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THE ISOLATION AND CHARACTERIZATION OF HOMEO BOX SEQUENCES IN THE MEXICAN AXOLOTL *AMBYSTOMA MEXICANUM*

BY

Mary Whiteley

A thesis submitted to the School of Graduate Studies and Research of the University of Ottawa in partial fulfillment of the requirements for the degree Doctorate in Philosophy in Biology

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Abstract

The homeo box is a 180 base pair DNA sequence that is found in some genes of Drosophila which are crucial to its embryogenesis. Hence, this sequence has been used extensively as a probe to search for genes which may play a role in the development of vertebrates. Sequences with homology to Drosophila homeo boxes have been found in a number of vertebrate species including mouse, man and frog.

The purpose of this thesis was to clone homeo box sequences from the Mexican axolotl, an animal which has had a distinguished history in the study of development. Clones cross-hybridizing with the Drosophila Antennapedia homeo box were obtained from a partial genomic library enriched for such sequences. One clone (pAhox1) contained a homeo box sequence (Ahox1) which is 66% homologous to the Antp sequence, and is most closely related to the mouse Hox-1.6 homeo box (84% identity). Total RNA from several embryonic stages was probed with a 560 bp KpnI fragment of pAhox1 containing the homeo box (HB-Kpn). This showed that transcripts hybridizing to this sequence were detected only in neurula, and tail bud axolotl embryos, suggesting that this sequence is developmentally regulated. Tissue specific expression of Ahox1 was examined in dissected neurula and

tail bud embryos. In the neurula, transcripts hybridizing to Ahox1 were found to be restricted to the posterior two thirds of the neural plate and in tail bud embryos from the anterior margin of the gill bulge to the pronephric duct.

Another clone (pAhox2) obtained from this library contained a partial homeo box sequence, and its homology to the Antp sequence was found to reside in the last 60 nucleotides of the homeo box, a region thought to be responsible for DNA binding.

One powerful method to study gene function in vertebrates has been the introduction of cloned DNA sequences into developing organisms by microinjection. Expression of exogenous sequences in fertilized axolotl eggs were therefore examined, by using constructs consisting of a eukaryotic promoter, fused with the E. coli lacZ coding sequence which could be detected by histochemical staining. Expression from several eukaryotic promoters was observed, and the strongest expression observed was with the mouse hsp68 promoter.

In order to examine the possible role of Ahox1, a construct was prepared with HB-Kpn fused to the mouse hsp68 promoter. When this construct was microinjected into fertilized axolotl eggs, defects in the anterior neural tissues were observed. These were first observed in the neurula stage of development, when Ahox1 expression is first

observed. The abnormalities observed in these embryos were in neural tissues not normally expressing Ahox1.

RESUME

Le "homeo box" est une séquence de 180 pb de l'ADN conservée dans certains gènes de drosophile qui sont important pour son développement. Donc, cette séquence a été très utilisée comme une sonde pour chercher des gènes qui seraient importants dans le développement des vertébrés. Des gènes avec un "homeo box" similaire a celui de drosophile ont été indentifiés chez plusieurs vertéebrés comme l'homme, la souris, et la grenouille.

Le but de cette thèse a été de cloner des séquences "homeo box" chez l'axolotl, un animal qui joue un rôle important dans l'étude du développement. Ces clones ont été obtenus d'une génothèque enrichie pour ces séquences. La génothèque a été criblée avec une sonde "homeo box" du gène Antennapedia de drosophile. Un de ces clones (pAhox1) contenait une séquence 66% homologue a celle de Antp. Comparée à toute les autres séquences de "homeo box" connues, celle de Ahox1 est plus homologue à celle de Hox-1.6 de la souris. Les transferts Northern de l'ARN total de plusieurs stades des embryons ont demontrés des transcripts specifiques durant les stades neural et bourgeon caudal. Donc, la régulation de ce gène est affectée par le développement.

Un autre clone obtenu de cette génotèque contient une sequence de "homeo box" partiellement homologue à la séquence de Antp dans les derniers 60 pb a l'extrémité 3' du "homeo box". Cette region du "homeo box" est suppposée être responsable pour les interactions régulatoire avec l'ADN.

Une méthode importante pour étudier la fonction des gènes chez les vertébrés est la microinjection des séquences clonées dans les embryons. Donc, l'expression de ces séquences ont été examinées dans les oeufs fertilisé des axolotls, aves des plasmides qui contenaient un promoteur eucaryotique, fusioné avec le gene lacZ de E. coli. On peut observer cette expression facilement avec un colorant histochimique. L'expression de plusieurs promoteurs a ete examinée et celle la plus forte a été observée avec celle du gene hsp68 de la souris.

Le rôle de Ahox1 durant le devéloppement a été examiné par la localisation de transcripts dans les embryons dissequés. Au neurale, les transcripts ont été trouvés dans la région (2/3) posterieur de la plaque médullaire.

Pour clarifie davantage le rôle de Ahox1 un construit fait du promoteur hsp68 de la souris joint avec un fragment (HB-Kpn) génomique qui contenait le "homeo box" de Ahox1 à été microinjecté dans les oeufs fertilisés des axolotls. Les résultats de cette experience ont demontrés

que les embryons avec ce clone sont défectueux dans les tissus neurals antérieurs. Ces defauts ont été observés premièrement au stade 15 quand l'expression de Ahox1 débute. Aussi les defauts ont été observés dans les cellules qui normalement n'expriment pas Ahox1.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER III

CHAPTER IV

ABBREVIATIONS

bp	base pair
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetate
µg	microgram
kb	kilobase (1000 bp)
mL	milliliter
PBS	phosphate buffered saline
pfu	plaque forming unit
PVPD	polyvinylpyrolidone
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	sodium citrate saline
Tris	tris(hydroxymethly) aminomethane

CHAPTER 1

INTRODUCTION

Developmental biology attempts to understand the processes by which a fertilized egg gives rise to a complex multicellular organism, composed of many different cell types organized into a specific body plan. How the body plan is formed (pattern formation), and how the different cell types arise from the interaction of cells brought together in close proximity during development (embryonic induction), are still largely unanswered questions.

Simple organisms, such as the fruit-fly (Drosophila melanogaster), have been popular for the study of development. The reasons for this are their short generation time, many progeny, and relatively small genome. In addition, there are many developmental mutants of Drosophila. More recently, molecular approaches to the study of development in this organism have given rise to a wealth of information concerning the interaction of genes during development. One of the most important discoveries was that some developmentally important genes contain a conserved 180 base pair DNA sequence called the homeo box (McGinnis et al., 1984a; Scott and Weiner, 1984). This sequence was subsequently found in a number of organisms ranging from sea urchin (Dolecki et al., 1986), to mouse

(McGinnis et al., 1984c; Colberg-Poley, 1985), frog (Carrasco et al., 1984; Muller et al., 1984) and man (Levine et al., 1984).

The study of development by combining molecular analysis with traditional techniques is currently an exciting area of research. Undoubtedly, this research will aid in our understanding of the complex processes of development.

A. The Mexican axolotl and development

Among vertebrates, the Mexican axolotl (Ambystoma mexicanum) has had a distinguished history as an experimental animal for studying development. Like other amphibians, it is particularly well suited for developmental studies in that a large number of eggs are produced which develop external to the mother, and embryos can be manipulated by microdissection and analyzed by standard biochemical techniques. In addition there are some 30 developmental mutants of the axolotl (reviewed by Armstrong, 1985).

The Mexican axolotl is a neotenous salamander, that maintains its larval characteristics while reaching sexual maturity. Consequently, the axolotl spends its adult life in water and is, therefore, easy to maintain in the laboratory. In addition, eggs can be obtained year round. The main disadvantage of the axolotl is its long generation time.

Like other urodeles, the axolotl has a large genome (ten times the size of the human genome). For molecular studies, this may appear to be a drawback. However, reassociation kinetics of genomic DNA suggests that the increase in genome size is not due to an abundance of highly repetitive DNA, but rather to an increase in all three classes of DNA (repetitive, moderately repetitive and unique) (Straus, 1971; Baldari and Amaldi, 1976).

At present, a great deal has been learned about the development of the axolotl, however, little is known about the molecular biology of its development. Therefore, the aim of this thesis was to clone developmentally relevant genes from the axolotl. One approach to cloning developmental genes in vertebrates has been to use homeo box probes from D. melanogaster. Success using this approach in other vertebrates indicated that it could be applied to the axolotl. One of the aims of this thesis was to clone homeo box sequences from the axolotl. One such sequence was isolated and characterized.

In addition to the cloning of developmental genes, it is important to be able to determine their role in development. To date, one of the difficulties in studying homeo box-containing genes in vertebrates has been the lack of assignment of mutants to these genes. One of the most powerful methods to study gene expression during development has been the re-introduction of cloned DNA sequences into developing embryos. Expression of exogenous DNA sequences

in the axolotl was therefore examined. Such sequences were expressed, thus establishing the usefulness of the axolotl for the study of gene expression during development. The role of a homeo box-containing gene in the axolotl was examined by microinjection of a construct consisting of a mouse heat shock promoter fused to an axolotl genomic DNA fragment containing a homeo box.

B. The homeodomain

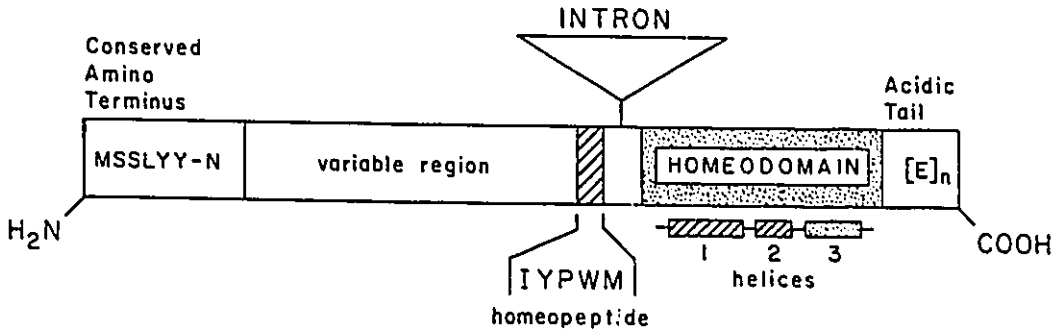
Soon after the discovery of the homeo box, the protein domain (called the homeodomain) encoded by this sequence was found to have homology with DNA regulatory proteins from yeast and bacteria (Laughon and Scott, 1984; Shepherd et al., 1984). Of particular interest was that this sequence shared some similarity to the proteins from the yeast mating type (MAT) genes. These genes are involved in the differentiation of yeast into different cell types (mating types α or a, or spores). The most conserved sequence beween the homeodomain and other regulatory proteins is the helix-turn-helix motif which is involved in DNA binding. This motif is found in the C-terminal half of the homeodomain. Antibodies directed against homeodomain proteins from Drosophila have been localized in the nucleus (DiNardo et al., 1985; Macdonald and Struhl, 1986), suggesting that these proteins have a regulatory function. In addition, in vitro DNA binding activity had been demonstrated for the

homeodomain protein from the engrailed gene of Drosophila (Desplan et al., 1985; Desplan et al., 1988).

The homeodomain constitutes part of a larger protein. The general features of a an Antp-like homeodomain protein are given in Fig. 1.1. The homeodomain is usually located in the C-terminus of these proteins. In general, there is little conservation of the primary protein sequence outside of the homeodomain. As noted in Fig. 1.1, however, some of these proteins contain MSSLYY-N at their amino terminus, and nearly all contain the pentapeptide IYPWM just upstream of the homeodomain (Mavilo et al., 1986). The variable region of these proteins is often rich in alanine, serine, glycine, proline or glutamine. In addition, some homeo box-containing genes contain a M repeat, which is a repeat of CAX codons (McGinnis et al., 1984a).

Other genes with extended homology to the homeo box have also been reported. These include mammalian transcription factors Oct-2 (Ko et al., 1988), Pit-1 (Ingraham et al., 1988), and Oct-1 (Sturm et al., 1988). The unc-86 gene from C. elegans, involved in differentiation of neural cells, is also included in this group, as it shows greater sequence homology to these transcription factors than to the Drosophila homeo box sequences. In addition to the homeodomain, these proteins also share another domain of about 75 amino acids upstream from the homeodomain. This region of sequence similarity is know as the pou box (pronounced "pow"). Since the homeodomains found in these

Fig. 1.1 Features of prototype Antennapedia-like homeodomain proteins. Note three α-helical regions in the homeodomain. The third α-helix is the DNA recognition helix. (Wright et al., 1989b)


INTRON
Conserved
Amino
Terminus
Acidic
Tail
MSSLYY-N
variable region
HOMEODOMAIN
[E]n
H2N
COOH
1
2
3
helices
IYPWM
homeopeptide

pou proteins are more similar to each other than they are to any of the known homeo box sequences from Drosophila, they are considered to be a separate class.

C. The homeo box genes of Drosophila and their role in development

A homeotic mutation causes one part of the body to be replaced by another. Genes directing the proper development of segments are called homeotic genes. In Drosophila, there are two clusters of homeotic genes which reside on the right arm of the third chromosome. These are the Antennapedia (ANT-C)(Struhl, 1982) and bithorax (BX-C)(Lewis, 1978) gene complexes. The Antp genes are essential for the proper development of the head, pro and mesothoracic segments, while the genes of the bithorax complex (BX-C) are responsible for the development of the metathoracic and abdominal segments. Cloning of the Antennapedia (Antp), Ultrabithorax (Ubx), and fushi tarazu genes from these complexes, revealed that they share a similar sequence of about 180 bp now known as the homeo box (Scott and Weiner, 1984). Fushi tarazu, which lies within the ANT-C gene complex, is involved in segmentation of the body. Many other segmentation genes contain a homeo box. For example, the cloning of the engrailed gene, whose product specifies the posterior compartments of segments, revealed that it contained a homeo box which was significantly diverged from the homeo box found in Antp (53%

DNA sequence identity) (Poole et al., 1985). Homeo boxes resembling the Antp homeo box are called Class I homeo boxes, while those resembling the engrailed homeo box are called Class II homeo boxes. Another class of homeo boxes similar to the one found in the paired (prd) gene of Drosophila has also been described (Frigerio et al., 1986).

In Drosophila, homeo box sequences have been found in about 20 genes involved in a variety of developmental processes. These include homeotic genes (Scott and Weiner, 1984; Regulski et al., 1987), segmentation genes (Laughon and Scott, 1984; Poole et al., 1985; Frigerio et al., 1986) genes involved in dorsal/ventral differentiation (Doyle et al., 1986), maternally transcribed genes which act to define anterior-posterior polarity (Mlodzik et al., 1985; Frigerio et al., 1986), and a gene involved in eye development (Tomlinson et al., 1988).

At the time of fertilization, the Drosophila egg contains maternally transcribed mRNAs and is already organized with respect to where the ultimate anteroposterior (head to tail), and dorsoventral axes will arise. Within the egg cytoplasm, there are two organizing centers, one anterior and one posterior, which determine these positions later in development. These organizing centers were discovered by removing cytoplasm within these regions and allowing the embryos to develop (reviewed by Nusslein-Volhard et al., 1987). Eggs lacking anterior cytoplasm were missing head structures, while those lacking

posterior pole plasm had defective abdominal structures. Some of these factors were identified as messenger RNAs. The maternally transcribed gene bicoid, which encodes a mRNA located in the anterior cytoplasm of wild type oocytes and early embryos, has recently been cloned and sequenced (Frigerio et al., 1986). This gene contains a homeo box. The protein product of this gene is distributed throughout the embryo in a gradient with its highest concentration being at the anterior end. This protein has the properties of a morphogen in that it can autonomously determine positions in the anterior half of the embryo (Driever and Nusslein-Volhard, 1988). Likely targets for the bicoid protein appear to be zygotically active genes like the gap genes (see below).

In Drosophila, the homeo box genes work in a defined sequence. The interplay between these genes can be detected by monitoring the distribution of transcripts of a wild type gene in a mutant embryo.

Genes affecting segmentation in Drosophila fall into three major groups depending on their mutant phenotype. These include gap (segments are missing), pair rule (alternate segments are missing) and segment polarity (a compartment within each segment is missing, and this is replaced with a mirror image duplication of the remaining compartment). Some of the gap genes are required for the correct expression of ftz (Carroll and Scott, 1986). Within the pair-rule genes, of which ftz is a member, there is a

hierarchy of gene expression. For example, the h (hairy) gene regulates the expression of ftz but the converse is not true (Howard and Ingham, 1986).

The controlling elements of homeo box-containing genes in Drosophila have also been studied. One method for examining the DNA sequences necessary for transcription is by analysis of altered cloned sequences introduced into developing Drosophila embryos by P-element-mediated transformation (Spradling and Rubin, 1982). Fusion of deleted or altered promoter sequences with the E. coli lacZ coding sequence, allows gene expression to be monitored histochemically. This approach was used to study the controlling elements of the ftz promoter (Hiromi et al., 1985). One such element is the "zebra" element which resides 0.62kb 5′ to the translation initiation site. This element is necessary for the proper expression of ftz in its striped pattern.

Possible models to explain the role of homeo box containing genes in development have been summarized by Scott and Carroll (1987). These include possibilities of how combinations of these gene products could produce different cell types. For example, different cell types could arise in different parts of a segment if the homeotic proteins are produced at different levels in the cells, or exist in different combinations. If, for example, these proteins act as different transcription factors, different combinations of these genes could serve to activate other

genes, or alternately, the interaction of previously expressed homeotic genes could modify presently expressed genes in the network. Of interest is that the yeast cell-type specific repressor α2 acts cooperatively with a generalized transcription factor (Keleher et al, 1988). If this example is extended to the homeo box containing genes, it is possible that the cooperative binding of selective combinations of these genes could regulate gene expression. In addition, these proteins could also influence the expression of other groups of homeo box-containing genes. Gradients of homeodomain proteins observed at very early stages of development (such as bcd) could set the process in motion. The initial pattern of signals distributed throughout the egg, could subsequently regulate other genes which control the fate of cells.

D. Homeo box containing genes in other organisms

1. Xenopus laevis

To date, approximately 10 homeo box containing genes have been isolated from Xenopus. Most of the homeo boxes described show a high degree of similarity with the Drosophila Antp homeo box sequence (Fritz and De Robertis, 1988). Homeo box sequences which are highly divergent from Antp have only recently been reported (Ruiz I Altaba and Melton 1989a; Wright et al., 1989a; Rosa, 1989). Although no homeo box genes with homology to engrailed have been isolated, an engrailed-related protein has been detected in

Xenopus embryos using a monoclonal antibody against the Drosophila invected (engrailed-related) homeodomain (Hemmati Brivanlou and Harland, 1989), suggesting that a gene containing an engrailed class homeo box exists.

The genes of Xenopus containing homeo box sequences have been characterized in terms of their gene organization (intron, exon etc.), and the localization of transcripts during development. Some of these genes share features with Drosophila homeo box genes. The gene Xhox-36, for example, codes for the conserved pentapeptide (Fig. 1.1), described by Mavilo et al., (1986). Other genes, for example, XlHbox-6, contain an opa or M-repeat (Sharpe et al., 1987). In addition, the organization of these genes is similar to the Drosophila genes in that the homeo box is located in the last exon, and thus the homeodomain is close to the C-terminus of the protein. One of the Xenopus homeo box containing genes, XlHbox 2, encodes both a homeo box containing and a homeo box-less transcript (Wright et al., 1987).

Attempts to characterize the developmental significance of these genes have focused on the localization of transcripts during development. The first homeo box-containing genes isolated from Xenopus, AC-1, later renamed Xlhbox 1, produces two predominant transcripts during neurulation (Carrasco et al., 1984). These transcripts have been shown to be initiated from two different promoters (Cho et al., 1988). Carrasco and Malacinski, 1987, demonstrated

that this gene is expressed in the cervical spinal cord of swimming tadpoles by in situ hybridization.

The gene Xlhbox 2 is expressed in high levels in oocytes (Müller et al., 1984). Although it is not known whether this gene is expressed in a gradient in the egg like the Drosophila bicoid gene, it is exciting to speculate that this may be one of the determinants that specifies the future fate of cells in development.

Xlhbox 1 and 2 are transcribed at all stages past the mid blastula transition, while other homeo box genes of Xenopus are transcribed during specific stages of early development (Fritz and De Robertis, 1988). For example, Xlhbox 3 is transcribed at the late neurula stage, Xlhbox 4 shows strongest expression in the tail bud stage, and Xlhbox 5 is transcribed during late neurula and early tail bud stages. Xlhbox 6 and Xhox 36 are interesting in that they are both expressed posteriorly during neurulation, and thus provide convenient markers to study development during this important stage of development.

Harvey et al. (1986), showed that the protein produced by the homeo box-containing gene Xhox 1A is located in the nucleus of cells indicating a regulatory function for this gene.

Recently, attempts have been made to define a role for homeo box-containing genes in Xenopus. The approach was to microinject excess copies of a homeo box (Xhox-1A) transcript (produced in vitro) (Harvey and Melton, 1988).

The rationale was that excess copies of the transcript would disturb the developmental equilibrium. The results from this study suggested that the Xhox-1A gene product was involved in somite formation. This was based on a reproducible phenotype observed in injected embryos. Somite segmentation can be considered somewhat analogous to the segmentation in Drosophila. Another approach (Cho et al., 1988) was to inject Xenopus embryos with antibodies directed against a homeodomain protein. This resulted in the ablation of part of the dorsal fin. It is clear that more experiments will be necessary to more precisely define the functions of homeo box containing genes in vertebrates.

2. Mouse

About 20 homeo box containing genes have been identified in the mouse (reviewed by Holland and Hogan, 1988), and most of these show a high degree of sequence similarity with the homeo box of Antennapedia. In many cases, the protein homology is even higher than the DNA homology. As in Drosophila, most of these genes are found in clusters on chromosomes. The major clusters are Hox 1 located on chromosome 6, Hox 2 on chromosome 11, Hox 3 on chromosome 15, and Hox 5 on chromosome 2.

The mouse Hox-1.6 gene contains a homeo box sequence which has diverged significantly from Antp. This homeo box sequence is 64% homologous with Antp at the nucleotide level, and is more divergent from Antp in its protein sequence (62%) (Baron et al., 1987). Hox-7.1 (Hill et al.,

1989) does not show significant homology (less than 50%) with either the Drosophila Antp, or engrailed homeo box sequences but is 57% homologous to Hox-1.6.

