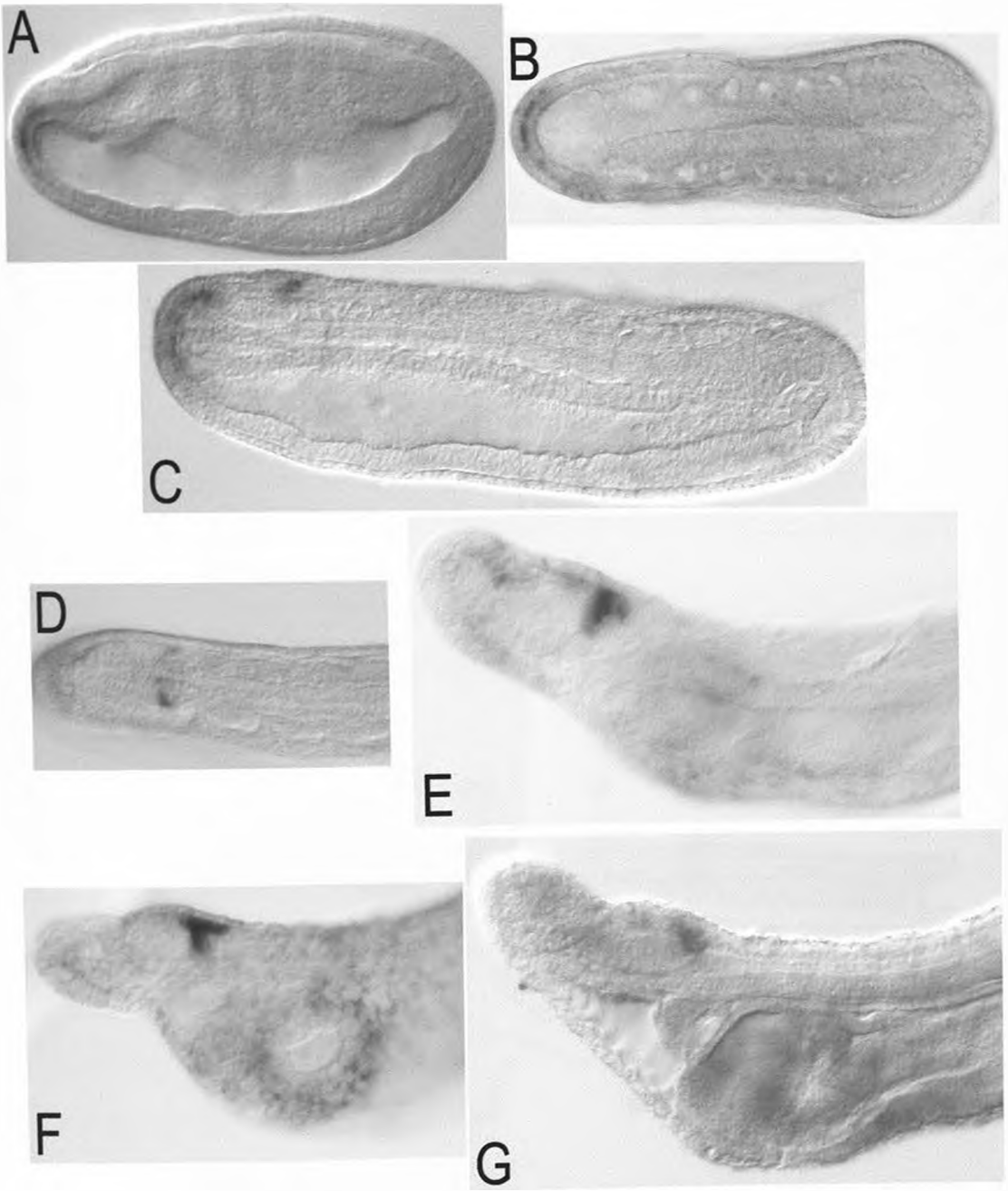

Figure 9

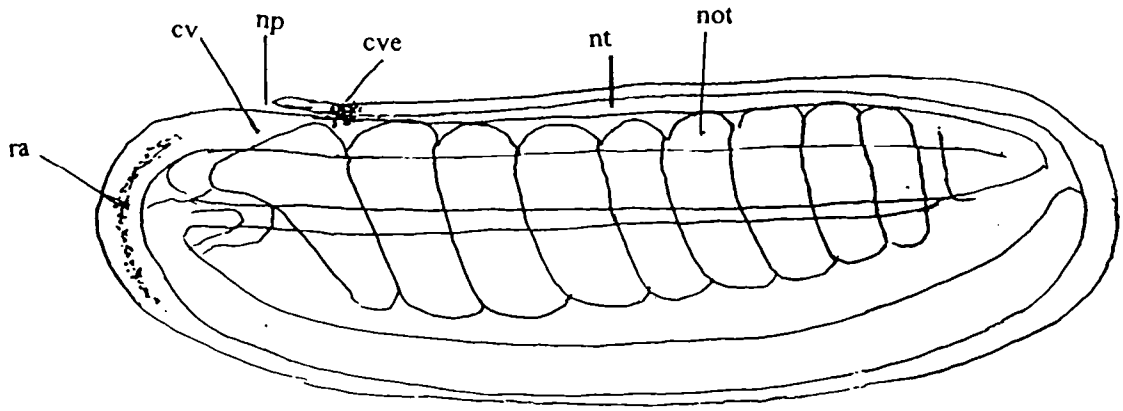

Figure 10

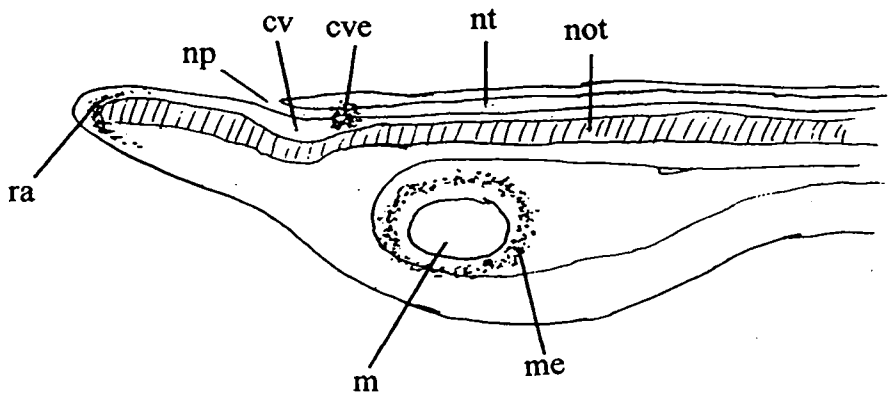

Figure 11