In addition to the Antp homeo box genes of mouse, genes with homology to the engrailed class of homeo box have been isolated from this genome (Joyner et al., 1985).

Attempts to characterize homeo box-containing genes in the mouse have focused on gene organization and localization of transcripts during development. Many of these studies have relied on in situ hybridization techniques due to the paucity of material available for RNA isolation. The information concerning the temporal and spatial distribution of mouse homeo box transcripts during development has recently been summarized in a review by Holland and Hogan (1988). Embryonal carcinoma cell lines have also been used to study homeo box gene expression (Colberg-Poley et al., 1985).

The engrailed-like homeo box genes of mouse have also been shown to be expressed embryonically. For example, Mo-en.1 (Joyner et al., 1985), now called En-1, is expressed maximally in 10.5 and 15 day old embryos.

In the mouse, more direct evidence is currently being presented on the role of homeo box genes in development. The production of transgenic mice has been a powerful method for studying gene expression. This technique has recently been used to examine the role of Hox-1.4 in development (Wolgemuth et al., 1989). Excess copies of Hox-1.4

introduced into transgenic mice caused abnormal development of the gut. This report is very important, as it provides evidence that vertebrate homeo box-containing genes play a role in development.

3. Human

As in the mouse and Drosophila, the human homeo boxes are clustered. Several human homeo box sequences which show a high degree of sequence conservation to the Antp homeo box have been isolated (Levine et al., 1984 ;Simeone et al., 1987; Boncinelli et al., 1988). Northern analysis using these homeo box sequences as probes has shown that genes containing these sequences are expressed during embryogenesis in a temporally and spatially restricted pattern (Simeone et al., 1987).

4. Other organisms

The discovery of the homeo box in a number of organisms with a segmented body plan led to the notion that the homeo box played a role in the segmented architecture of the body. However, shortly after this, homeo box sequences were described in non-segmented organisms such as the sea urchin (Dolecki et al., 1986). A homeo box sequence of the Antp class, and one of the engrailed class (Dolecki and Humphreys, 1988) have been isolated from the sea urchin. These genes have a significant degree of sequence similarity with their Drosophila counterparts. In addition, homeo box sequences have been described in genes with developmental significance in the nematode worm, C. elegans (Way and

Chalfie, 1988). Other vertebrates with homeo box sequences include the newt, Notophthalmus viridescens (Savard et al., 1988), the rat (Falzon and Chung, 1988), and zebrafish (Njoistad et al., 1988).

E. Prospects for studying homeo box gene function in vertebrates:the use of transgenic animals

One of the most promising approaches to understanding the function of homeo box containing genes involves the microinjection of DNA sequences into developing organisms. These studies have aided in the understanding of the sequences necessary for the correct temporal and spatial expression of genes. The most notable studies have been in the mouse (reviewed by Palmiter and Brinster, 1985) and Drosophila (Spradling and Rubin, 1982). The introduction of altered DNA sequences allows the function of normal sequences to be evaluated.

Amphibian species are ideal for such studies due to the large size of their eggs, which facilitates microinjection, large number of progeny, and the ease with which the eggs can be analyzed by standard biochemical techniques. However, the main drawback is the long generation time, if germ line transmission of sequences is required. Some success has been obtained with the injection of exogenous sequences into frog (Xenopus laevis) embryos. Injected rRNA genes were correctly expressed (Busby and Reeder, 1983). However, this was generally not the case

with genes transcribed by RNA polymerase II, even when the injected sequences were from Xenopus (Rusconi and Schaffner, 1981, Etkin et al. 1984; Bendig and Williams, 1983; Andres et al. 1984). Only recently has the correct spatial and temporal expression of some genes been reported in Xenopus. These included a cardiac α-actin gene from Xenopus borealis (Wilson et al., 1986), and a gastrula specific gene from Xenopus laevis (Krieg and Melton, 1985). Etkin and Pearman, (1987) reported that animals which had been successfully transformed with foreign DNA were mosaic, with no apparent correlation to integration at a given time or in a given germ layer. The reason for this seemed to be that the DNA was incorporated during early cleavage, but not before first cleavage. Germ line transmission of DNA sequences was reported by Etkin and Pearman, (1987) but the mosaic distribution of DNA sequences does not guarantee this.

In the axolotl, the interval from fertilization to first cleavage is about six hours. This may provide a distinct advantage for the production of transgenic animals, as there should be sufficient time for integration of the DNA at the one cell stage. If this is the case, then the study of homeo box gene function in the axolotl by the introduction of altered homeo box genes or by deregulation of gene expression by a change in copy number of a homeo box gene may provide a valuable method for studying the role of the homeo box genes in development.

CHAPTER II

HOMEO BOX SEQUENCES IN THE AXOLOTL

A. Rationale

The discovery of homeo box sequences in vertebrate genomes homologous to those in Drosophila was an important step in the identification of genes which may play a role in the development of these organisms. Human, mouse, and frog genomes all contain homeo box sequences which have a high degree of homology to the Drosophila Antp homeo box at both the nucleotide and protein levels. In these genomes, homeo box sequences with greater that 80% nucleotide identity and even higher protein homology have been reported (mouse: Colberg-Poley et al, 1985; human: Levine et al., 1985; frog: Fritz and De Robertis, 1988). The success obtained with the Antp probe in isolating homeo box sequences from other genomes suggested that it would be suitable for detecting homeo box sequences in other vertebrates such as the axolotl (Ambystoma mexicanum). There was no a priori reason to suggest that the use of a probe from a more closely related organism was warranted, or would offer any advantage over the Antp probe.

The purpose of these experiments was to isolate homeo box sequences from the axolotl. The first step was therefore, to determine appropriate hybridization conditions

to detect these sequences with the Drosophila Antp probe. Genomic Southern transfers were prepared from axolotl DNA digested with a number of restriction enzymes. Restriction enzyme fragments containing a number of cross-hybridizing sequences were used to construct a partial genomic library from which homeo box sequences could be cloned, and characterized.

B. Materials and methods

1. Isolation of axolotl genomic DNA

Axolotl embryos were obtained from spawnings performed at the University of Ottawa axolotl colony. Ambystoma tigrinum, and Ambystoma maculatum embryos were obtained from the Charles D. Sullivan company, Nashville Tn. Genomic DNA was prepared from embryos (neurula or tail bud) essentially as described by Etkin et al. (1984). One embryo (neurula or tail bud) was homogenized in one mL of homogenization lysis buffer (300 mM NaCl, 100 mM Tris HCl (pH 7.5), 10 mM EDTA, 2% SDS). Proteinase K (Boehringer Mannheim Biochemicals) was added to a final concentration of 1 mg/mL. Proteinase K digestion was for 30 minutes at room temperature. The DNA was then purified by extractions with phenol, phenol:chloroform and chloroform. The DNA was then ethanol precipitated, and resuspended in 30 μL/ embryo of TE (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). Approximately 20 μg of genomic DNA was obtained from one embryo.

2. Isolation of Drosophila DNA

Genomic DNA was isolated from Drosophila melanogaster (Oregon R strain) as follows. 100 mg of flies were homogenized, using a Dounce homogenizer, in 1 mL of buffer containing 10 mM Tris HCl (pH 7.5), 60 mM NaCl, 10 mM EDTA, and 0.2 mg/mL proteinase K. The Dounce homogenizer was then rinsed with one mL of 0.2 M Tris HCl pH 8.0, 30 mM EDTA, 2% SDS, and 0.2 mg/mL proteinase K. The homogenate was incubated at 37°C for one hour, and extracted with an equal volume of phenol, and then phenol:chloroform. The DNA was precipitated with ethanol, and resuspended in 2 mL of 8 mM Tris HCl pH 7.6, 100 mM NaCl, and treated with RNAse at 100 μg/mL for one hour at 37°C, and then proteinase K (50 μg/mL) for one hour at 37°C. The DNA was extracted with phenol and phenol:chloroform and ethanol precipitated as above, and resuspended in 0.2 mL of TE.

3. DNA probes

The plasmid, pAM18 containing the Drosophila Antp homeo box sequence was purchased from Amersham. The 175 bp DNA fragment containing the homeo box was isolated from this plasmid by restriction enzyme digestion with BamHI and HindIII. The fragment was purified from low melting point agarose gel by heating the gel slice to 65°C, adding an equal volume of TE, and extracting this with phenol and phenol:chloroform. The DNA was then ethanol precipitated.

The probe for Ahox1 was a 560 bp KpnI fragment (see section C. 3, page 38). The probe for ribosomal DNA was prepared as follows. Total RNA (15 μg) from axolotl oocytes was electrophoresed on a 1% low melting point agarose gel. The 18 and 28S ribosomal RNAs were purified from the gel as described above (materials and methods 3.).

4. Genomic DNA hybridizations

Approximately 10 μg of axolotl genomic DNA was digested with a 4 fold excess of restriction enzyme in the buffer recommended by the manufacturer. Digests were done at 37°C for 4 to 6 hours. Parallel digests were performed with bacteriophage lambda DNA as an internal standard to test for the completeness of digestion. This was evaluated by running a sample of these digests on an agarose gel containing ethidium bromide. Digests of mouse (gift from G. Pari) and Drosophila DNA were performed as above. Genomic digests were electrophoresed on a 0.8% agarose gel with TBE (89 mM Tris HCl, 89 mM boric acid, 3.4 mM EDTA, pH 8.3) as running buffer. Electrophoresis was done overnight at 22V. The gels were stained with ethidium bromide, and photographed under ultraviolet transillumination. Molecular weight markers (lambda DNA digested with HindIII) were included in the gels. Drosophila DNA (1 μg digested with EcoRI) was also included on the gels. For mouse genomic DNA, 5 μg of DNA was loaded on a lane of the gel.

The genomic DNA was transferred from the agarose gels to Hybond N (Amersham) nylon membranes by alkaline capillary blotting after acid hydrolysis (Reed and Mann, 1985).

Reduced stringency conditions were as follows: Membranes were prehybridized overnight at 37°C, in a solution containing 43% deionized formamide, 5X SSC, 50 mM sodium phosphate(pH 6.5), 5X Denhardt's solution (0.05% BSA, 0.05% ficoll,0.05% PVPD), 100 μg/mL heat denatured sheared herring sperm DNA and 0.5% SDS. Hybridization was performed in the same solution for 48 hours using a DNA probe labelled by the random primer labelling method (Feinberg and Vogelstein, 1983). 25 ng of DNA was labelled with 50 μCi of α-^{32}P-dCTP at 3000 Ci/mM. One to two ng of labelled DNA was added per mL of hybridization solution. For the rDNA probe, the ribosomal RNAs (25 ng) were labelled in a standard random primer labelling reaction with 5 U of reverse transcriptase overnight at room temperature. The ribosomal RNAs were denatured at 71°C for three minutes prior to the labelling reaction. Low stringency conditions were as described above except that the formamide concentration was lowered to 30%. Washing conditions for both low and reduced stringency were 2X SSC, 0.1% SDS at 37°C for 4 to 6 hours. High stringency conditions were as above except that the formamide concentration was raised to 50%, and the temperature of hybridization was 42°C.

5. Preparation and screening of enriched genomic library

Fifty μg of axolotl genomic DNA was digested with 200 units of EcoRI at 37°C for 4 hours and electrophoresed on a 1% preparative low melting point agarose gel with TBE as running buffer overnight at 20 volts. Three to four kb EcoRI genomic DNA fragments were cut out of the gel. The gel slice was melted at 65°C, and divided into 200 μL amounts in 1.5 mL eppendorf tubes. An equal volume of TE was added to the tubes, and the DNA was purified by phenol, and phenol:chloroform extraction followed by ethanol precipitation. Approximately 40 ng of these fragments were ligated to 250 ng of EcoRI digested and phosphatased lambda gt11 DNA (Promega Biotech). The DNA was then packaged using extracts obtained from Promega Biotech. 100,000 pfu were obtained. 80,000 pfu were screened under low stringency conditions described above. Plaque lifts were prepared using Hybond N according to the manufacturer. The phage were plated on E.coli strain LE392. Positive clones were picked, and rescreened to purity.

6. Subcloning and restriction mapping of lambda clones

Lysates of phage lambda plaques were prepared as follows. A single plaque was added to 5mL of NZ broth (1% NZ amine (ICN biochemicals),0.5% NaCl, 0.5% yeast extract, 0.1% casamino acids, 15 mM $MgSO_4$ pH 7.5). 50 μL of overnight culture (E. coli, LE392) was added to the phage in NZ broth, and the culture was grown with shaking for 4 to 6 hours at

37°C, or until lysis occurred. A few drops of chloroform were added to the lysate, and cellular debris were removed by centrifugation in a Sorvall SS34 rotor at 8000 rpm for 15 minutes .

Lambda DNA was prepared from the phage lysate as follows. Two mL of the lysate was incubated with 10 μL of DNAse I (2 mg/mL in 0.1 M Tris HCl pH 7.4) for one hour at 37°C. The phage were then precipitated on ice for one hour by the addition of 0.3 mL of 5 M NaCl, and 0.75 mL of 40% PEG (polyethylene glycol). The phage were pelleted by centrifugation in a Sorvall SS34 rotor at 5000 rpm for 10 minutes. The pellet was resuspended in 0.2 mL of ice cold SM (0.1 M NaCl, 15 mM $MgSO_4$, 20 mM Tris HCl pH 7.5), and the phage DNA was then purified by sequential extractions with an equal volume of phenol, phenol:chloroform, and chloroform. The DNA was then ethanol precipitated overnight, and resuspended in a final volume of 50 μL. Contaminating RNA was removed by digestion with RNAse at a final concentration of 50 μg/mL for 10 minutes at 37°C.

The cloned inserts were isolated from the phage DNA by digesting 10 μL of the above DNA with EcoRI followed by electrophoresis in a low melting point agarose gel, and recovering the insert DNA from this as described above.

The insert DNA was then subcloned into a plasmid (pAM18 , Amersham corp.) digested with EcoRI, and phosphatased by the addition of 1 unit of calf intestinal phosphatase (Boehringer Mannheim Biochemicals) for 30 minutes at 37°C

following restriction enzyme digestion. The phosphatase was heat inactivated (65°C, 10 minutes), and removed from the plasmid DNA by phenol and phenol:chloroform extraction, followed by ethanol precipitation. Ligation of the insert and vector was as follows. 4 ng of vector was ligated with 12 ng of insert in a final volume of 10 μL, in ligation buffer (30 mM Tris HCl (pH 8.0), 7 mM $MgCl_2$, 1 mM EDTA, 0.2 mM ATP (adenosine 5'-triphosphate), 0.1 mM DTT, 250 μg/mL BSA). Ligation was for 2 hours at room temperature. Clones were then obtained by transforming E. coli (C600) with the ligation mix. Competent E. coli cells were prepared by a modification of the method of Hanahan (Alexander et al., 1984).

Plasmid DNA was prepared by the "boiling method" (Holmes and Quigley, 1981). The DNA prepared by this method was further purified by precipitation with 0.5 M NaCl, 20% PEG overnight at 4°C.

Restriction maps were generated from fragment length determinations from single and double digests of plasmid DNA. Small restriction fragments hybridizing to the Antp probe were identified by low stringency hybridization described above.

7. DNA sequencing

Restriction fragments hybridizing to the Antp probe were subcloned into either M13mp19 or M13mp8 for sequencing. A DNA sequencing kit was obtained from Amersham and DNA

template was prepared as described by the manufacturer. Sequencing was done by the chain termination method (Sanger et al., 1977) using ^{35}S-dATP and Klenow enzyme.

8. RNA isolation

RNA was isolated from axolotl embryos by the acid phenol-guanidinium method (Chomcznski and Sacchi, 1987) as follows. 50 embryos were homogenized in a Dounce homogenizer in 5 mL of solution A (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The homogenate was transferred to a 12 mL polypropylene tube and the following were added sequentially. 0.5 mL of 2 M sodium acetate pH 4, 5 mL of water saturated phenol, and 1.0 mL of chloroform-isoamyl alcohol (49:1). The mixture was shaken vigorously for 10 seconds and placed on ice for 15 minutes. The aqueous and organic phases were then separated by centrifugation in a SS34 rotor at 10,000 rpm for 20 minutes. The aqueous phase was removed, and the RNA was precipitated by the addition of 5 mL of isopropanol followed by incubation at -20°C for two hours. The RNA was pelleted, and resuspended in 0.5 mL of solution A, and reprecipitated with isopropanol as above. Contaminating polysaccharides were removed from RNA preparations by precipitation with LiCl (3M final concentration). The pellet from the LiCi precipitation was then ethanol precipitated. Approximately 250 μg of RNA was obtained per 50 embryos. The embryos were staged according

to Bordzilovskaya et al. (1989) (see Appendix I for details).

9. Northern blot analysis

RNA was separated on a 1% agarose, 2.2 M formaldehyde gel as described by Maniatis et al. (1982). The RNA was transferred to Hybond N nylon membranes (Amersham) with 20 X SSC as transfer solution. Hybridization was performed at 42°C in a solution containing 50% formamide, 5X SSC, 50 mM sodium phosphate (pH 6.5), 0.5% SDS, 100 μg/mL heat denatured herring sperm DNA, 5X Denhardt's solution. Washing conditions were as described by Thomas (1980).

C. Results

1. Detection of homeo box sequences in the Ambystoma species

Sequences with homology to the Drosophila Antp homeo box have been detected in genomes from a variety of species using conditions of reduced stringency (43% formamide, 37°C). Studies by McGinnis (1985) and Holland and Hogan (1986) examined a number of genomes for the presence of Antp-like homeo box sequences using these hybridization conditions, and a genome was considered positive for Antp sequences if the observed cross-hybridizing fragments did not correspond to positions of genomic repeats. McGinnis (1985), had first reported that spurious hybridization signals to the Antp homeo box could occur at positions of

genomic repeats, and some of these repeats were identified as rDNA sequences.

In this study, the presence of Antp sequences in the axolotl and other Ambystoma species was examined using conditions of reduced stringency (Fig. 2.1). DNA from A. mexicanum, A. tigrinum, and A. maculatum was digested with EcoRI, and probed with the Antp homeo box sequence. Both wild type, and albino axolotl DNA was included in this analysis, as the albino mutant was introduced into the axolotl from A. tigrinum (Humphrey, 1967). In A. mexicanum, (wild type and albino) and A. tigrinum two prominent fragments were observed (3.5 and 3.2 kb) (Fig. 2.1). In A. maculatum, three hybridizing fragments were observed (4.0, 3.3, and 2.0 kb). The two prominent bands in A. mexicanum, corresponded to the positions of genomic repeats as determined by ethidium bromide staining of a gel showing a higher DNA concentration (Fig. 2.2). In A. tigrinum, one of the prominent hybridizing fragments corresponded to the position of a genomic repeat. In A. maculatum, none of the hybridizing fragments corresponded to the position of a genomic repeat. In all three species, weakly hybridizing fragments were also observed.

In order to determine if any of the hybridizing fragments corresponded to ribosomal DNA sequences, the Antp probe was removed from the membrane (Hybond N) according to the manufacturer's specifications, and the filter was rehybridized with cDNA prepared from A. mexicanum ribosomal

RNA (18 and 28S). The ribosomal RNA probe hybridized at the same positions as some of the weaker hybridizing fragments in A. mexicanum and A. tigrinum (Fig 2.1).

The prominent bands in axolotl DNA were located between 3 and 4 kb, and therefore, to accurately determine if these corresponded to the positions of genomic repeats observed in the ethidium bromide stained gel (Fig. 2.2), a probe of 3-4 kb fragments was prepared, and hybridized to the same blot. The results of this experiment confirmed that the bands hybridizing to Antp probe were in identical positions to genomic repeats. In the case of A. tigrinum, a band which corresponded to a genomic repeat also showed hybridization at this position.

Analysis of the three Ambystoma species, therefore, showed that under conditions of reduced stringency, only A. tigrinum and A. maculatum showed hybridizing fragments which did not correspond to positions of genomic repeats. These genomes were therefore considered positive for the presence of highly conserved copies of Antp.

The possibilities for A. mexicanum are that it does contain highly conserved copies of the Antp homeo box, which fortuitously hybridize at the same positions as genomic repeats, or that the Antp homeo box gives spurious hybridization to genomic repeats, and this genome does not contain highly conserved copies of Antp. These possibilities could be resolved by examining genomic Southerns using other restriction enzymes.

Fig. 2.1. Detection of Antp-like sequences in various Ambystoma species using reduced stringency hybridization conditions.
Genomic DNA was digested to completion with EcoRI, separated on a 0.8% agarose gel, transferred to Hybond N nylon membrane and hybridized to various probes as described below. The filter was exposed for 6 days (Antp probe), or overnight (ribosomal cDNA and 3-4 kb EcoRI axolotl genomic fragments) to Kodak XAR-5 film with intensifying screen at -70°C.

(A) Re-probing of the same blot as (B), using 3-4 kb EcoRI fragments as probe. Note that the positions of genomic repeats correspond to fragments hybridizing in lane 1, and one of the fragments in lane 3.
Lane 1 A. mexicanum (wild type)
2 A. mexicanum (albino mutant)
3 A. tigrinum
4 A. maculatum

(B) Hybridization of Ambystoma species genomic DNA with the Drosophila Antp probe using conditions of reduced stringency (43% formamide, 37°C). Each lane contains approximately 10 μg of DNA.

(C) Re-probing of the same blot as (A), using cDNA prepared from 18 and 28S ribosomal RNA as probe. Note that the position of rDNA sequence corresponds to weaker signals in (A), for A. mexicanum and A. tigrinum.

(D) Hybridization of D. melanogaster genomic DNA with the Antp probe. Reduced stringency conditions were used. The Antp homeo box is located on the fragment showing strong hybridization below the 2 kb size marker.

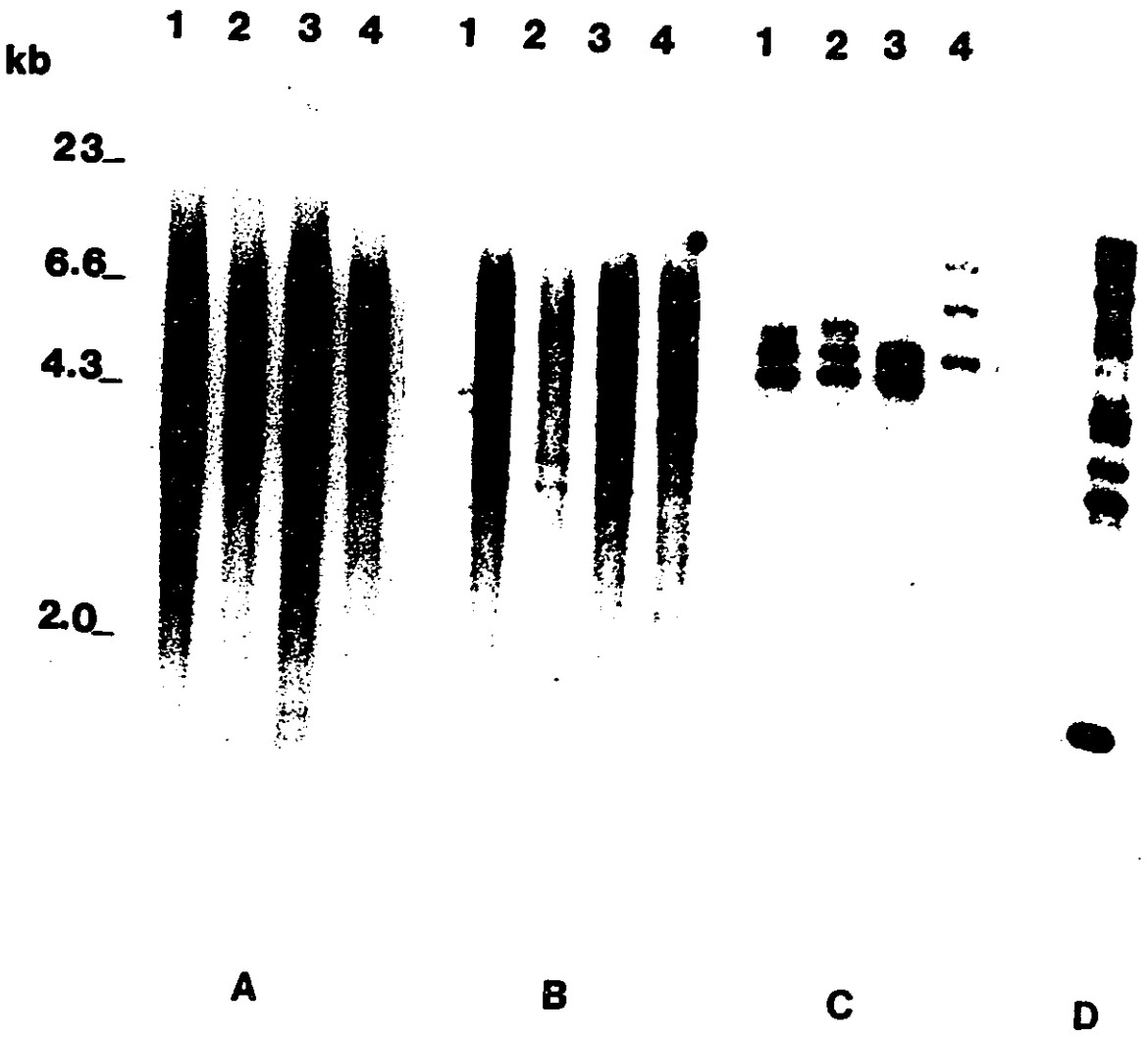

kb
1 2 3 4
1 2 3 4
1 2 3 4
23
6.6
4.3
2.0
A
B
C
D

Fig. 2.2 Agarose gel electrophoresis of Ambystoma species genomic DNA digested with EcoRI.

Genomic DNA was digested to completion with EcoRI, and electrophoresed on a 0.8% agarose gel. The gel was then stained with ethidium bromide.

Lane 1. A. mexicanum (wild type)

2. A. mexicanum (albino mutant)

3. A. tigrinum

4. A. maculatum

Arrows point to positions of genomic repeats which hybridize with the Antp probe

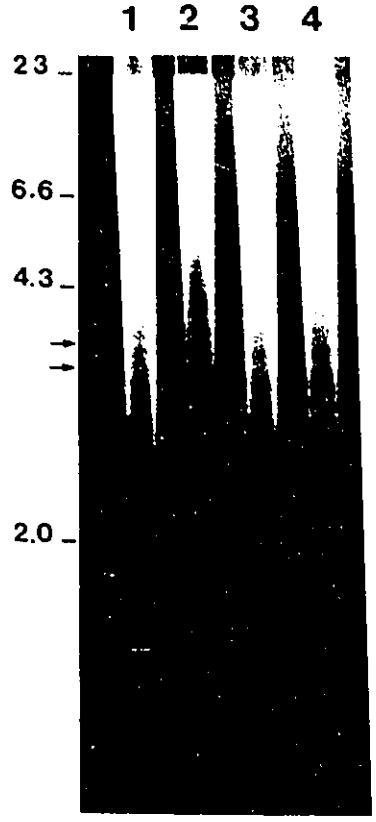
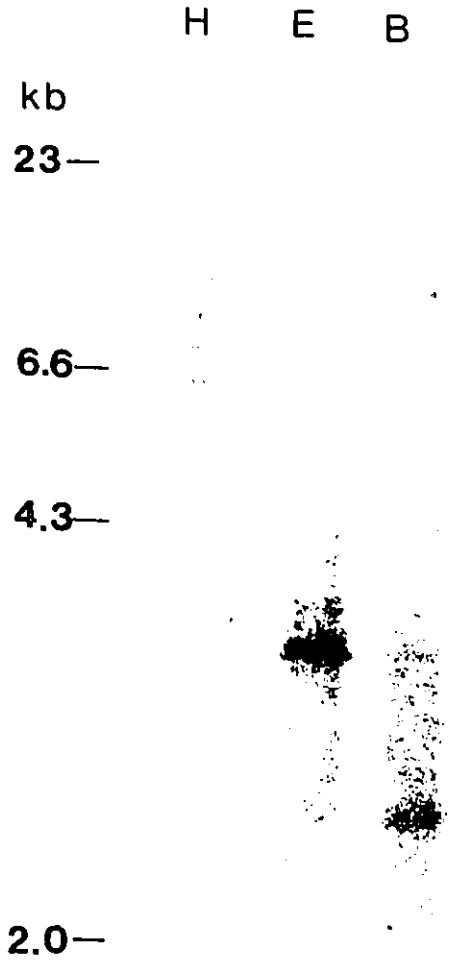

1 2 3 4
23
6.6
4.3
2.0

Fig. 2.3. Detection of genomic fragments of axolotl DNA hybridizing with the Drosophila Antennapedia probe using low stringency hybridization.

Genomic DNA (10 μg) was digested to completion with HindIII (H) EcoRI (E), or BamHI (B), separated on a 0.8% agarose gel, transferred to Hybond N nylon membrane and hybridized with the Drosophila Antp


H
E
B
kb
23
6.6
4.3
2.0

Based on this analysis, the axolotl genome does not appear to contain highly conserved copies of Antp, at least at the nucleotide level. A. maculatum and A. tigrinum do, however, contain low numbers of Antp-like sequences.

At lower stringency (30% formamide, 37° C), a number of fragments hybridizing to Antp were detected with axolotl DNA (Fig 2.3) This suggests that there are homeo box related sequences in this genome.

2. Isolation of homeo box sequences from the Mexican axolotl

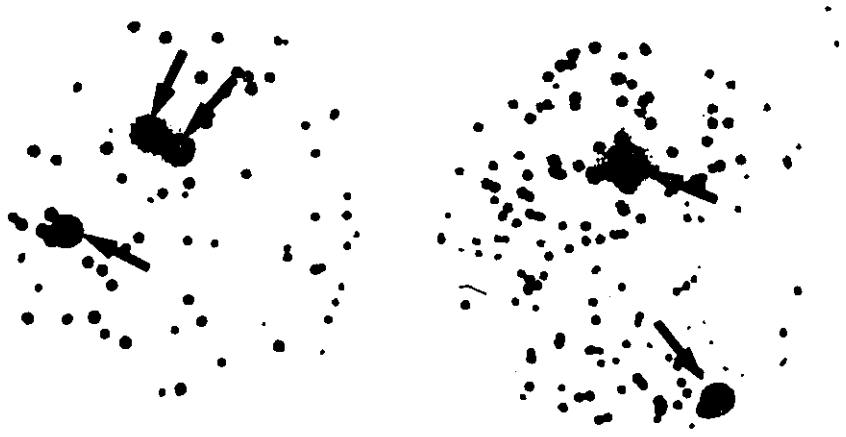
Under low stringency hybridization conditions, many of the fragments cross-hybridizing with the Antp probe were found in the 3-4 kb range in EcoRI digested axolotl DNA (Fig. 2.3). This DNA also included the fragments which hybridized with Antp at positions of genomic repeats. A partial genomic library was prepared in lambda gt11 using the same preparation of 3-4 kb fragments that were used as a probe in Fig. 2.1. 80,000 plaques were screened using low stringency hybridization, and 5 positive clones were obtained and purified (Fig. 2.4). One clone contained an 4 kb insert. The other four clones were found to be the same based on restriction enzyme digestion, and they contained a 3.5 kb insert. These inserts were subcloned into a plasmid vector , and restriction

Fig. 2.4. Screening of partial genomic library with the Drosophila Antp probe. A genomic library of 3-4 kb EcoRI axolotl DNA fragments was prepared in the bacteriophage lambda vector gt11.

(A) First screening of the library. 20,000 pfu were plated on a 150 mM agar plate containing NZ media. Plaque lifts were prepared using Hybond N nylon membranes according to the manufacturer. Hybridization with the Antp probe were done using low stringency hybridization (30% formamide, 37°C). Positive plaques are indicated by an arrow.

(B) Second screening of plaques. Regions of the first screening plate surrounding positive plaques were excised and put into SM buffer. Plaques were plated at a density of about 100 per plate and rescreened as above. Positive purified plaques are indicated with an arrow.

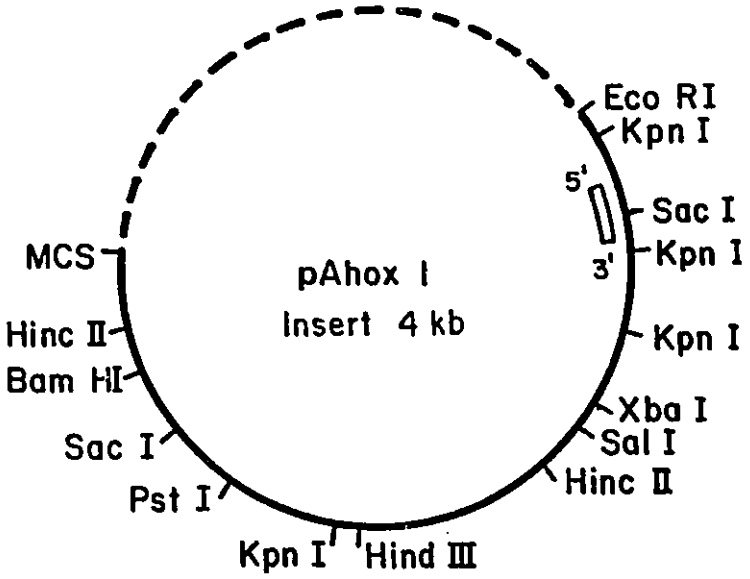
A

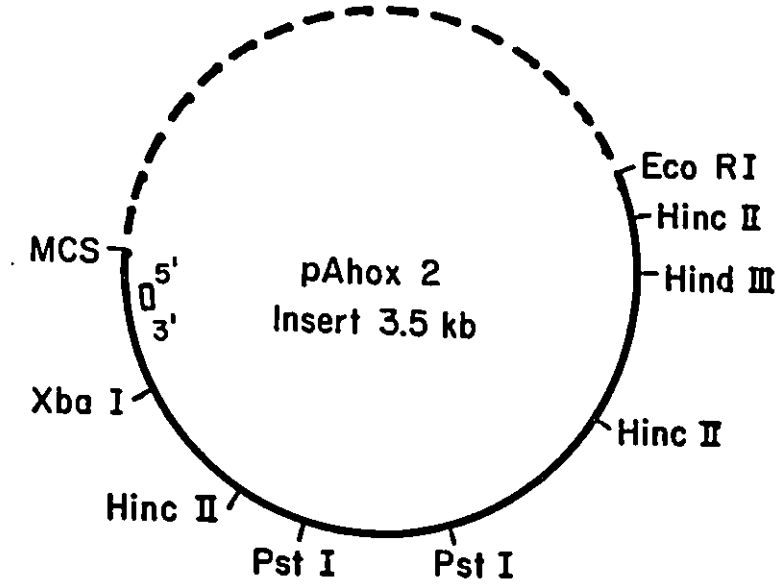


B

Fig. 2.5. Restriction maps of pAhox1 and pAhox2

EcoRI inserts from the bacteriophage lambda positive clones (Fig. 2.4) were subcloned into the plasmid vector pTZ19R, and restriction maps were generated from single and double digest data. The region of the clone showing homology to the Drosophila Antp probe is indicated by an open box.





Homeo box homology
MCS Multiple cloning site
Vector (2.9 kb)

mapped (Fig. 2.5) The plasmid clone containing the 4 kb insert was called pAhox1, and the one with the 3.5 kb insert was called pAhox2. Fragments showing homology with the Antp probe were identified by low stringency Southern transfer hybridization. The smallest homeo box containing fragment from pAhox2 was a 550 bp XbaI fragment, and from pAhox1 a 560 bp KpnI fragment. The 560 bp KpnI fragment contained a SacI site dividing this fragment into 200 and 360 bp fragments. The KpnI fragment was therefore sequenced in both directions by subcloning the KpnI fragment in both orientations into M13mp19, and also by cloning the SacI-KpnI fragments into M13mp19.

3. DNA sequence analysis of putative homeo box containing clones

DNA sequencing of the putative homeo box-containing region of pAhox2 revealed that hybridization with the Antp probe was due to homology to the last 60 nucleotides of the homeo box. This region of homology was called Ahox2. The homology was 57% in this region with greatest sequence conservation being in the region coding for the second helix of the helix-turn-helix of the homeo box (Fig. 2.6). Since this clone did not contain a complete homeo box sequence it was not characterized further, except that rehybridization of the homeo box sequence against axolotl DNA using reduced stringency conditions showed that this sequence was not

found in high copy number, and it hybridized to a single 3.5 kb EcoRI fragment (not shown).

pAhox1 did contain a complete homeo box sequence. This homeo box was called Ahox1. Ahox1 has an overall homology to the Antp sequerce of 66% at the DNA level and 62% at the amino acid level (in the putative reading frame). Comparison of Ahox1 with homeo box sequences from human, mouse, frog, and Drosophila showed that it was most similar to the homeo box of the mouse Hox-1.6 gene (Baron et al., 1987), and the Drosophila labial (lab) gene (Mlodzik et al., 1988)(Table 2.1). Hox-1.6 and lab homeo box sequences also show extensive sequence divergence from the Antp sequence. The overall homology of Ahox1 and Hox-1.6 was 84% at the nucleotide level, and 90% at the protein level for the putative reading frame of Ahox1. Ahox1 is 79% homologous to lab at the nucleotide level, and 85% at the protein level. Figs 6 and 7 show a comparison of Ahox1 with the Antp, Hox-1.6, and lab homeo box nucleotide and protein sequences. The 560 bp KpnI fragment of pAhox1 (designated HB-Kpn) containing the homeo box contains an open reading frame of 184 amino acids in the reading frame of the homeo box (see chapter IV, Fig. 4.4).

Fig. 2.6 Comparison of the nucleotide and putative amino acid sequences of Ahox2 and the Drosophila Antp homeo box. (A) DNA sequence of Ahox2 aligned 5′ to 3′ with the last 60 nucleotides of the Antp homeo box. Nucleotide homology (indicated by asterisks) was limited to this region of the homeo box. The sequence was determined from the 550 bp XbaI fragment of pAhox2 cloned into M13mp19 in two orientations. (B) Putative protein sequence derived from Ahox2 and compared to the sequence of the Drosophila Antp protein, and the yeast Matα1 protein sequence showing homology in the DNA recognition helix of the helix-turn-helix region of the homeo box.

(A)

```
                                                                                                                  180
      121
ANTP  ACG GAG CGC CAG ATA AAG ATT TGG TTC CAG AAT CGG CGC ATG AAG TGG AAG AAG GAG AAC
       *   *      ***   *   **    *  *** *** **  **  *** *** * *   *   *   * * *  *   *
AHOX2 TCC TTG GCT CAG GTG AAA GTC TGG TTC CAC AAC CGG CGC ACG TTG AAG TGG AGG CAC TCC
```

(B)

```
                                                                                                                  60
      41
ANTP  THR GLU ARG GLN ILE LYS ILE TRP PHE GLN ASN ARG ARG MET LYS TRP LYS LSY GLU ASN
                  ***     ***     *** ***     *** *** ***
AHOX2 SER LEU ALA GLN VAL LYS VAL TRP PHE HIS ASN ARG ARG THR LEU LYS TRP ARG HIS SER
                  *** ***     *** *** ***
MAT 1           LEU GLN VAL ARG VAL TRP PHE ILE
                --- --- --- --- --- --- --- ---
                      HELIX
```

Fig. 2.7. Comparison of the nucleotide sequence of Ahox1 homeo box with the Drosophila Antp, lab and the mouse Hox-1.6 homeo box nucleotide sequences.

The DNA sequence was determined by subcloning the 560 bp KpnI fragment of pAhox1 into M13mp19 in two orientations, and by subcloning the SacI KpnI fragments into M13mp19. The sequence was thus obtained in two directions.

(A) DNA sequence of Ahox1 homeo box aligned 5′ to 3′ with Drosophila lab and the mouse Hox-1.6 homeo box sequences. Common nucleotides are indicated by a star.

(B) DNA sequence of Ahox1 homeo box aligned with the Drosophila Antp homeo box sequence. Common nucleotides are indicated by a star.

(A)

```
                                                                                0
AHOX1                                                        GAG TAC GGC TTG GGG AGC CAG
--------------------------------------------------------------------------------------
      1                                                                        60
LAB   AAC AAC TCC GGC CGG ACG AAC TTC ACC AAC AAG CAG CTG ACC GAG CTG GAA AAA GAG TTC
       *  ***   *   * *** **  *** *** *** * * *** *** ***  ** *** *** **  **  *** ***
AHOX1 CAG AAC AGC ATC CGG ACC AAC TTC ACC ACC AAG CAG CTG TCC GAG CTG GAG AAG GAG TTC
      *   ***      *  **  *** **  *** *** *** *** *** ***  *  *** *** *** *** *** ***
HOX1.6 CCC AAC GCA GTG CGC ACC AAT TTC ACC ACC AAG CAG CTC ACA GAG CTG GAG AAG GAG TTC

--------------------------------------------------------------------------------------
      61                                                                       120
LAB   CAC TTC AAT CGC TAC TTG ACG CGG GCG CGC CGC ATT GAA ATC GCC AAT ACG TTG CAG CTT
      *** *** **   *  ***  ** **  *** **  ***  *   *  **  *** ***     **   **  ** **
AHOX1 CAC TTC AAC AGG TAC CTG ACC CGG GCC CGC AGG GTG GAG ATC GCC GCC ACC CTG GAG CTC
      *** *** *** * * *** **  **  **  **  *** *** *** *** **  *** **   ** **   ** ***
HOX1.6 CAC TTC AAC AAG TAC CTT ACA CGA GCG CGC AGG GTG GAG ATT GCC GCG TCC CTA CAG CTC
--------------------------------------------------------------------------------------
      121                                                                      180
LAB   AAT GAA ACG CAG GTC AAA ATC TGG TTC CAG AAC CGC CGC ATG AAG CAG AAG AAG CGC GTC
      **  **  **  *** *** **  *** *** **  *** *** **  **  *** *** *** *** *** **  *
AHOX1 AAC GAG ACC CAG GTC AAG ATC TGG TTT CAG AAC CGG CGG ATG AAG CAG AAG AAG CGG GAG
      **  *** *** *** **  *** *** *** **  *** **  **  **  *** *** *** *** *** **  ***
HOX1.6 AAT GAG ACC CAG GTG AAG ATC TGG TTC CAG AAT CGC CGC ATG AAG CAG AAG AAG CGT GAG
--------------------------------------------------------------------------------------
AHOX1 AAG GAG GCC TGG CCC CCT CCA
      181
```

(B)

```
                                                                                0
AHOX1                                                        GAG TAC GGC TTG GGG AGC CAG
--------------------------------------------------------------------------------------
      1                                                                        60
ANTP  CGC AAA CGC GGA AGG CAG ACA TAC ACC CGG TAC CAG ACT CTA GAG CTA GAG AAG GAG TTT
      *   **   **      **     *   * * ***      *  ***         *** **  *** *** *** **
AHOX1 CAG AAC AGC ATC CGG ACC AAC TTC ACC ACC AAG CAG CTG TCC GAG CTG GAG AAG GAG TTC
--------------------------------------------------------------------------------------
      61                                                                       120
ANTP  CAC TTC AAT CGC TAC TTG ACC CGT CGG CGA AGG ATC GAG ATC GCC CAC GCC CTG TGC CTC
      *** *** **   *  ***  ** *** **      **  ***  *  *** *** ***   *  ** ***     ***
AHOX1 CAC TTC AAC AGG TAC CTG ACC CGG GCC CGC AGG GTG GAG ATC GCC GCC ACC CTG GAG CTC
--------------------------------------------------------------------------------------
      121                                                                      180
ANTP  ACG GAG CGC CAG ATA AAG ATT TGG TTC CAG AAT CGG CGC ATG AAG TGG AAG AAG CGT GAG
      *   ***   * ***  *  *** **  *** **  *** **  *** **  *** ***   * *** *** **  ***
AHOX1 AAC GAG ACC CAG GTC AAG ATC TGG TTT CAG AAC CGG CGG ATG AAG CAG AAG AAG CGG GAG
--------------------------------------------------------------------------------------
AHOX1 AAG GAG GCC TGG CCC CCT CCA
      181
--------------------------------------------------------------------------------------
```

Fig. 2.8 Comparison of the predicted amino acid sequence of the Ahox1 homeo box with the Drosophila Antp, lab, and the mouse Hox1.6 homeo box amino acid sequences

(A) Predicted amino acid sequence of Ahox1 aligned with the amino acid sequence of the Drosophila lab and the mouse Hox-1.6 homeo domains. Amino acids shared with Ahox1 are indicated by three stars.

(B) Predicted amino acid sequence of Ahox1 aligned with the amino acid sequence of the Drosophila Antp homeo domain. Amino acids shared with Ahox1 are indicated by three stars.

(A)

```
        1                                                                  20
LAB     Asn Asn Ser Gly Arg Thr Asn Phe Thr Asn Lys Gln Leu Thr Glu Leu Glu Lys Glu Phe
            *** ***     *** *** *** *** ***     *** *** ***     *** *** *** *** *** ***
AHOX1   Gln Asn Ser Ile Arg Thr Asn Phe Thr Thr Lys Gln Leu Ser Glu Leu Glu Lys Glu Phe
            ***         *** *** *** *** *** *** *** *** ***     *** *** *** *** *** ***
HOX1.6  Pro Asn Ala Val Arg Thr Asn Phe Thr Thr Lys Gln Leu Thr Glu Leu Glu Lys Glu Phe

--------------------------------------------------------------------------------
        21                                                                 40
LAB     His Phe Asn Arg Tyr Leu Thr Arg Ala Arg Arg Ile Glu Ile Ala Asn Thr Leu Gln Leu
        *** *** ***     *** *** *** *** *** *** ***     *** *** ***     *** ***     ***
AHOX1   His Phe Asn Lys Tyr Leu Thr Arg Ala Arg Arg Val Glu Ile Ala Ala Thr Leu Glu Leu
        *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***     *** ***
HOX1.6  His Phe Asn Lys Tyr Leu Thr Arg Ala Arg Arg Val Glu Ile Ala Ala Ser Leu Gln Leu

--------------------------------------------------------------------------------
        41                                                                 60
LAB     Asn Glu Thr Gln Val Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Gln Lys Lys Arg Val
        *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
AHOX1   Asn Glu Thr Gln Val Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Gln Lys Lys Arg Glu
        *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
HOX1.6  Asn Glu Thr Gln Val Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Gln Lys Lys Arg Glu

--------------------------------------------------------------------------------
(B)

        1                                                                  20
ANTP    Arg Lys Arg Gly Arg Gln Thr Tyr Thr Arg Tyr Gln Thr Leu Glu Leu Glu Lys Glu Phe
                        ***             ***         ***         *** *** *** *** *** ***
AHOX1   Gln Asn Ser Ile Arg Thr Asn Phe Thr Thr Lys Gln Leu Ser Glu Leu Glu Lys Glu Phe
--------------------------------------------------------------------------------
        21                                                                 40
ANTP    His Phe Asn Arg Tyr Leu Thr Arg Arg Arg Arg Ile Glu Ile Ala His Ala Leu Cys Leu
        *** *** ***     *** *** *** ***     *** ***     *** *** ***         ***     ***
AHOX1   His Phe Asn Lys Tyr Leu Thr Arg Ala Arg Arg Val Glu Ile Ala Ala Thr Leu Glu Leu
--------------------------------------------------------------------------------
        41                                                                 60
ANTP    Thr Glu Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys Glu Asn
            ***     ***     *** *** *** *** *** *** *** *** *** ***     *** ***
AHOX1   Asn Glu Thr Gln Val Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Gln Lys Lys Arg Glu
--------------------------------------------------------------------------------
```

Table 2.1. Percentage of Nucleotide and amino acid identity of Ahox1 and other homeo boxes

HOMEO BOX	NUCLEOTIDE HOMOLOGY	AMINO ACID HOMOLOGY	REFERENCE
Mouse			
Hox 1.1	66%	58%	Kessel et al., 1987
Hox 1.2	65%	60%	Colberg-Poley et al.,1985
Hox 1.3	65%	65%	Odenwald et al., 1987
Hox 1.4	68%	67%	Duboule et al., 1986
Hox 1.5	72%	63%	McGinnis et al., 1984c
Hox 1.6	84%	90%	Baron et al., 1987
Hox 1.7	63%	61%	Rubin et al., 1987
Hox 2.1	64%	62%	Krumlauf et al., 1987
Hox 2.3	62%	55%	Meijlink et al., 1987
Hox 3	56%	55%	Awgulewitsch et al., 1986
Human			
Hu1	68%	62%	Levine et al., 1984
Hu2	62%	57%	Levine et al., 1984
HHOc.8	61%	52%	Simeone et al., 1987
HHOc.1	58%	57%	Simeone et al., 1987
Xenopus			
XLHBox1	56%	58%	Fritz & DeRobertis., 1988
XLHBox2	56%	62%	Wright et al., 1987
XLHbox3	60%	60%	Fritz & DeRobertis, 1988
XLHbox4	57%	62%	Fritz & DeRobertis, 1988
XLHBox5	59%	62%	Fritz & DeRobertis, 1988
XHOX1A	65%	63%	Harvey et al., 1986
Drosophila			
Antp	65%	60%	Schneuwly et al., 1986
Ftz	65%	57%	Laughon & Scott, 1984
Cad	54%	<50%	Mlodzik et al., 1987
en	58%	51%	Poole et al., 1986
eve	61%	52%	Macdonald et al., 1986
prd	51%	<50%	Frigerio et al., 1986
lab	79%	85%	Mlodzik et al. 1988

Fig. 2.9. Determination of copy number of Ahox1

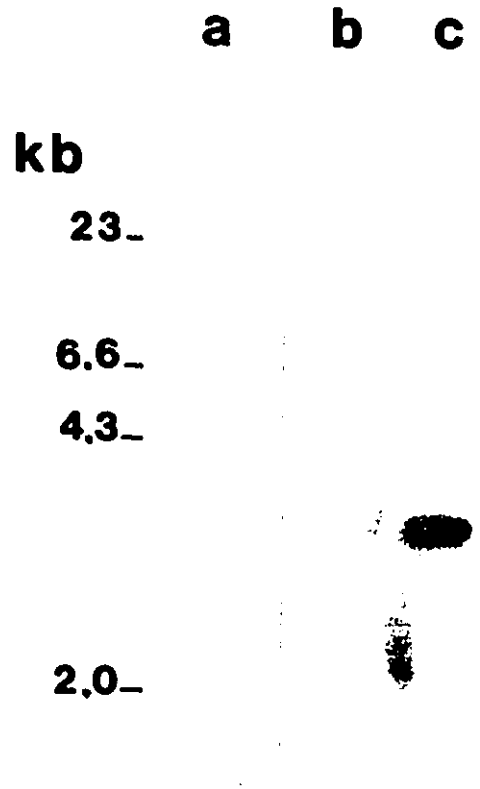
Axolotl genomic DNA (10 μg) was digested to completion with EcoRI, electrophoresed on a 0.8% agarose gel, transferred to Hybond N nylon membranes, and hybridized to HB-Kpn. High stringency hybridization conditions were used (50% formamide, 42°C).

As an internal standard, the plasmid pTZ19R containing HB-Kpn linearized with EcoRI, was run on the same gel. One and ten copy number equivalents/genome of this plasmid were run on the gel.

Lane a: axolotl genomic DNA

Lane b: 1 copy/genome internal standard (0.6 pg)

Lane c: 10 copies/genome internal standard (6.0 pg)


a
b
c
kb
23
6.6
4.3
2.0

4. Copy number of Ahox1

The copy number of Ahox1 was determined by Southern analysis using a plasmid (pTZ19R) containing HB-Kpn as an internal control. As shown in Fig. 2.9., the 4.0 kb fragment containing Ahox1 is present in this genome in single copy. This experiment was performed using high stringency conditions and two EcoRI fragments were observed hybridizing to the HB-Kpn probe. Since there is no EcoRI site in HB-Kpn, there are two highly conserved copies of Ahox1 in this genome.

5. Genomic hybridizations using Ahox1 homeo box as a probe

Since Ahox1 and the mouse Hox1.6 homeo box sequences show a high degree of homology, mouse and axolotl genomic DNA was examined for the presence of Ahox1-like sequences. Reduced stringency genomic hybridizations (43% formamide, 37°C) using HB-Kpn as a probe were performed on axolotl and mouse genomic DNA digested with EcoRI (Fig.2.10). Highly conserved cross-hybridizing sequences to Ahox1 were found in both the axolotl and mouse genomes. In the mouse, one of these is presumably the Hox-1.6 gene. The Hox-1.6 gene is located on an 8 kb EcoRI fragment. A hybridizing fragment of this molecular weight is seen in Fig. 2.10. Moreover, sequences detected in the mouse genome with the Antp probe were different from those detected with the Ahox1 probe.

Reduced stringency hybridization of Drosophila genomic DNA, digested with HindIII, with the HB-Kpn probe

revealed a single cross-hybridizing fragment of about 6 kb, corresponding to the lab gene. No cross-hybridization to the Antp sequence could be detected (see Fig.2.1), and thus the low stringency hybridization conditions used to isolate Ahox1 were appropriate.

6. Detection of Ahox1 sequences in other Ambystoma species

A genomic Southern was prepared with DNA from several Ambystoma species, and this was probed with HB-Kpn. This Southern was hybridized using reduced stringency conditions. The genomic DNA was digested with EcoRI. The results of this experiment are shown in Fig. 2.11. Two common hybridizing bands are seen in all of the Ambystoma species, and a third band is seen in Ambystoma maculatum.

7. Detection of transcripts of Ahox1 during axolotl development

Northern blots were prepared from total RNA from axolotl embyos at 6 developmental stages (see Appendix I for description of stages). Transcripts were detected using the HB-Kpn probe. Two transcripts were detected in RNA from neurula and tail bud embryos, suggesting a stage specific pattern of expression of transcripts containing the Ahox1 homeo box. These transcripts were also detected to a lesser extent in stage 35 (pre-hatching) embryos. The sizes of the transcripts were approximately 1.6 and 2.1 kb (Fig. 2.12).

Fig. 2.10. Hybridization of axolotl, mouse, and Drosophila DNA under reduced stringency conditions with HB-Kpn.
Genomic DNA was digested to completion, electrophoresed on a 0.8% agarose gel, transferred to Hybond N nylon membranes, and hybridized with HB-Kpn. Mouse and axolotl DNA was digested with EcoRI, and Drosophila DNA was digested with HindIII.
Conditions of reduced stringency were used. The filters were exposed to Kodak XAR-5 film at -70°C for five days.
Lane a,b,d : hybridization of Drosophila melanogaster (Dm), Axolotl (Am), and mouse (Mo) genomic DNA with the Ahox1 homeo box probe.
Lane c: hybridization of mouse (Mo) genomic DNA with the Drosophila Antp homeo box probe.

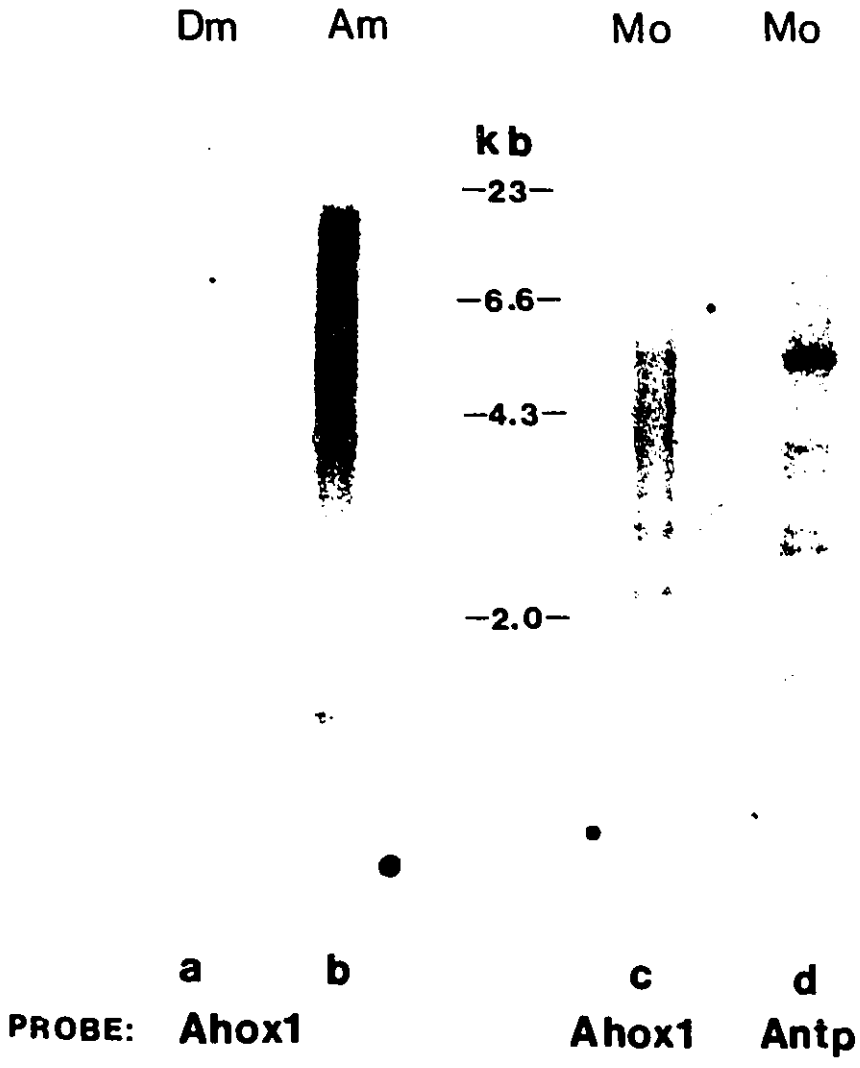

Dm
Am
Mo
Mo
kb
23
6.6
4.3
2.0
a
b
c
d
PROBE:
Ahox1
Ahox1
Antp

Fig. 2.11. Hybridization of various Ambystoma species with HB-Kpn.

Genomic DNA (10 μg) was digested to completion with EcoRI, electrophoresed on a 0.8% agarose gel, transferred to Hybond N nylon membrane, and hybridized with the KpnI fragment of Ahox1 containing the homeo box sequence under reduced stringency.

Lanes a: A. maculatum

b: A. tigrinum

c: A. mexicanum (wild type DNA)

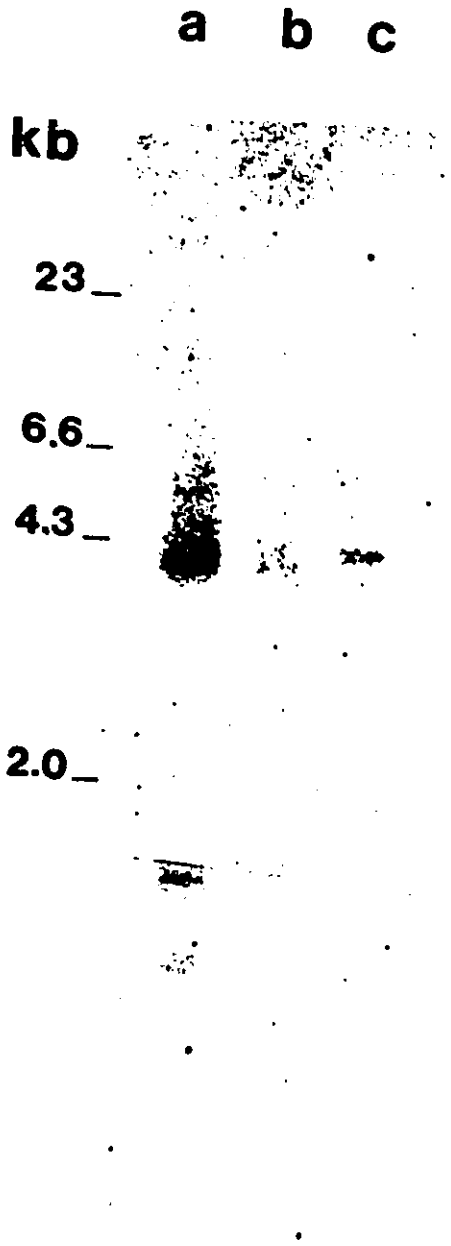

a
b
c
kb
23
6.6
4.3
2.0

Fig. 2.12. Northern analysis of transcripts containing the Ahox1 sequence throughout axolotl development.

Northern transfer hybridization was performed using total RNA from 6 different stages of embryogenesis (see appendix 1). 15 μg of total RNA was loaded per lane, and hybridized under high stringency to HB-Kpn.

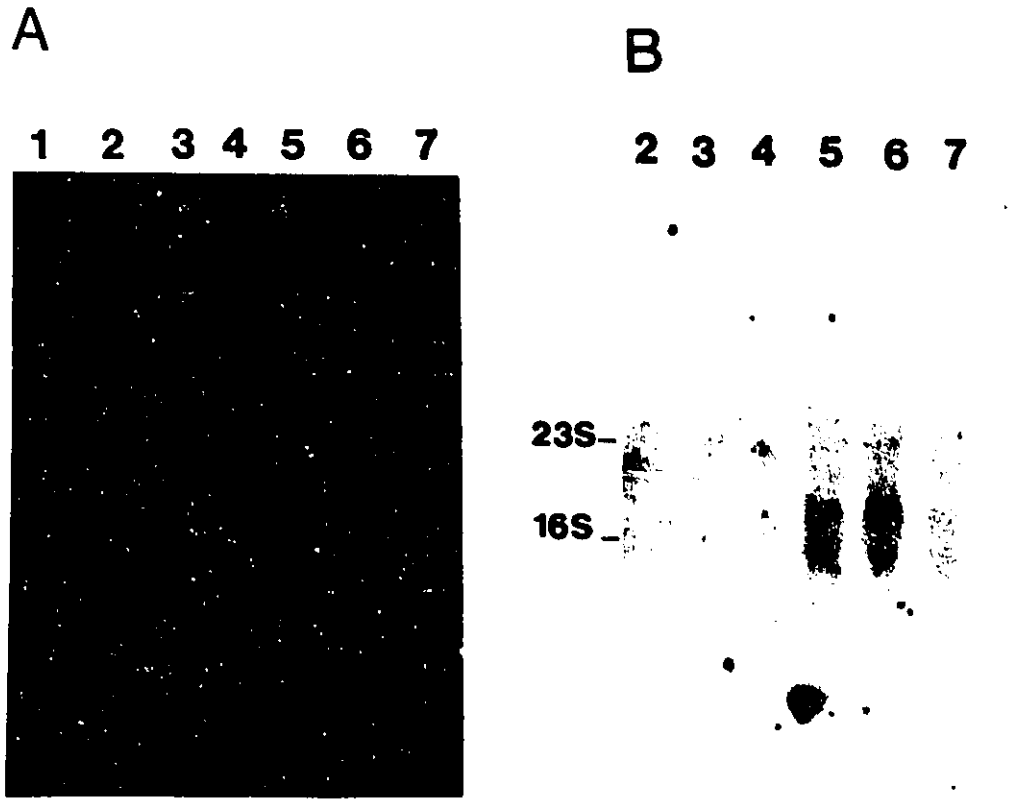
(A). Ethidium bromide stained gel of total RNA from axolotl embryos

1. E. coli 23 and 16S ribosomal standards
2. RNA from oocytes (stage 1)
3. RNA from stage 8 embryos (blastula)
4. RNA from stage 10 3/4 embryos (gastrula)
5. RNA from stage 15 embryos (neurula)
6. RNA from stage 25 embryos (tail bud)
7. RNA from stage 35 embryos (pre-hatching)

(B) Northern blot of gel in (A), probed with HB-Kpn

RNA samples in lanes 2-7 are as described in (A)

Hybridizing transcripts are seen in RNA from stage 15 (neurula) and stage 25 (tail bud) embryos, and to a lesser extent in stage 35 (pre-hatching) embryos. Transcript sizes (1.6 and 2.1 kb) were determined by their mobility versus E. coli 23S and 16S ribosomal RNAs.


A
1 2 3 4 5 6 7
B
2 3 4 5 6 7
23S
16S

D. Discussion

To date, all vertebrate genomes examined contain multiple highly conserved copies of homeo boxes similar to the Drosophila Antp sequence, with the conservation often being higher at the protein than the nucleotide level. For example, the chicken genome has about nine EcoRI fragments which hybridize to the Antp probe, while human, mouse and Xenopus have about six (McGinnis et al., 1984b; Carrasco et al., 1984). Compared to other vertebrate genomes, the Ambystoma species appear to have lower numbers of Antp-like sequences. Whether A. mexicanum, in fact, contains highly conserved copies of this sequence remains to be determined.

The role of homeo box sequences in development has been the subject of investigation for the past several years. In Drosophila, it is clear that genes containing this sequence play an important role in embryogenesis. This conclusion is largely due to the number of mutant genes available.

Initially, it was proposed that the conservation of Antp-like homeo box sequences in very diverse organisms could be correlated with the possession of a segmented body plan (McGinnis, 1985). However, shortly after this proposal, Antp-like homeo box sequences were reported in the unsegmented sea urchin (Dolecki et al., 1986). In another study, Holland and Hogan (1986) reported both the presence and absence of Antp homeo box sequences in a number of

organisms with varied developmental strategies. In addition, they reported Antp-like homeo box sequences in molluscs, whereas two different strains of molluscs were previously reported as negative (McGinnis 1985). It would appear then that Antp sequences are involved in more fundamental developmental processes, perhaps acting as regulatory genes in deciding between developmental fates. In organisms considered negative for the presence of Antp homeo box sequences, alternative sequences which are significantly diverged from the Antp homeo box may have evolved to perform a cognate function, or the conservation of the Antp sequences may be at the protein level, and hence they are not detected in genomic Southerns with reduced stringency conditions. The Ambystoma species may contain a number of sequences which would be categorized as an Antp class homeo box at the protein level.

Lower stringency hybridization of axolotl DNA did, however, show a number of cross-hybridizing sequences with the Antp probe. Cloning and sequencing of a homeo box from the axolotl genome confirmed that low stringency hybridization conditions (30% formamide, 37°C) required to detect these sequences were appropriate for detecting this sequence, which is only 66% homologous to Antp. This conclusion was reinforced by the failure of the Ahox1 probe to cross-hybridize with Antp under reduced stringency conditions. In fact, under such conditions, a single cross-hybridizing fragment was observed when Drosophila

genomic DNA was probed with Ahox1. This HindIII fragment corresponds to a fragment containing the homeo box of the labial gene as predicted from the restriciton map of this region of the genome given by Mlodzik et al., (1988). The labial homeo box is 79% homologous to Ahox1, but is only 64% homologous to Antp.

The Ahox1 homeo box sequence was found to be most closely related to the Hox-1.6 homeo box sequence of mouse. Both of these are significantly diverged from Antp at the protein level, showing even lower homology than at the nucleotide level. On the other hand, Ahox1 and Hox-1.6 have higher homology to each other at the protein level than the nucleotide level. Of particular interest is the homology between Ahox1 and Hox-1.6 in the first 14 amino acids of the homeo box sequence. Here, these sequences are 70% homologous at the protein level compared to 21% with Antp. Perhaps the 5′ region of these homeo box sequences may have a functional role in their specificity.

The complete cDNA sequence for the gene ERA1 (which corresponds to the Hox-1.6 gene) has recently been reported (LaRosa and Gudas, 1988), permitting a comparison with the sequence of HB-Kpn outside the homeo box (see chapter 4, Fig. 4.4.) Though no significant homology was found, this lack of homology would, of course, be expected if the KpnI fragment contained intronic sequences. The ERA-1/Hox- 1.6 gene has an intron just upstream from the homeo box, but the

KpnI fragment contains no consensus sequence for a 3' splice acceptor site (6PyNCAG) in this region.

Likewise, there is no significant homology with the sequence from the lab gene outside the homeo box, except that both Ahox1 and lab code for a stretch of polyglycine 5' to the homec box. Again, however, more information on the genomic organization of HB-Kpn is required before firm conclusions can be drawn.

Highly conserved sequences cross-hybridizing to Ahox1 were found in both axolotl and mouse genomes. In the mouse, one of these sequences is presumably the Hox-1.6 gene. Also, sequences in the mouse genome detected with the Ahox1 probe were different from those detected with the Antp probe.

In addition to the presence of Ahox1-like sequences in the mouse, Ahox1 sequences were also detected in other closely related Ambystoma species. All three species had a similar pattern of EcoRI fragments hybridizing to the Ahox1 sequence. A. maculatum had an additional hybridizing fragment of about 1 kb in size. This suggests that not only was the Ahox1 homeo box conserved among these species, but that the EcoRI fragments containing these sequences were also conserved.

The copy number of the 4.0 kb EcoRI fragment containing the Ahox1 homeo box was determined to be one. This is of significance since it indicates that the hybridization observed with the Antp probe was not due to the selective

by the same gene. The presence of multiple transcripts has been observed for several homeo box-containing genes. In the case of the ERA-1/Hox-1.6 gene, the homeo box sequence is not translated from one of the transcripts due to alternate splicing which causes an in-frame stop codon. In the case of the Xenopus homeo box gene XlHbox2 (Wright et al., 1987), one transcript contains a homeo box while the other does not. A further example of multiple transcripts for a homeo box containing gene is the Xenopus homeo box containing gene, Xlhbox1, which produces two predominant transcripts during neurulation from two different promoters (Cho et al., 1988), both of which contain the homeo box. In the case of Ahox1, transcripts were detected with the HB-Kpn probe which includes sequences outside the homeo box. The reason for detection of two transcripts remains to be determined. The fact that these transcripts only occur at specific stages implies a developmental function for their gene products.

The development of the Mexican axolotl has been extensively studied, and it is now important to identify the genes involved in these processes. The Ahox1 probe reported here will be an invaluable aid for identifying some of these genes.

presence of highly diverged Antp sequences in higher copy number than conserved Antp-like sequences. Also, it confirms the hypothesis that unique sequences in urodeles contain single copy genes (Baldari and Amaldi, 1976). The increase in genome size in these amphibians is due to an increase in all classes of DNA (repetitive, intermediate repetitive, and unique). As a result of the increases in all three DNA classes, the unique class has more sequence complexity than in smaller genomes. The success of isolating a homeo box sequence is encouraging in light of the large genome size, and may help put to rest the fears that the large size of the axolotl's genome would be a serious impediment to gene isolation.

The other clone, pAhox2, which did not contain a complete homeo box, may be a regulatory gene based on its similarity with the DNA recognition helix of the homeo box. Although this clone was 3.5 kb in size, which corresponds to one of the positions for an EcoRI genomic repeat, it was not present in high copy number, nor did it show sufficient homology to Antp to detected the Antp sequence in Drosophila DNA with conditions of reduced stringency.

Two transcripts homologous to the Ahox1 probe were detected in the both neurula and tail bud stages of development. The detection of multiple transcripts could be due to cross-hybridization of the Ahox1 homeo box with the products of two genes containing highly related homeo box sequences (Fig. 2.7), or these transcripts could be produced

CHAPTER III

EXPRESSION OF EXOGENOUS SEQUENCES IN THE AXOLOTL

A. Rationale

The introduction of cloned DNA sequences into developing organisms by microinjection has been very useful in the study of gene expression. In particular, the reintroduction of altered DNA sequences has aided in the understanding of the sequences necessary for the correct temporal and spatial expression of genes. In vertebrates, the lack of developmental mutations for specific genes will ultimately make it necessary to utilize gene transfer techniques to understand developmental processes.

The objectives of this study were to examine the possibility of using fertilized axolotl eggs to express injected DNA sequences. This was tested by using a variety of eukaryotic promoters fused with an easily identifiable reporter sequence gene (*E.coli lacZ*). In addition, the use of such gene expression as a cell lineage marker was examined, as it is often useful to follow the fate of cells or groups of cells during development.

In the axolotl, the interval to first cleavage is about six hours and should provide sufficient time for integration of injected sequences at the one cell stage. In addition, the axolotl's regenerative capabilities provide a convenient

means of following continued expression of injected sequences without having to sacrifice the animal.

B. MATERIALS AND METHODS

1. Plasmids used for microinjection

All plasmids used in this study consisted of a eukaryotic promoter, the E. coli lacZ coding sequence, and a SV40 polyadenylation site, cloned into a plasmid vector. The eukaryotic β-galactosidase expression vector pCH110 was purchased from Pharmacia. This plasmid contains the SV40 early promoter. The Drosophila copia promoter obtained from the plasmid pcopneo (Rio and Rubin, 1985) was cloned into pCH110 which had been linearized with HindIII (nucleotide position 0)and NcoI(nucleotide position 7130) . The promoter was inserted into this vector by ligation in the presence of Klenow enzyme and dNTP's. This plasmid was called pLTR110. The plasmid pCAB17, containing the mouse PGK (phosphoglycerate kinase) promoter, was obtained from Dr. M. McBurney, University of Ottawa. The construct containing the mouse hsp68 (heat shock) promoter was a gift from Dr. J. Rossant, University of Toronto. This plasmid is called pKP110. The plasmid DNAs were linearized prior to injection. pKP110 and pCAB17 were linearized with XbaI, and pCH110 and pLTR110 were linearized with ScaI. Restriction maps of the plasmids used in this study are given in Fig. 3.1.

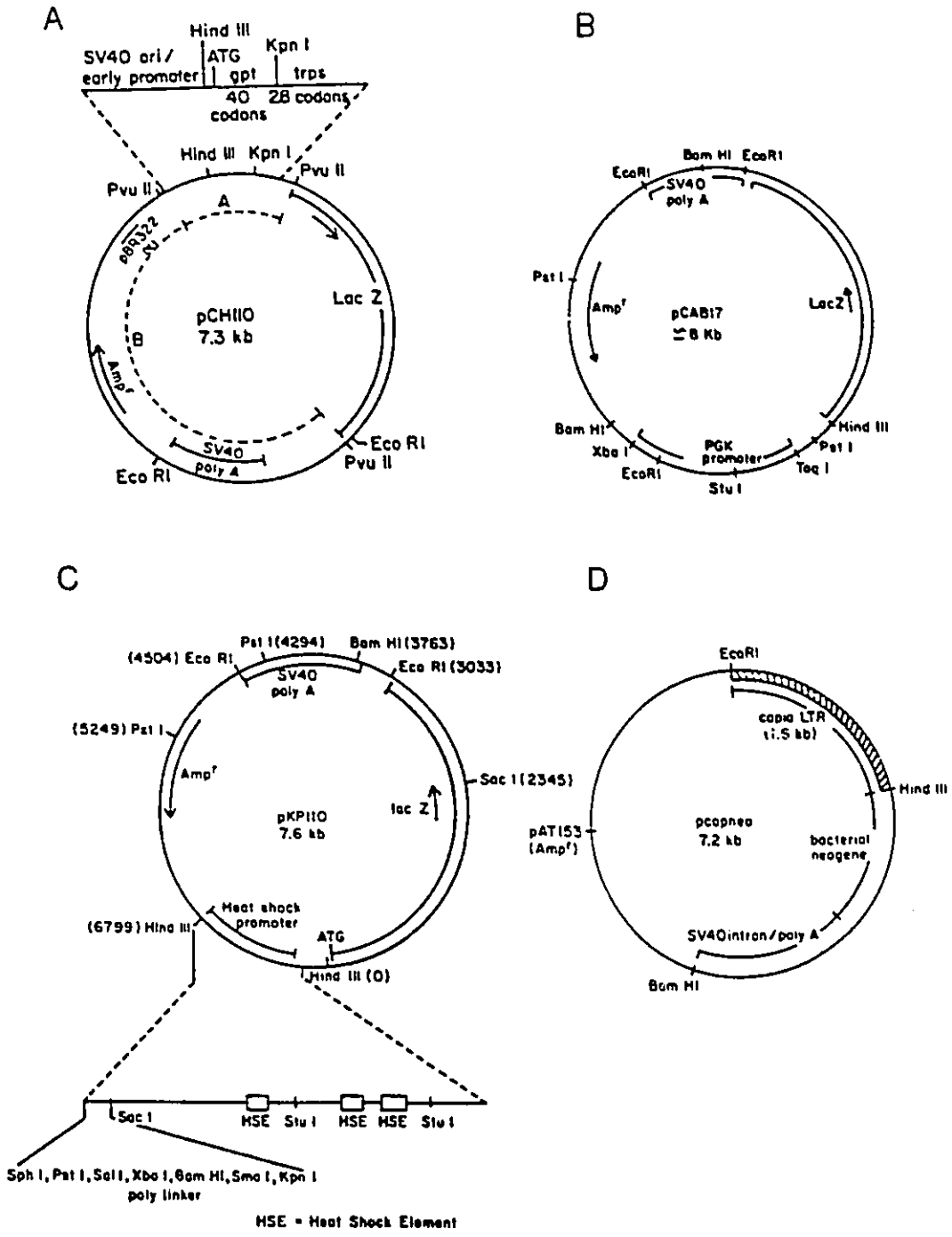
Fig. 3.1 Restriction maps of lacZ fusion construct plasmids

A. pCH110 containing the SV40 early promoter

B. pCAB17 containing the mouse phosphoglycerate kinase promoter

C. pKP110 containing the mouse hsp68 promoter

D. pcopneo containing the Drosophila copia promoter, which was cloned into pCH110


A
Hind III
Kpn I
SV40 ori / early promoter
ATG
gpt
trps
40 codons
28 codons
Hind III
Kpn I
Pvu II
Pvu II
pBR322 ori
A
B
pCH110
7.3 kb
Lac Z
Amp^r
SV40 poly A
Eco RI
Pvu II
Eco RI
B
Bam HI
EcoRI
EcoRI
SV40 poly A
Pst I
Amp^r
pCAB17
≃8 Kb
LacZ
Bam HI
Xba I
EcoRI
PGK promoter
Stu I
Taq I
Pst I
Hind III
C
Pst I (4294)
Bam HI (3763)
(4504) Eco RI
Eco RI (3033)
SV40 poly A
(5249) Pst I
Amp^r
Sac I (2345)
pKP110
7.6 kb
lac Z
Heat shock promoter
(6799) Hind III
ATG
Hind III (0)
Sac I
HSE
Stu I
HSE
HSE
Stu I
Sph I, Pst I, Sal I, Xba I, Bam HI, Sma I, Kpn I
poly linker
HSE = Heat Shock Element
D
EcoRI
copia LTR
(1.5 kb)
Hind III
pAT153
(Amp^r)
pcopneo
7.2 kb
bacterial neogene
SV40intron/poly A
Bam HI

2. Microinjection of axolotl eggs

Axolotl eggs were obtained from spawnings performed at the University of Ottawa axolotl colony. Both albino and wild type embryos were used. The eggs were manually dejelled using watchmaker's forceps, and were microinjected within 30 minutes of egg deposition. The microinjection was performed according to the method of Stephens et al. (1981). The eggs were injected in 25% Steinberg's solution (Asashima et al., 1989) containing 5% ficoll. Approximately one nanogram of DNA (in water), was injected into each egg. Following injection, the eggs were allowed to develop overnight in the Steinberg's-ficoll solution, and were subsequently transferred to 25% Holtfreter's media (Asashima et al., 1989) containing 0.01% penicillin and streptomycin. The embryos were staged according to Bordzilovskaya et al. (1989).

3. Histochemical staining of embryos for β-galactosidase activity

Embryos or tail tips were fixed overnight at 4°C in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. Following fixation, the embryos (tail tips) were rinsed three times over 24 hours in PBS, and were stained in the solution described by Sanes et al. (1986) containing 400 μg/mL X-Gal (4-Cl-5-Br-3-indolyl-β-galactosidase). Staining was done at room temperature for 2 to 16 hours.

4. Isolation of DNA from axolotl embryos

DNA was isolated from embryos essentially as described by Etkin et al. (1984), except that one embryo was homogenized per mL of lysis buffer. Approximately 20-30 μg of genomic DNA was obtained from one tail bud embryo. For the isolation of DNA from embryos shortly after microinjection, an uninjected neurula was used as a source of carrier DNA.

5. Southern transfer DNA hybridization

Restriction enzyme digested genomic DNA,(5 μg) was electrophoresed through a 0.7% agarose gel and was transferred to Hybond N (Amersham) by the method of Mann and Reed, (1985). The DNA was hybridized to vector DNA TaqI fragments labelled by the random primer method (Feinberg and Vogelstein, 1983). The filters were hybridized overnight at 42°C in 50% deioninzed formamide, 5X SSC, 50 mM sodium phosphate (pH 6.5), 5X Denhardt's solution, 100 μg/mL heat denatured sheared herring sperm DNA and 0.5% SDS.

C. RESULTS

1. Expression of the E. coli lacZ gene from various promoters

Four eukaryotic promoters were chosen for these experiments. These included the SV40 early promoter, the

Drosophila copia promoter, the mouse PGK (phosphoglycerate kinase) promoter, and the mouse hsp68 (heat shock) promoter.

Embryos were injected at the one cell stage and allowed to develop to late neurula or tail bud stages. They were then stained for β-galactosidase activity. In some cases, various early and late stages were also monitored.

β-galactosidase activity was detected in axolotl embryos from all except the copia promoter. Various responses were observed from the other three promoters. The expression from the SV40 promoter was restricted to the brain and neural tissues in the tail bud embryos (stage 25). The staining in these cells was not very intense. Other stages were not tested. The mouse PGK promoter gave labelling in all quadrants of the embryo, though not all cells were labelled. Occasionally, a patch of expression was observed. Intense β-galactosidase staining was observed in these embryos, with the blue color reaction being obvious within 20 minutes of adding the X-Gal substrate. Due to the dramatic activity observed in tail bud embryos with this promoter, other stages were also tested for β-galactosidase activity. In all cases up to late tail bud (stage 32), the same pattern of expression was observed, that is, not all cells were expressing the E.coli lacZ gene. This promoter functioned equally well in both wild type and albino embryos (Fig. 3.2).

The best results were obtained with the mouse heat shock promoter. Surprisingly, embryos which were heat

shocked (33°C, 90 minutes) did not show any difference in expression from embryos which had not been heat shocked. This construct gave a high level of gene expression as judged by intense β-galactosidase staining, and was expressed in a large proportion of cells (Fig. 3.3). In some cases the embryos showed a patch of expression. No β-galactosidase activity was detected in uninjected embryos. Table 3.1 summarizes the results of microinjection of this plasmid into embryos at the one cell stage from three different spawnings.

Persistent expression of injected DNA was evidenced by expression of β-galactosidase in three month old animal tail tips. These animals had been injected with the plasmid pCH110 (SV40 promoter) or pCAB17 (mouse PGK promoter). Four out of five animals injected with pCH110 tested positive for β-galactosidase activity. One out of four animals injected with pCAB17 tested showed β-galactosidase activity. Animals injected with the mouse hsp68 construct are currently being raised, and they too will be tested for continued expression of injected DNA sequences.

Fig. 3.2 Examples of β-galactosidase activity in embryos injected with the plasmid pCAB17 (mouse PGK promoter) (A) Albino embryo (tail bud) injected with the plasmid pCAB17 at the one cell stage showing cells labelled both internally and externally. (B) Wild type embryo (tail bud) injected into one cell at the two cell stage with the plasmid pCAB17 showing a sector of β-galactosidase expression on the head.

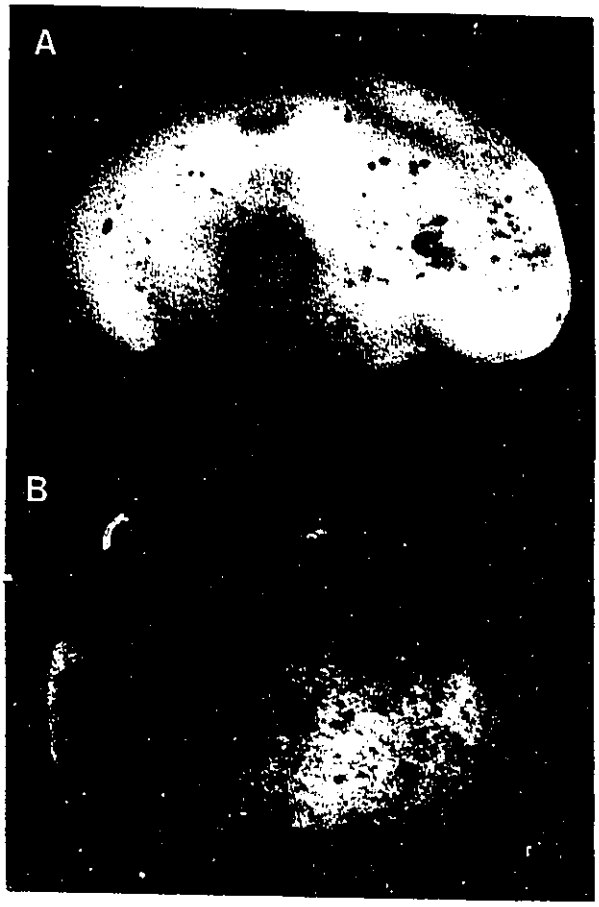

A
B

Fig. 3.3. Examples of β-galactosidase activity in embryos injected with the plasmid pKP110 (mouse hsp68 promoter). (A) Wild type embryo (late neurula) injected with the plasmid pKP110 at the one cell stage showing intense staining in a large proportion of cells. (B) Wild type embryo (tail bud) injected with the plasmid pKP110 into one cell at the two cell stage. Note the intense staining of most of the cells in the head. The embryos shown here were not heat shocked.

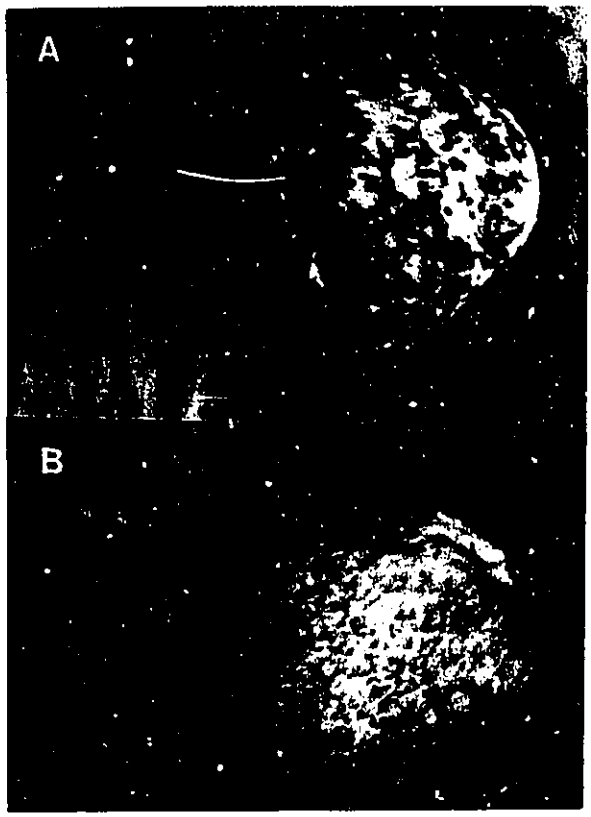

A
B

Table 3.1. Microinjection of pKP110 at the one cell stage

Exp#	1	2	3	control
Total	50	50	50	50
Infertile	8	9	4	13
Abortive	12	18	9	7
Exogastrula	7	5	10	6
Normal	23	18	27	24
Blue	7	10	11	-
BlueP*	2	1	3	-
%Blue	30	55	41	-

Embryos were injected with 1 ng of XbaI linearized pKP110, and were allowed to develop to late neurula or early tail bud stages of development. They were then stained for β-galactosidase activity. Each experiment represents a different spawning. Blue refers to the number of embryos which were blue in all quadrants: blueP* is the number of embryos with a patch of β-galactosidase activity: In the control experiment, embryos were injected with 75 nL of water.

Table 3.2. Microinjection of pKP110 in two and four cell stage embryos

Exp#	1	2	3
Total	45	20	15
Exogastrula	12	7	3
Normal	33	13	12
BlueP*	17	7	10
%Blue	51	53	83

Embryos were injected with pKP110 into a cell as described above, and were allowed to develop to late neurula, or early tail bud stages of development, and were then stained for β-galactosidase activity. Each experiment represents a different spawning. BlueP* is the number of embryos with a blue patch of β-galactosidase activity, and %blue is the percentage of embryos showing β-galactosidase activity.

2. Microinjection of DNA into 2 and 4 cell stage embryos: application as a cell lineage marker

The plasmid pCAB17 (PGK promoter) or pKP110 (hsp68 promoter) was injected into 2 and 4 cell stage embryos to determine if expression of β-galactosidase could be used to follow the fate of cells. With two cell embryos, the pattern of expression ranged from almost one half of the embryo being labelled to a sector or patch of the embryo being labelled (Fig. 3.2). When one cell was injected at the four cell stage, a patch of expression was also observed. With the PGK promoter, not all cells in the patch were labelled, however with the mouse heat shock promoter, most of the cells were labelled (Fig. 3.3). The results of microinjection of embryos at the two and four cell stage with pKP110 are given in Table 3.2.

3. Detection of DNA sequences in microinjected eggs

Embryos injected with pKP110 were tested for β-galactosidase activity at stage 33 by staining one half of each embryo with X-gal, and DNA was prepared from the other half. DNA from three positive embryos and from one negative embryo were then examined for the presence of plasmid DNA (Fig.3.4). The results show that the embryos which expressed β-galactosidase activity contained the plasmid

DNA. Digestion of genomic DNA with XbaI gave a single hybridizing fragment of 7.6 kb corresponding to linearized plasmid DNA. Some background hybridization was noted in the embryos containing injected DNA. The reason for this is not clear, particularly since background hybridization was observed above the 7.6 kb linearized plasmid DNA. However, it may be due, in part, to degraded DNA from high molecular weight forms. Some degraded DNA may have been generated during the DNA isolation procedure. Alternately, this degraded DNA may have been generated by unstable forms of the injected DNA (such as tail-to tail, or head-to-head concatemers).

Digestion of genomic DNA with EcoRI gave two hybridizing fragments, which would be expected if the plasmid DNA was concatemerized (see Fig.3.1), as head-to-tail repeats. If the injected DNA had remained in a linear form, three fragments would have been obtained. This injected DNA migrated with high molecular weight DNA in undigested samples (data not shown). The copy number of all three embryos analyzed was approximately 100 copies/genome, although in other experiments as few as 10 and greater than 100 copies/genome have been observed.

In another experiment, high molecular weight plasmid DNA was observed within three hours of microinjection (Fig. 3.5). This DNA was migrating above the 23 kb molecular weight marker, indicating that the injected DNA was being converted to a higher molecular weight form (either as

linearized or circular multimers). Alternatley, this DNA may represent open circular forms of the plasmid. Plasmid DNA was also observed below the injected linear DNA (lowest band Fig. 3.5) This band is likely supercoiled plasmid DNA. This experiment provides evidence that shortly after injection the DNA is undergoing ligation, and is not simply existing as injected linear molecules.

D. DISCUSSION

To date, most gene expression studies using amphibians have centered around the frog, Xenopus laevis. In Xenopus, the short time before first cleavage leads to random distribution and integration of injected sequences in cleaving nuclei resulting in animals which are mosaic (Etkin and Pearman, 1987). By contrast, the DNA injected into axolotl eggs appears to be distributed in a non-random fashion, either by entering the nucleus at the one cell stage, or, less frequently, a single nucleus after a few cell divisions. As a consequence of this, the embryos are labelled in all quadrants or in a patch which arose from a single blastomere. Embryos injected at the two or four cell stage also gave a patch of β-galactosidase expression. Embryos with two or more non-contiguous labelled patches or sectors were not observed.

Fig. 3.4. Detection of DNA in stage 33 embryos injected with the plasmid pKP110.

Genomic DNA (5μg) was digested to completion, electrophoresed on a 0.7% agarose gel, transferred to Hybond N nylon membranes, and hybridized with random TaqI vector fragments. The filters were exposed to Kodak XAR-5 film overnight at -70°C.

Lanes 1,2,3: Genomic DNA from three individual embryos showing β-galactosidase activity. The DNA was digested with XbaI.

Lane 4: Genomic DNA from an embryo not showing β-galactosidase activity. The DNA was digested with XbaI.

Lanes 5,6,7 Genomic DNA from three individual embryos showing β-galactosidase. The DNA was digested with EcoRI.

Lane 8: Genomic DNA from an embryo not showing β-galactosidase activity. The DNA was digested with EcoRI

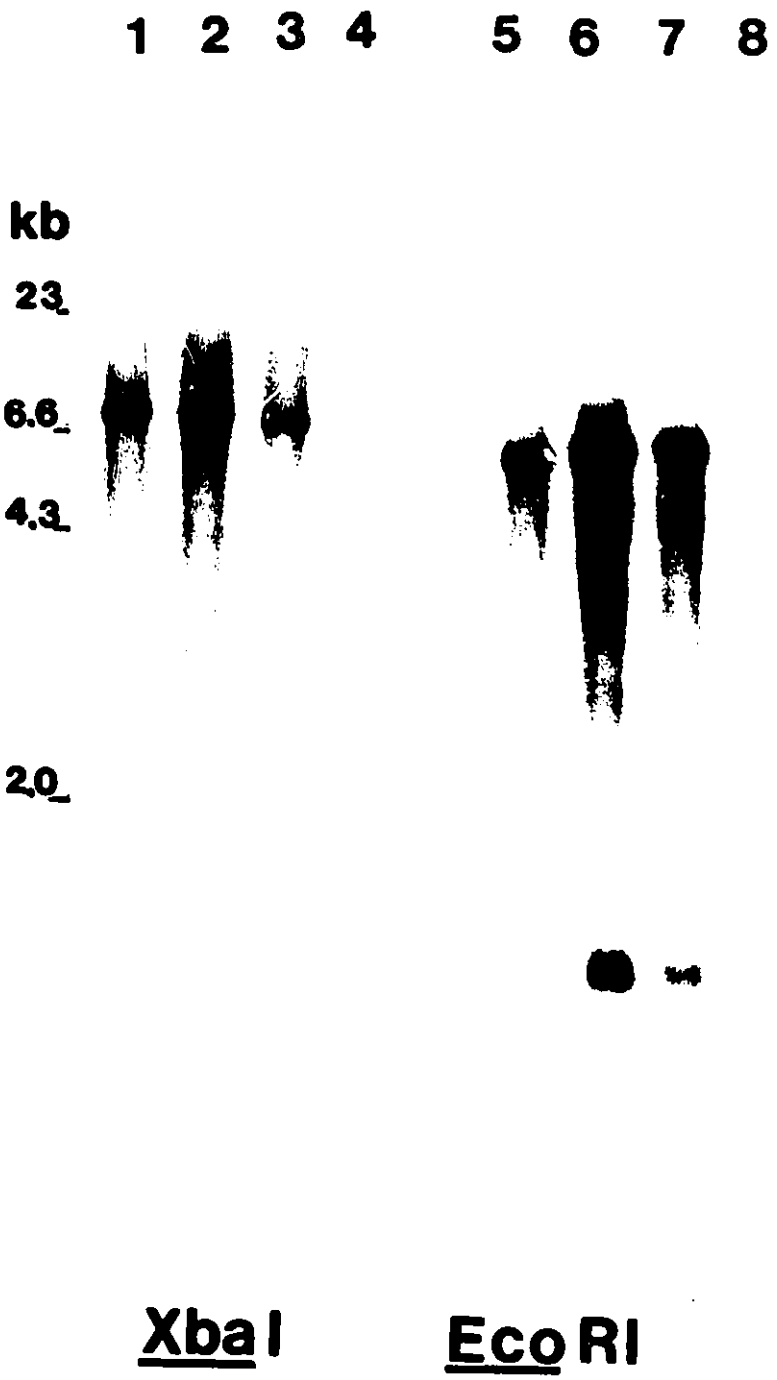
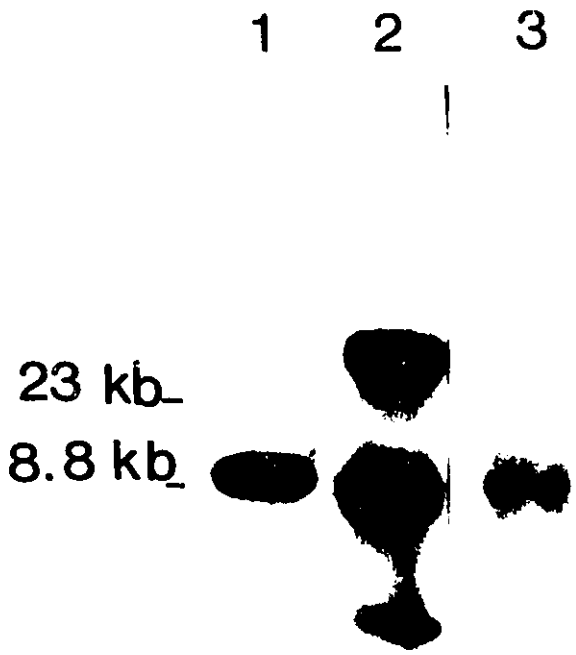

1 2 3 4 5 6 7 8
kb
23
6.6
4.3
2.0
Xba I
Eco RI

Fig. 3.5. Detection of DNA in microinjected embryos. Southern transfers were prepared and hybridized as described in Materials and Methods. Lane 1 contains 0.25 ng of linearized plasmid DNA. Lane 2 shows the state of the DNA 3 hours after microinjection. The injected DNA at this point has been converted into high molecular weight concatemers or circular molecules, with some of the DNA remaining linear. Lane 3 shows the detection of DNA in a tail bud embryo. Genomic DNA (5 μg) was digested with ScaI prior to electrophoresis. The amount of DNA represented as plasmid is approximately 50 copies per genome as judged from internal standards.


1
2
3
23 kb
8.8 kb

The most likely conclusion from these results is that the DNA is integrated into the chromosomes of a single cell, and this DNA is then passed to its descendants. This conclusion is based largely on the patterns of expression obtained with the mouse heat shock promoter, in which most cells were labelled. The failure of some cells to express β-galactosidase could be due to a loss of integrated sequences, or a failure of expression by some cells containing the DNA. Similar patterns of patchy expression of *lacZ* constructs has been reported in tissue culture cells (MacGregor *et al.*, 1987), for pKP110 in transgenic mice (Kothary *et al.*, 1989). The reason that not all cells express *β-galactosidase* in these cases is due to inefficient translation from a bacterial translational start site. If the observed patterns of expression were due to partitioning of the DNA among blastomeres early in development, this would almost inevitably lead to the labelling of non-contiguous sectors. Since these were never observed, the distribution of sequences by partitioning seems unlikely.

Although constitutive expression from the *hsp68* promoter was somewhat surprising, the response of the axolotl transcriptional machinery to foreign promoter elements could not have been predicted.

With the mouse PGK promoter, not all cells were labelled. However, the pattern of expression was otherwise similar to that obtained with the heat shock promoter, either with cells labelled throughout the embryo, or within a single quadrant or patch. Again, non-continuous labelled patches were not observed. The non-uniform labelling with the PGK promoter may be due to the presence of a bacterial translation initiation site as mentioned above. The selective labelling of neural tissues with the SV40 promoter may be due to tissue specific expression, rather than selective presence of the DNA in those tissues.

One of the factors responsible for the non-random distribution of the injected DNA may well be the long interval to first cleavage. Southern transfer DNA hybridization experiments demonstrated that injected plasmid DNA is converted to a higher molecular weight form within three hours of injection. This is long before first cleavage, and should allow sufficient time for integration at this stage. Absolute proof of integration is only demonstrated by germ line transmission, but the high proportion of cells expressing β-galactosidase with the heat shock promoter suggests that there is a good chance for this. In addition, the detection of β-galactosidase activity in three and five month old animals also indicated the persistence of microinjected DNA sequences. Animals are currently being raised to test for germ line transmission.

Genomic Southerns prepared from stage 33 embryos expressing β-galactosidase show that the injected DNA is concatemerized as head-to-tail repeats and is present in high copy number. This bias towards the head-to-tail concatemers is similar to that observed in the mouse (Gordon and Ruddle, 1985), and it may be that the other forms are unstable. In addition, the sizes of restriction fragments were as predicted, and therefore, no obvious gross rearrangements of the DNA had occurred.

In the past, cell lineage markers such as vital dyes have been used to follow the fate of a cell or group of cells. The disadvantage with this type of marker is that it diffuses from the point of application and fades with time. Fluorescent dyes conjugated to large molecules such as dextrans have also been used (Gimlich and Braun, 1985; Smith et al. 1985). These markers have proven useful in that they are retained for much longer periods of time than vital dyes, but localization of these compounds requires specialized fluorescent microscopes. Intrinsic markers such as triploidy are difficult and laborious to score, and heterospecific combinations require that both organisms are distinguishable but are similar in developmental rate and pattern. Ideally, the constitutive expression of an easily identifiable marker such as β-galactosidase would provide advantages over previously used cell lineage markers in that progeny cells arising from a founder cell could be located visually with histochemical staining of whole embryos, and

then more precisely in sectioned material. For internally labelled structures, albino embryos are ideal, although with the strong expression from the heat shock or PGK promoters, wild type embryos can be used. As seen from the results, cells expressing β-galactosidase arose from a single cell which is extremely important for the application of this technique as a cell lineage marker.

Ideally, germ line transmission of exogenous sequences would provide a permanent source of lineage marker which could be used for the construction of chimeras. If not germ line transmitted, chimeras could still be constructed directly from microinjected eggs. Although not all cells showed β-galactosidase activity with the promoters used in this study, the fate of groups of cells could still be followed. Success with β-galactosidase as a lineage marker has already been achieved in both the rat and the mouse (Sanes et al. 1986; Turner and Cepko, 1987).

The study of gene expression has been greatly advanced by techniques which allow the reintroduction of cloned DNA sequences into developing organisms. Since a variety of foreign promoters are functional in axolotl embryos, the use of this organism may further advance the study of development and gene expression.

CHAPTER IV

ECTOPIC EXPRESSION OF HB-KPN

A. Rationale

One method to study gene function in vertebrates is by introducing excess copies of the gene exogenously into the developing embryos. In this way gene expression is deregulated, and if this results in a reproducible phenotype, some inferences may be made as to the role of the gene in development.

Ectopic gene expression has been used successfully in Drosophila to examine the role of segmentation and homeo box-containing genes (Struhl, 1985, Ish-horowitz and Pinchin, 1987). In general, mutations cause the alteration of a gene product so that it is no longer functional (loss-of-function mutants). For example, the Drosophila segmentation gene, fushi tarazu, is normally expressed in the even-numbered parasegments. Loss-of function mutants of ftz result in embryos with missing even-numbered parasegments. Gain-of-function ftz mutants, produced by ectopic expression of ftz, (Struhl, 1985), however, result in the loss of the odd numbered parasegments. Hence, the result of over-expression of this developmental gene (and others) is the production of a phenotype which is reciprocal to its loss-of-function mutant. Tissues affected by gain-of-function mutants are those not normally expressing the

gene. Hence, inferences could be made concerning the function of the gene in normal development.

Such an approach has also successfully been used for mouse oncogenes and growth factors (Stewart et al., 1984 ; Ruther et al., 1987; Leder et al., 1986). In the frog Xenopus laevis, attempts to use such techniques have been hindered by the lack of an effective method of introducing exogenous genes into embryos, and mosaic gene expression. However, an alternate approach has been used to circumvent this problem (Harvey and Melton, 1988). This was to microinject excess copies of mRNA (produced in vitro), into fertilized eggs. In this example, the mRNA from a homoeobox-containing gene, Xhox-1A, was injected, and a reproducible phenotype was observed. This technique has also been successful with another Xenopus homeo box-containing gene, Xhox3, (Ruiz I Altaba, and Melton, 1989b).

In chapter III, exogenous DNA sequences were shown to be expressed in the axolotl. If this system is to be used to perturb development, then the injected DNA sequences must be present in high copy number in a large proportion of cells in the embryo. The results show that these criteria were met, and thus the axolotl may be a useful organism to study gene function in development. In addition, since the embryos develop externally to the mother, lethal phenotypes produced by ectopic expression can be easily observed.

The purpose of the experiments in this chapter was to examine the possible role of Ahox1 in development by

ectopically expressing HB-Kpn which contains an open reading frame, in the reading frame of the homeo box. The promoter used was the mouse hsp68 promoter, which has strong and constitutive expression in the axolotl (Chapter III).

The first step was to determine what tissues in neurula and tail bud embryos were expressing transcripts containing Ahox1. This information was crucial to the understanding of phenotypes produced by overexpression, since defects should be produced in tissues not normally expressing Ahox1. The most severe defects caused by ectopic expression of HB-Kpn were first noted during neurulation. Embryos were observed with abnormal neural plates which later in development resulted in extreme defects in the head. Minor defects were also noted, which arose from embryos with apparently normal neural plates. These included missing or underdeveloped eyes.

B. Materials and Methods

1. Isolation of RNA from dissected embryos.

Dissections of embryos were performed in 100% Steinberg's solution using sharpened tungsten needles and hair loops. The dissected tissues were rinsed three times in Steinberg's solution and transferred to sterile Eppendorf tubes. The Steinberg's solution was pipetted off and the tissues were immediately frozen at -80°C.

The RNA isolation procedure was essentially as described in chapter II, except that it was scaled down such

that three embryo pieces were homogenized in 0.5 mL of guanidinium solution. All isolation procedures were performed in 1.5 mL Eppendorf tubes.

2. Northern and Southern blot analysis

Northern blots of RNA from dissected embryos were performed as described in chapter II. Genomic Southerns from microinjected embryos were performed as in chapter III.

3. Preparation of constructs for microinjection

Standard cloning procedures were used. The constructs were prepared as outlined in Figs. 4.1, and 4.2.

4. Microinjection of fertilized embryos.

Microinjection was performed as described in chapter III. The constructs were linearized prior to microinjection. pHSPHB and pKP110 were linearized with XbaI, and pHSPHB was linearized with Tth111I. XbaI linearizes the plasmid 5′ to the heat shock promoter, and Tth111I cuts in the plasmid vector sequence.

C. Results

1. Localization of tissues containing Ahox1 transcripts

Transcripts hybridizing to Ahox1 were previously identified in neurula and tail bud embryos (chapter II). To more precisely identify the tissues expressing Ahox1, RNA was isolated from dissected neurulae and tail bud embryos.

In stage 16 neurulae embryos, transcripts were localized in the neural plate (see Fig. 4.3(A)), and the amount of hybridizing transcripts in the nerual plate was approximately equal to that in a whole neurula, although the relative amount of RNA in the neural plate was less than in either the plateless embryo or the whole embyro (Fig. 4.4 (A). To determine if different regions of the neural plate contained these transcripts, a blot was prepared using RNA from three different sections of the neural plate as shown in Fig 4.3(B). No Ahox1 transcripts were present in the anterior third of the neural plate, but they were present in the middle and posterior plate regions. All three neural plate RNA samples contained approximately equal amounts of RNA as judged from ethidium bromide staining of the gel (Fig. 4.4 (B)). Equivalent numbers of embryos pieces were examined versus whole embryos. The amount of transcript detected in the dissected pieces appeared to be equal to the control amounts from total embryos, and hence all the transcripts containing Ahox1 could be accounted for.

In stage 25 tail bud embryos, Ahox1 transcripts were found to be localized in the embryo from the anterior margin of the gill bulge to the pronephric duct (Fig. 4.3(C)). Approximately equal amount of RNA were present in each of the dissected regions of the tail bud embryos (Fig. 4.4(C)).

Some spurious hybridization was observed in these northern blots at the positions of rRNAs, and is probably due to the large amount of the rRNA in the lanes. In

addition, other homeo box sequences, including the Hox-1.6 homeo box have also been shown to weakly cross-hybridize with rRNAs (Baron et al., 1987).

2. Constructs for microinjection

In chapter III, the mouse hsp68 promoter was shown to give high levels of constitutive expression in the axolotl. HB-Kpn contains an open reading frame of 184 amino acids in the reading frame of the homeo box with a stop codon (TAG) 11 nucleotides from the 3' end. This fragment was therefore cloned to be expressed under the control of the mouse hsp68 promoter (Fig. 4.1) The resulting fusion protein should produce a protein with a homeodomain. This protein would be initiated from an internal AUG (see discussion). The SV40 polyadenylation site is present in this construct (pHSPHB). The DNA sequence of the resulting clone from the HindIII site (site of insertion of the mouse hsp68 promoter) to the polyadenylation site is given in Fig. 4.5).

A control construct was prepared in which the last 60 bp of the homeo box (containing the DNA recognition helix) was deleted (Fig. 4.2).

Fig. 4.1 Construction of pHSPHB

(A). The lacZ sequences were removed from the plasmid pCH110, by digestion of this plasmid with PvuII, and ligation of fragments A and B (pCHZ-).

(B) HB-Kpn containing the homeo box of Ahox1 was cloned into the KpnI site of pCHZ- to give the plasmid pSVHB.

(C). The heat shock promoter from pKP110 (chapter III), was cloned into the unique HindIII site of pSVHB.

(D) Restriction map of pHSPHB used for microinjection.

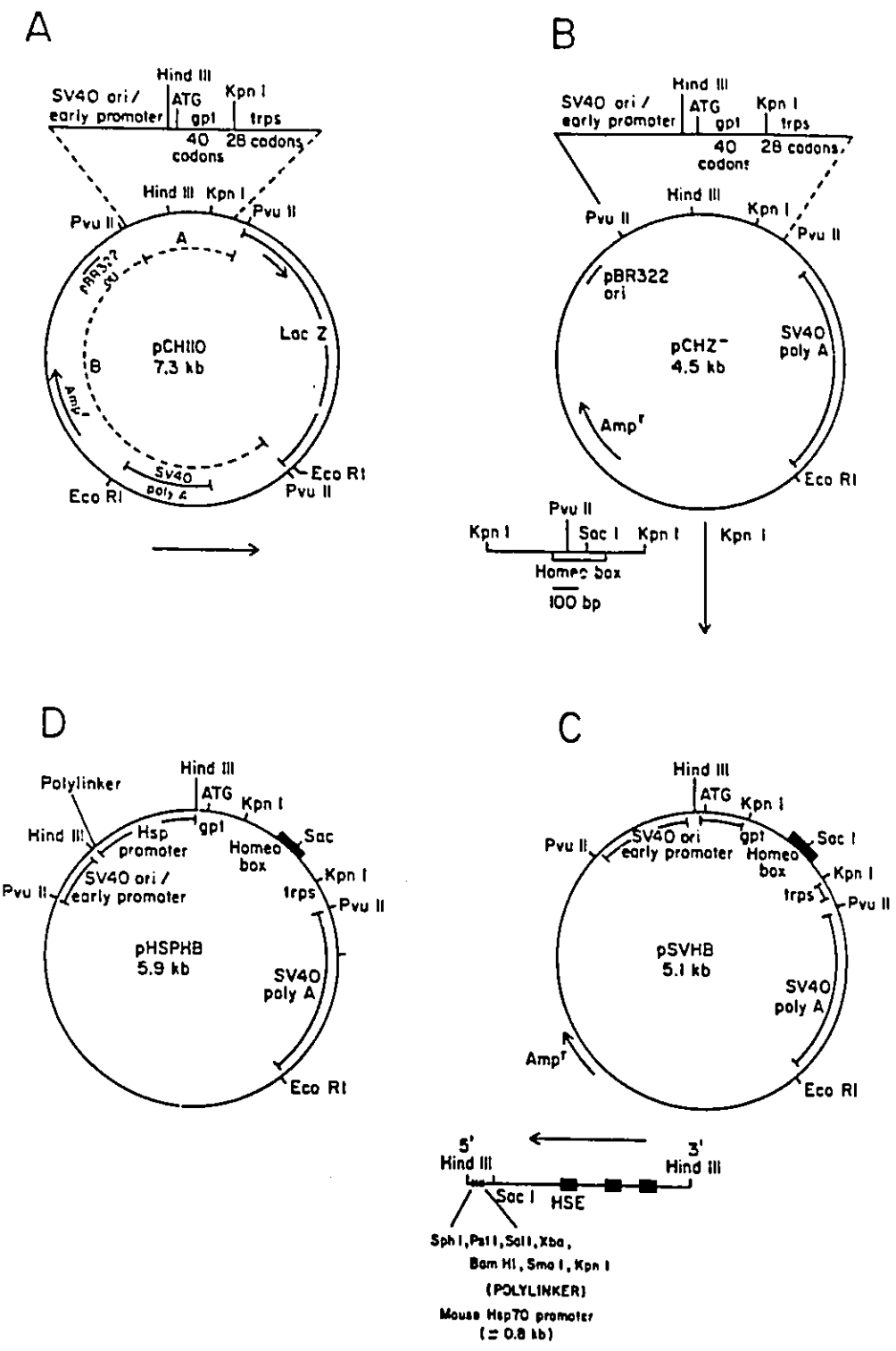

A
Hind III
SV40 ori / early promoter
ATG
Kpn I
gpt
trps
40 codons
28 codons
Hind III Kpn I
Pvu II
Pvu II
pBR322 ori
A
Lac Z
pCH110
7.3 kb
B
Amp r
SV40
poly A
Eco RI
Pvu II
Eco RI
B
Hind III
SV40 ori / early promoter
ATG
Kpn I
gpt
trps
40 codons
28 codons
Pvu II
Hind III
Kpn I
Pvu II
pBR322 ori
pCHZ⁻
4.5 kb
SV40 poly A
Amp r
Eco RI
Pvu II
Kpn I
Sac I
Kpn I
Kpn I
Homeo box
100 bp
D
Polylinker
Hind III
ATG
Kpn I
Hind III
Hsp promoter
gpt
Homeo box
Sac
Pvu II
SV40 ori / early promoter
Kpn I
trps
Pvu II
pHSPHB
5.9 kb
SV40 poly A
Eco RI
C
Hind III
ATG
Kpn I
Pvu II
SV40 ori early promoter
gpt
Homeo box
Sac I
Kpn I
trps
Pvu II
pSVHB
5.1 kb
SV40 poly A
Amp r
Eco RI
5'
Hind III
3'
Hind III
Sac I
HSE
Sph I, Pst I, Sal I, Xba, Bam HI, Sma I, Kpn I
(POLYLINKER)
Mouse Hsp70 promoter
(= 0.8 kb)

Figure 4.2. Construction of pHSPHBΔ

(A) Fragment A of pHSPHB containing the heat shock promoter to the SacI site in the homeo box was ligated with fragment B containing the SV40 polyadenylation site to the PvuII site in the vector (including Amp^r) to give the plasmid pHSPHBΔ.

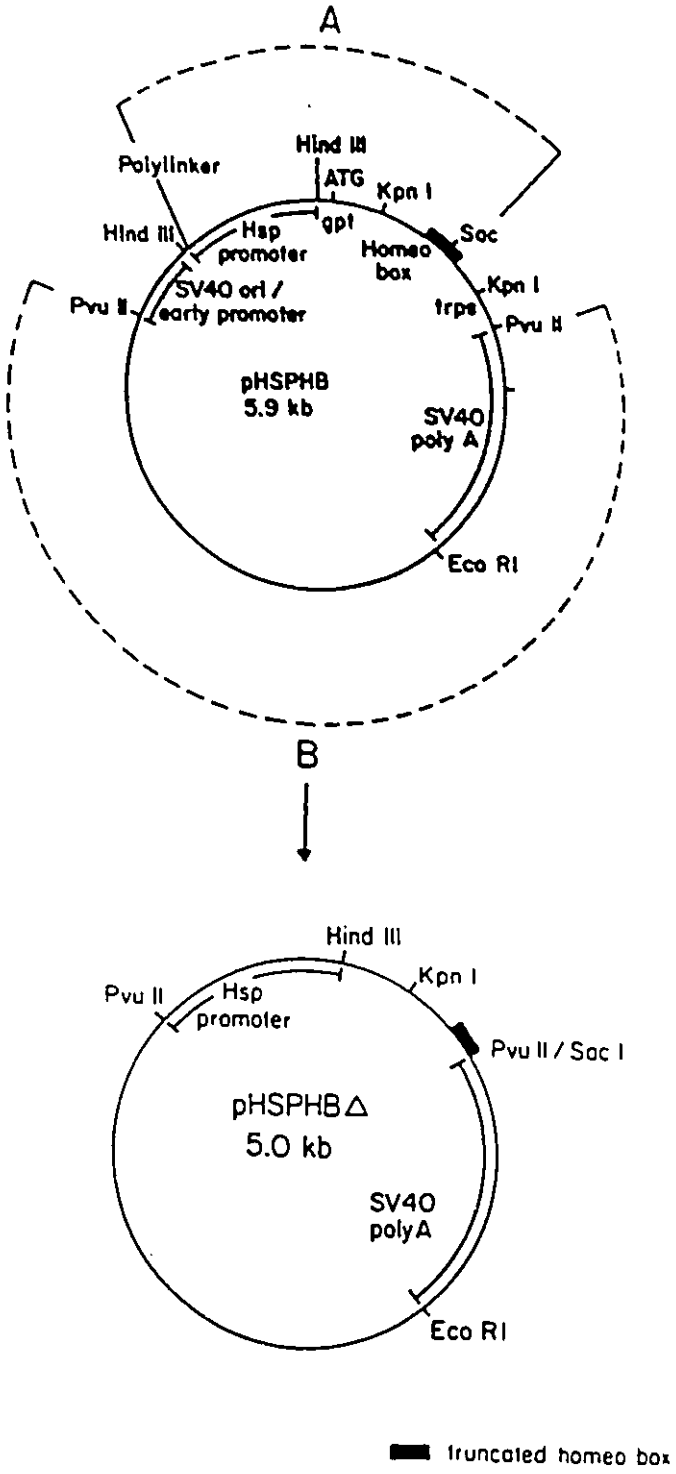

A
Polylinker
Hind III
ATG
Kpn I
Hind III
Hsp
promoter
gpt
Homeo
box
Soc
Kpn I
Pvu II
SV40 ori /
early promoter
trpe
Pvu II
pHSPHB
5.9 kb
SV40
poly A
Eco RI
B
Hind III
Kpn I
Pvu II
Hsp
promoter
Pvu II / Soc I
pHSPHBΔ
5.0 kb
SV40
polyA
Eco RI
truncated homeo box

Fig. 4.3 Localization of transcripts of Ahox1 in dissected neurula and tail bud embryos

Northern blots were prepared from embryos as described below. Blots were hybridized with the HB-Kpn. Following hybridization the filters were exposed to Kodak XAR film at -70°C for 3 to 5 days.

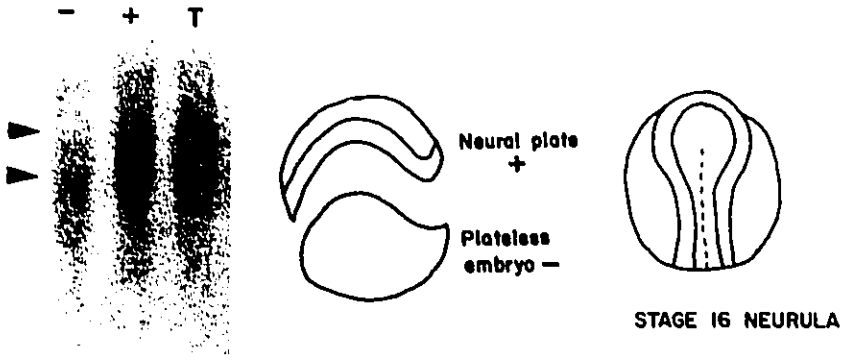
(A) Detection of transcripts of Ahox1 in stage 16 neurula embryos. T is total RNA from three stage 16 embryos.+ is total RNA from three neural plates and - is RNA from three plateless embryos.

(B) Localization of transcripts of Ahox1 in the neural plate. G is total RNA from three gastrula embryos (negative control) T is RNA from three stage 16 embryos. PT is RNA from three posterior neural plates: MT is RNA from three middle neural plates: AT is RNA from three anterior neural plates.

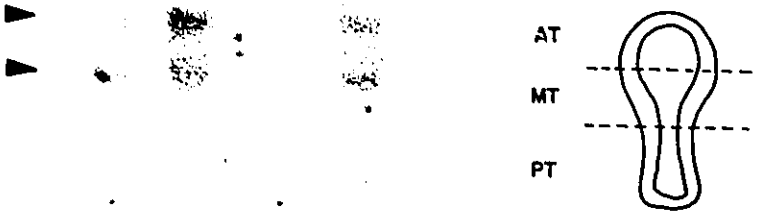
(C) Localization of transcripts of Ahox1 in tail bud embryos. Three tail bud embryos were dissected as shown. T is total RNA from three stage 25 embryos: G is total RNA from three gastrula embryos. 1,2,3 are the regions shown. Transcripts of Ahox1 are present in region 2.

Arrows point to location of 2.2 and 1.6 kb transcripts of Ahox1.

A


- + T
Neural plate +
Plateless embryo –
STAGE 16 NEURULA

B


PT MT AT T G
AT
MT
PT

C

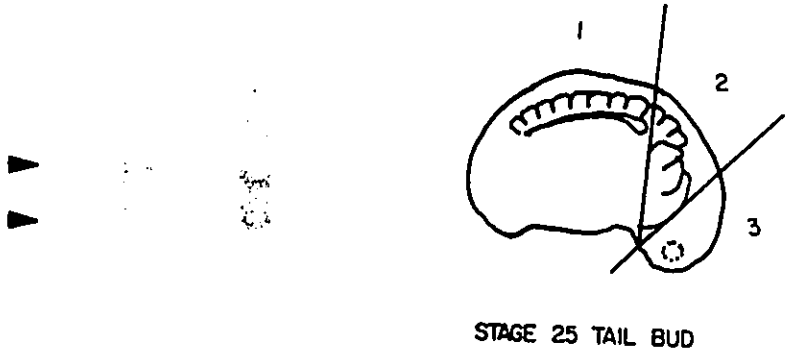

1 2 3 T G
1
2
3
STAGE 25 TAIL BUD

Fig. 4.4. Relative amounts of RNA in dissected embryos as judged by fluorescence in ethidium bromide stained agarose gels.

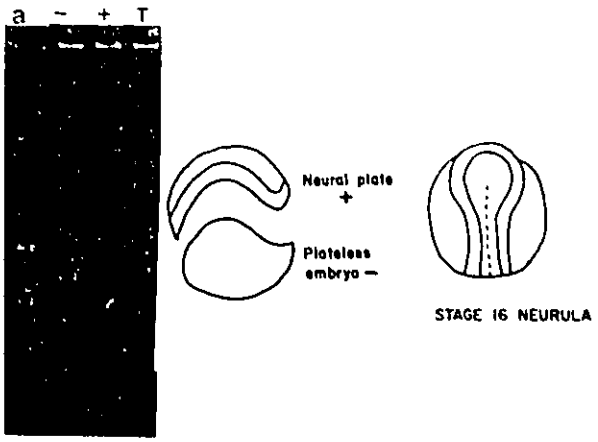
RNA samples are as described in Fig. 4.3. Lane a in all panels is an E. coli ribosomal RNA standard

(A) RNA from dissected stage 16 neurula. T is total RNA from three stage 16 embryos. + is total RNA from three neural plates and - is RNA from three plateless embryos.

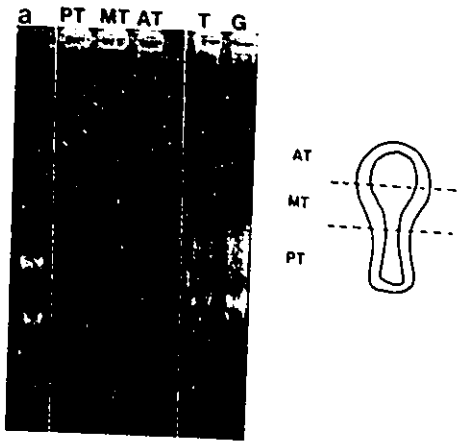
(B) RNA from dissected neural plates. G is total RNA from three gastrula embryos (negative control) T is RNA from three stage 16 embryos. PT is RNA from three posterior neural plates: MT is RNA from three middle neural plates: AT is RNA form three anterior neural plates.

(C). RNA from dissected tail bud embryos. T is total RNA from three stage 25 embryos: G is total RNA from three gastrula embryos: 1,2,3 are the regions shown in right panel.

A


a
−
+
T
Neural plate
+
Plateless
embryo −
STAGE 16 NEURULA

B


a
PT
MT
AT
T
G
AT
MT
PT

C

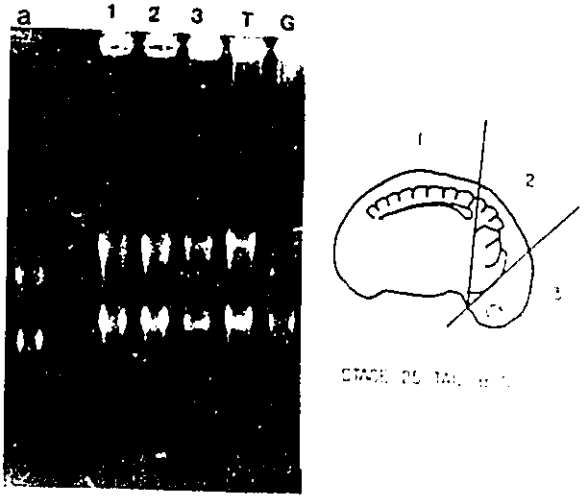

a
1
2
3
T
G
1
2
3

Fig. 4.5 Nucleotide sequence of construct containing the HB-KPN

Nucleotide sequence of pHSPHB from the HindIII site (insertion site of hsp68 promoter) to the SV40 polyadenylation site. In frame ATG's and stop codons prior to initiation of fusion protein are underlined. The homeo box is enclosed in a box. The polyadenylation site (position 1410) is also enclosed in a box. HB-Kpn was inserted into this clone at nucleotide 319.

```
                  15                  30                  45                  60
AAG CTT GGA CAC AAG ACA GGC TTG CGA GAT ATG TTT GAG AAT ACC ACT TTA TCC CGC GTC
                  75                  90                 105                 120
AGG GAG AGG CAG TGC GTA AAA AGA CGC GGA CTC ATG TGA AAT ACT GGT TTT TAG TGC GCC
                 135                 150                 165                 180
AGA TCT CTA TAA TCT CGC GCA ACC TAT TTT CCC CTC GAA CAC TTT TTA AGC CGT AGA TAA
                 195                 210                 225                 240
ACA GGC TGG GAC ACT TCA CAT GAG CGA AAA ATA CAT CGT CAC CTG GGA CAT GTT GCA GAT
                 255                 270                 285                 300
CCA TGC ACG TAA ACT CGC AAG CCG ACT GAT GCC TTC TGA ACA ATG GAA AGG CAT TAT TGC
                                                         MET GLU ARG HIS TYR CYS
                 315                 330                 345                 360
CGT AAC CCG TGG CGG TCT GGT ACC CCC ACC ACT GCA TGC AGC CTT CCT GGG GGG GGG GGG
ARG LYS PRO TRP ARG SER GLY THR PRO THR THR ALA CYS SER LEU PRO GLY GLY GLY GLY
                 375                 390                 405                 420
GGC TGG CTG TGG GGT GGG ACC TGT GTA CAG GCG ATG GGG AGA GGT GGT CAT CGT CCC AGT
GLY TRP LEU TRP GLY GLY THR CYS VAL GLN ALA MET GLY ARG GLY GLY HIS ARG PRO SER
                 435                 450                 465                 480
GAT GGC CAC CTC TCT CCA TCG CCA TGC TGC GCT TTT TTC TCA AGG TGC CTA TGT TGT TTC
ASP GLY HIS LEU SER PRO SER PRO CYS CYS ALA PHE PHE SER ARG CYS LEU CYS CYS PHE
                 495                 510                 525                 540
TTT GGC TTG TAT GCC CTT TCC ACA TCT CTC GCA CTG ATA TCG TCT CTG TTT TCT TTA TTG
PHE GLY LEU TYR ALA LEU SER THR SER LEU ALA LEU ILE SER SER LEU PHE SER LEU LEU
                 555                 570                 585                 600
ACA GCT AAG CTG TCC GAG TAC GGC TTG GGG AGC CAG CAG AAC AGC ATC CGG ACC AAC TTC
THR ALA LYS LEU SER GLU TYR GLY LEU GLY SER GLN GLN ASN SER ILE ARG THR ASN PHE
                 615                 630                 645                 660
ACC ACC AAG CAG CTG TCC GAG CTG GAG AAG GAG TTC CAC TTC AAC AAG TAC CTG ACC CGG
THR THR LYS GLN LEU SER GLU LEU GLU LYS GLU PHE HIS PHE ASN LYS TYR LEU THR ARG
                 675                 690                 705                 720
GCC CGC AGG GTG GAG ATC GCC GCC ACC CTG GAG CTC AAC GAG ACC CAG GTC AAG ATC TGG
ALA ARG ARG VAL GLU ILE ALA ALA THR LEU GLU LEU ASN GLU THR GLN VAL LYS ILE TRP
                 735                 750                 765                 780
TTT CAG AAC CGG CGG ATG AAG CAG AAG AAG CGG GAG AAG GAG GGC CTG GCC CCC TCC AAT
PHE GLN ASN ARG ARG MET LYS GLN LYS LYS ARG GLU LYS GLU GLY LEU ALA PRO SER ASN
                 795                 810                 825                 840
GGA CCT CAG GGC AGA GCC AGA GAG GCC AGC GAG GCC TCG GAC CAA TCC AGC TCC CCA TCC
GLY PRO GLN GLY ARG ALA ARG GLU ALA SER GLU ALA SER ASP GLN SER SER SER PRO SER
                 855                 870                 885                 900
CCC TAC GCT TCT CCC GAC TCG TTC TCA TCG TGA ATG GTA CCG GTG GGT GAA GAC CAG AAA
PRO TYR ALA SER PRO ASP SER PHE SER SER ***
                 915                 930                 945                 960
CAG CAC CCC GCG ATA TTG CCC AGC GTT TCA ACG CGC TGT ATG GGC GAG ATC GAT CCC GTC
                 975                 990                1005                1020
GTT TTA CAA TCG ACG GTT TCC ATA TGG GGA TTG GTG GCG ACG ACT CCT GGA GCC CGT CAG
                1035                1050                1065                1080
TAT CGG CGT TTC GCC AGC TGA GCG CCG GTC GCG ACC ATT ACC AGT TGG TCT GGT GTC AAA
                1095                1110                1125                1140
AAT AAT AAT AAC CGG GCA GGC CAT GTC TGC CCG TAT TTC GCG TAA GGA AAT CCA TTA TGT
                1155                1170                1185                1200
ACT ATT TAA AAA ACA CAA ACT TTT GGA TGT TCG GTT TAT TCT TTT TCT TTT ACT TTT TTA
                1215                1230                1245                1260
TCA TGG GAG CCT ACT TCC CGT TTT TCC CGA TTT GGC TAC ATG ACA TCA ACC ATA TCA GCA
                1275                1290                1305                1320
AAA GTG ATA CGG GTA TTA TTT TTG CCG CTA TTT CTC TGT TCT GCT ATT ATT CCA ACC GCT
                1335                1350                1365                1380
GTT TGG TCT GCT TTC TGA CAA ACT CGG AAC TTG TTT ATT GCA GCT TAT AAT GGT TAC AAA
                1395                1410
TAA AGC AAT AGC ATC ACA AAA TTT CAC AAA TAA AGC AT
```

3. Phenotypes from microinjection of pHSPHB

The most striking effects in embryos injected with pHSPHB were first observed during neurulation. At this time, embryos with abnormal neural plates were observed (Fig. 4.6). The extent (width) of the plate was diminished, and the neural plate showed no obvious keyhole shape (Fig. 4.6 (B)). Normally, the neural plate is wider at the anterior end, as demonstrated in Fig. 4.6 (A). This region of the plate normally gives rise to the brain. Two abnormal embryos are shown in Fig. 4.6 (B). The abnormal embryo on the left, is an early neurula, prior to the raising of the neural folds, while the one on the right shows that folds are formed along the length of the plate. Embryos with severely deformed neural plates developed into embryos with severely reduced heads (not shown). The embryos also are rounded, with distended bellies. These embryos have somites, and normal heart beat, but they begin to disintegrate after stage 36.

Histological examination of an abnormal neurula revealed that the mesoderm and endoderm layers were not affected in pattern. The neural plate appeared to be normal except in width at the anterior end (not shown).

Table 4.1. Results of microinjection

Exp#	1		2		3	
	pHSPHB	pKP110	pHSPHB	pHSPHB$_\Delta$	pHSPHB	pHSPHB$_\Delta$
Total	50	25	84	84	68	65
Infertile	8	5	58	61	18	18
Abortive	11	4	4	6	22	13
Exogastrulae	6	3	0	2	0	3
Normal	9	13	11	15	14	31
Abnormal plate/head	5	0	6	0	4	0
Minor defects	11	0	5	0	10	0

Fig. 4.6. Defects caused by ectopic expression of pHSPHB

(A) Normal axolotl neurula embryo (stage 15)

(B) Abnormal neurula observed in embryos injected with pHSPHB. The embryo on the left is at an early stage of neurulation and has no obvious neural folds, while the embryo on the right shows raised neural folds. At later stages of development, these embryos have severely reduced heads.

(C) Normal axolotl larva

(D) Larva showing reduced eye development

(E) Larva with missing eyes


A
B
C
D
E

Less severe effects have also been observed. These include diminished or missing eyes (Fig 4.6 (D) and (E)). These embryos arise from apparently normal neurulae. Histological examination of these embryos showed no internal eye development in the case of the embryos missing eyes, and for the embryos with diminished eyes, there was a small optic cup and lens.

None of the phenotypes observed with embryos injected with pHSPHB were observed in control embryos injected with either the pKP110 (parent plasmid containing the hsp promoter), or pHSPHBΔ (Table 4.1) The results from these experiments show that the phenotypes produced by microinjection of pHSPHB are reproducible and consistent.

4. Detection of injected DNA sequences in microinjected embryos

Genomic DNA was prepared from one abnormal and one normal embryo injected with pHSPHB, and a pool of three normal embryos injected with pHSPHBΔ . Approximately 5 μg of genomic DNA was digested to completion with EcoRI, and hybridized to the HB-Kpn. The results from this experiment show that injected DNA sequences are present in the embryos with a copy number of about 100/genome (Fig. 4.7 (A)). As was demonstrated previously (Chapter III), DNA injected into axolotl embryos is present as head-to-tail concatemers, and hence one EcoRI fragment was observed. In the case of pHSPHB, only the abnormal embryo showed the presence of

injected DNA. A pool of three pHSPHBΔ embryos was used, as it had been previously shown (Chapter III), that between 30 and 50% of injected embryos contain injected DNA.

In another experiment, a correlation between presence of injected plasmid DNA and abnormal phenotype was shown for pHSPHB. In this experiment 4 abnormal and one normal embryo were analyzed. All abnormal embryos contained plasmid DNA while the normal embryo did not.

5. Detection of transcripts from microinjected plasmids

RNA was isolated from three abnormal and three normal neurula embryos injected with pHSPHB and from three normal neurula embryos injected with pHSPHBΔ. Northern blots were prepared and hybridized to the HB-Kpn (Fig. 4.7 (B)). In the abnormal neurulae injected with pHSPHB, a 1.4 kb transcript was observed to hybridize with the HB-Kpn probe, corresponding to the transcript produced from this construct. A 1.4 kb transcript was not observed in the normal neurula.

In embryos injected with pHSPHBΔ, a transcript with the expected 1.1 kb size was detected (not shown). The amount of transcript detected in these embryos was less than in the abnormal neurulae (pHSPHB), as not all the pHSPHBΔ embryos may contain injected DNA sequences (see above).

Fig. 4.7 Detection of DNA and RNA in microinjected embryos

(A) Detection of DNA in embryos injected with pHSPHB and pHSPHBΔ. DNA (5μg) was digested with EcoRI, electrophoresed on a 0.8% agarose gel, transferred to nylon membrane and probed with HB-Kpn. The filter was exposed to Kodak XAR film overnight at -70°C

Lane 1 :DNA from a pool of the normal embryos injected with pHSPHBΔ

Lane 2 :DNA from one abnormal embryo injected with pHSPHB.

Lane 3 : XbaI linearized pHSPHB equivalent to 100 copies/genome.

(B) Detection of transcripts in abnormal embryos injected with pHSPHB

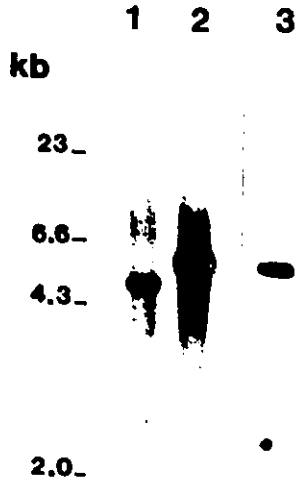
Northern blots of total RNA were prepared from embryos injected with pHSPHB, and probed with HB-Kpn. The filter was exposed to Kodak XAR film overnight at -70° C.

Lane 1: Total RNA from three normal gastrula embryos (negative control)

Lane 2: Total RNA from three normal neurula embryos injected with pHSPHB

Lane 3: Total RNA from three abnormal neurulae injected with pHSPHB. Arrow points to 1.4 kb transcript produced from the hsp68 promoter, showing abundance of this message versus endogenous levels of Ahox1, which are barely visible in this exposure. Longer exposure did, however reveal the normal 1.6, and 2.2 kb transcripts in lane 2, and the 2.2 kb endogenous transcript was seen in lane 3. The 1.6 kb endogenous transcript of Ahox1 in lane 3 was masked by the 1.4 kb transcript produced by pHSPHB in longer exposures of this northern.

A



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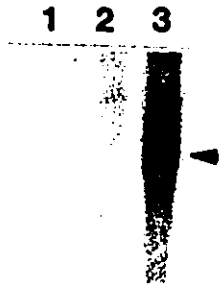


D. Discussion

Perhaps the most significant feature of genes containing the homeo box is that they play a crucial role in embryogenesis in Drosophila. In vertebrates, there is some evidence that genes containing this sequence play a critical role in development. Genes containing a homeo box are restricted both temporally and spatially during development. In Xenopus, ectopic expression of homeo box genes via injection of excess copies of homeo box mRNA suggest that these genes play a role in significant developmental processes such as segment formation (Harvey and Melton, 1988) and determination of position in the mesoderm along the anterior-posterior axis (Ruiz I Altaba and Melton, 1988b).

In this study, the role of a homeodomain was examined in the axolotl. This was accomplished by microinjection of a plasmid construct which would ectopically produce a protein containing the axolotl homeodomain encoded by Ahox1. Although, at present, it is not known whether the sequences outside of the homeo box are part of the coding region of a gene, or intronic (see chapter II), expression of this protein caused a reproducible phenotype. Proteins containing a homeo domain are thought to behave as transcription factors, directing development by regulation of other genes. Some homeodomain proteins have been shown to activate transcription (Driever et al., 1989; Kuziora and McGinnis, 1988), while others

repress transcription (Jaynes and O'Farrell, 1988; Biggin and Tjian, 1989). The DNA sequence specificity is however, believed to be determined by the homeodomain (Kuziora and McGinnis, 1989; Hanes and Brent, 1989). The current view of how homeodomain proteins control developmental fates is that different cell types contain combinations of these transcription factors as a result of the temporal and spatial expression of these genes (Akam, 1987; Scott and Carroll, 1987; Jaynes and O'Farrell, 1988). These combinations then act either competitively, or synergistically to regulate transcription. The protein produced by pHSPHB, may interfere with development by binding to DNA in a sequence specific fashion determined by the homeodomain, thereby interferring with the normal combination of transcription factors required to activate genes in the anterior neural plate. This interference may be strictly passive, since it is not clear whether or not the protein sequence outside of the homeodomain is able to mediate activation or repression of transcription (since the DNA sequence for this region may be intronic). If indeed, the protein outside of the homeodomain were part of a gene, it is possible that this protein might have the ability to activate or repress transcription, as it has been shown that a partial length bicoid protein, containing the homeodomain can activate transcription, and rescue bicoid mutants (Driever et al., 1989).

The DNA sequence of the fusion construct used for microinjection is given in Fig. 4.5. As in some other artificial constructs prepared for expression in eukaryotes, initiation of translation of this protein must occur from an internal AUG. There are many examples of translation from an internal AUG. Some of these include sequences fused to the SV40 early promoter such as the mouse dihydrofolate reductase cDNA (Subramani et al., 1981), the bacterial CAT (chloramphenicol acetyltransferase) sequences (Gorman et al., 1982), and the bacterial neo gene sequences (Southern and Berg, 1982) In addition, some natural examples exist, especially in mRNAs of viral origin (Kozak, 1986a). In order to achieve efficient internal translation, the internal AUG should lie in a favorable context with respect to the consensus sequence for initiation (CCACCAUGG), with particular emphasis on the +3 and -4 nucleotides (underlined) of this sequence (Kozak, 1986b). There are six ATGs present in the DNA sequence prior to the ATG which codes for the begining of the desired fusion protein. Four of these are followed by in-frame termination codons. Of the other two, only one (nucleotide 230) is in good context for initiation, and this will reduce translation of the desired fusion protein by five fold. The AUG for the fusion protein is also in a favorable context for initiation. Based on previous studies on translation initiation a fusion protein containing the homeo box domain should have been produced from this construct.

In order to make meaningful interpretations from the overexpression of a developmental gene, it is important to understand its normal temporal and spatial expression. If the result of such ectopic expression is the production of the reciprocal phenotype to that of a known mutant as has been demonstrated in Drosophila, then the affected tissues should be those not normally expressing the gene. Transcripts hybridizing to Ahox1 are not found in the anterior third of the neural plate during neurulation, nor in the most anterior head region during tail bud. Overexpression of a protein containing the Ahox1 homeodomain should thus affect these tissues. This is indeed what was observed. The effects of microinjection of pHSPHB were first noted during neurulation, when Ahox1 transcripts are first normally detected. No global consequences were observed from earlier expression, although transcription from the hsp68 promoter begins after the mid-blastula transition (unpublished). Hence it would appear that the Ahox1 homeo box does not interact aberrantly with other regulatory genes prior to neurulation. Most notably, the anterior neural plate is affected. Tail bud embryos show an unusually rounded shape. This may be due to the physical constraints imposed by the abnormal neural plate or a failure of the body axis to lengthen. All effects observed later than neurulation are in tissues not normally expressing Ahox1. These include reduced heads, and in less severe cases, missing or diminished eyes. Hence, it would appear that the

transcripts with the Ahox1 homeo box which first appear during neurulation are involved in the specification of posterior neural structures, and most likely the posterior parts of the brain.

How the nerual plate is affected is a matter of speculation. Normally, a neural plate is induced from part of the ectoderm which is induced by tissue which has invaginated through the dorsal lip of the bastopore during gastrulation. Whether the abnormal neural plate is formed as a result of a failure of the ectoderm to respond to normal signals from the underlying tissue or whether the signal to induce the neural plate is altered, remains to be determined. Isolation of other neural markers may be useful in resolving some of these questions. If for example, molecular markers for anterior or posterior neural plate tissues were available, one could determine if the regionalization in these abnormal neural plates was altered (ie., is the anterior neural plate now been specified as posterior).

Of importance is that the phenotypes produced from microinjection of pHSPHB were reproducible, and were not found in control injections. There was also a correlation with these phenotypes with presence of injected plasmid DNA. Although the percentages of abnormal phenotypes are somewhat lower than obtained with the technique of mRNA injection used in Xenopus, (Harvey and Melton, 1988) they are sufficient to draw valid conclusions. The technique

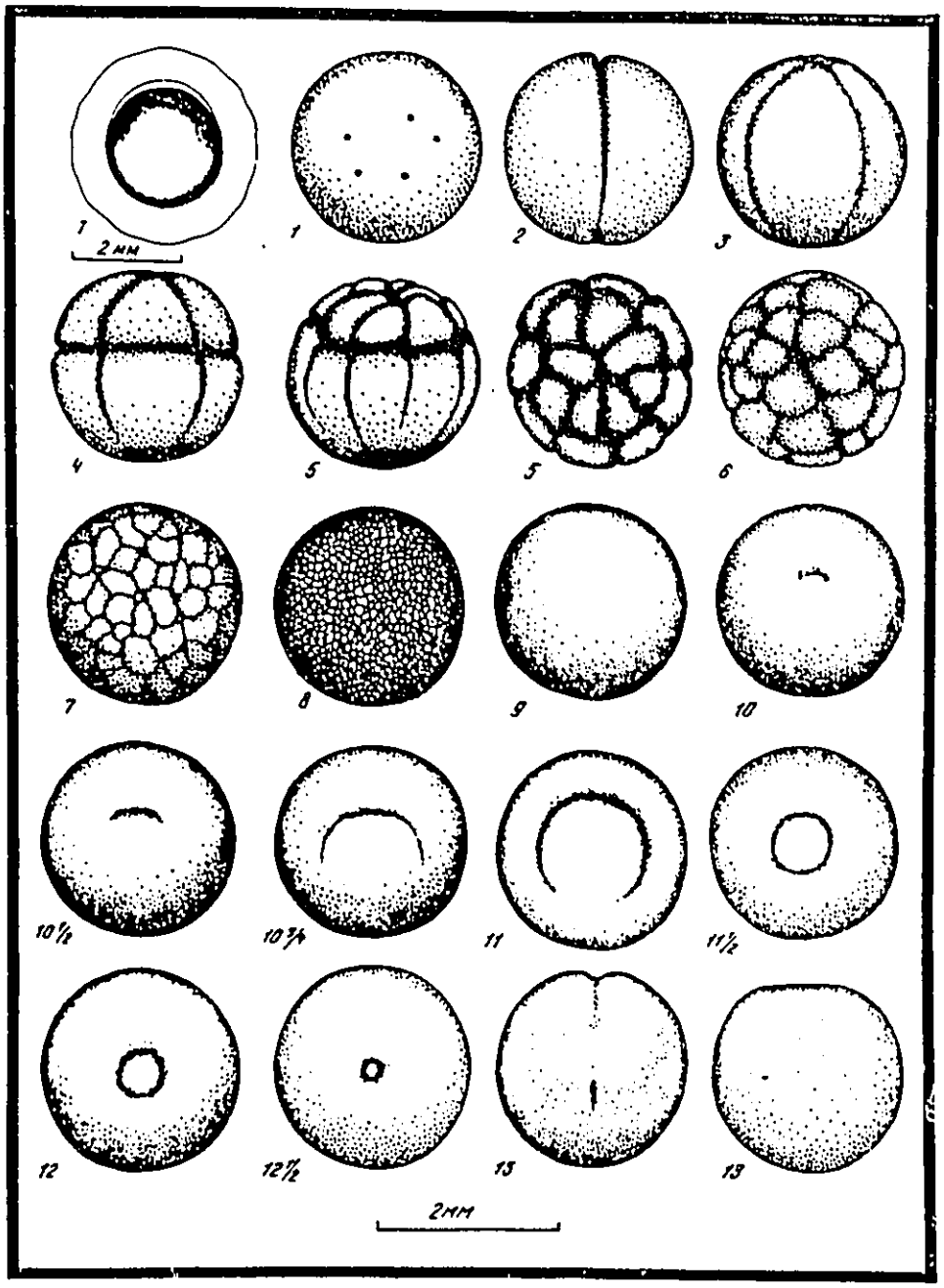
described here may provide an added advantage over mRNA injection in that it has been previously shown (Chapter III) that injected DNA sequences persist in high copy number late in development (at least stage 33), whereas injected mRNA may not remain at high levels for this length of time in development.

In this study, a homeo box containing gene was shown to be expressed regionally, in the developing nervous system of the axolotl. It is interesting to note that the homeo box sequence, Ahox1, shows similarities to the Drosophila labial gene. The labial gene is also expressed in parts of the developing head. Labial mutants have missing parts of the head. Although it is tempting to speculate that these genes may have evolved similar functions it is difficult to compare the development of the central nervous system of Drosophila with the development of the brain in an amphibian. Labial gene expression is also found in the posterior midgut primodium. Although no parallel expression was observed in the axolotl, it may be that there is expression in the intestine of adults as is observed for the mouse Hox-1.6 gene.

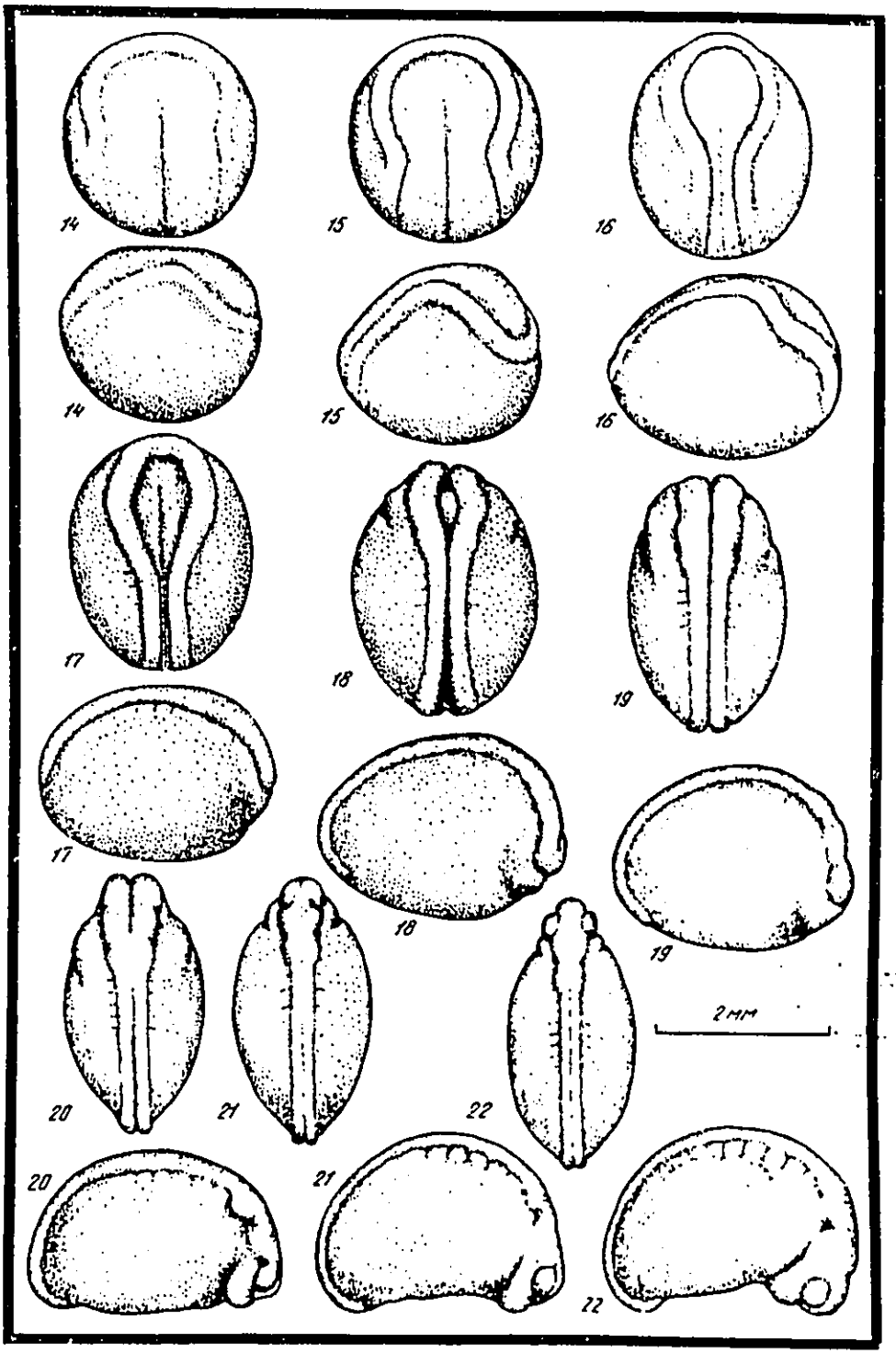
Clearly the axolotl offers advantages for the study of gene expression in development due to the ease with which exogenous sequences can be manipulated in this organism. Hence, this system can be used to test the significance of other developmentally regulated sequences.

APPENDIX I

Stages of Normal Development of *Ambystoma mexicanum*



Bordzilovskaya et al. (1989)



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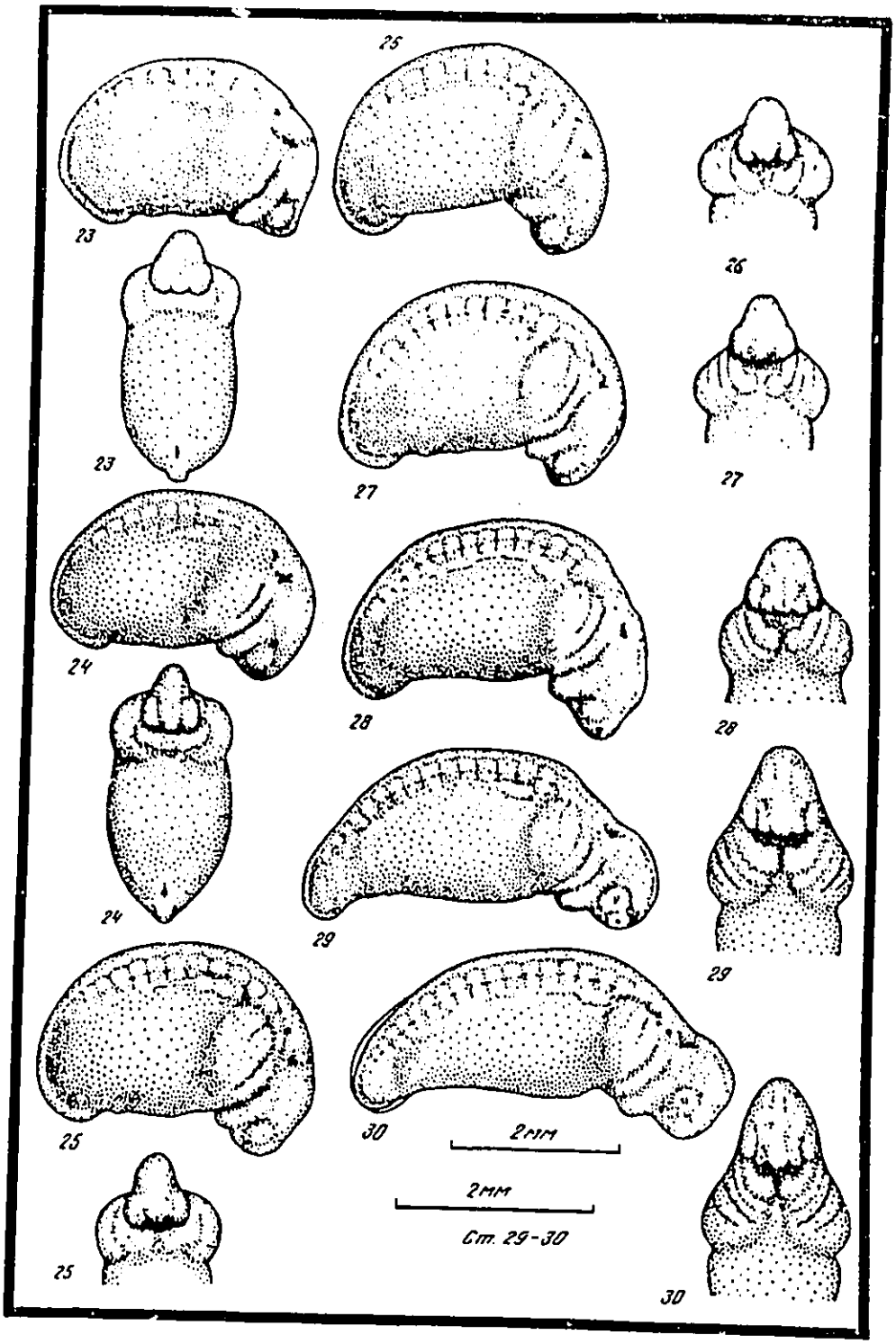

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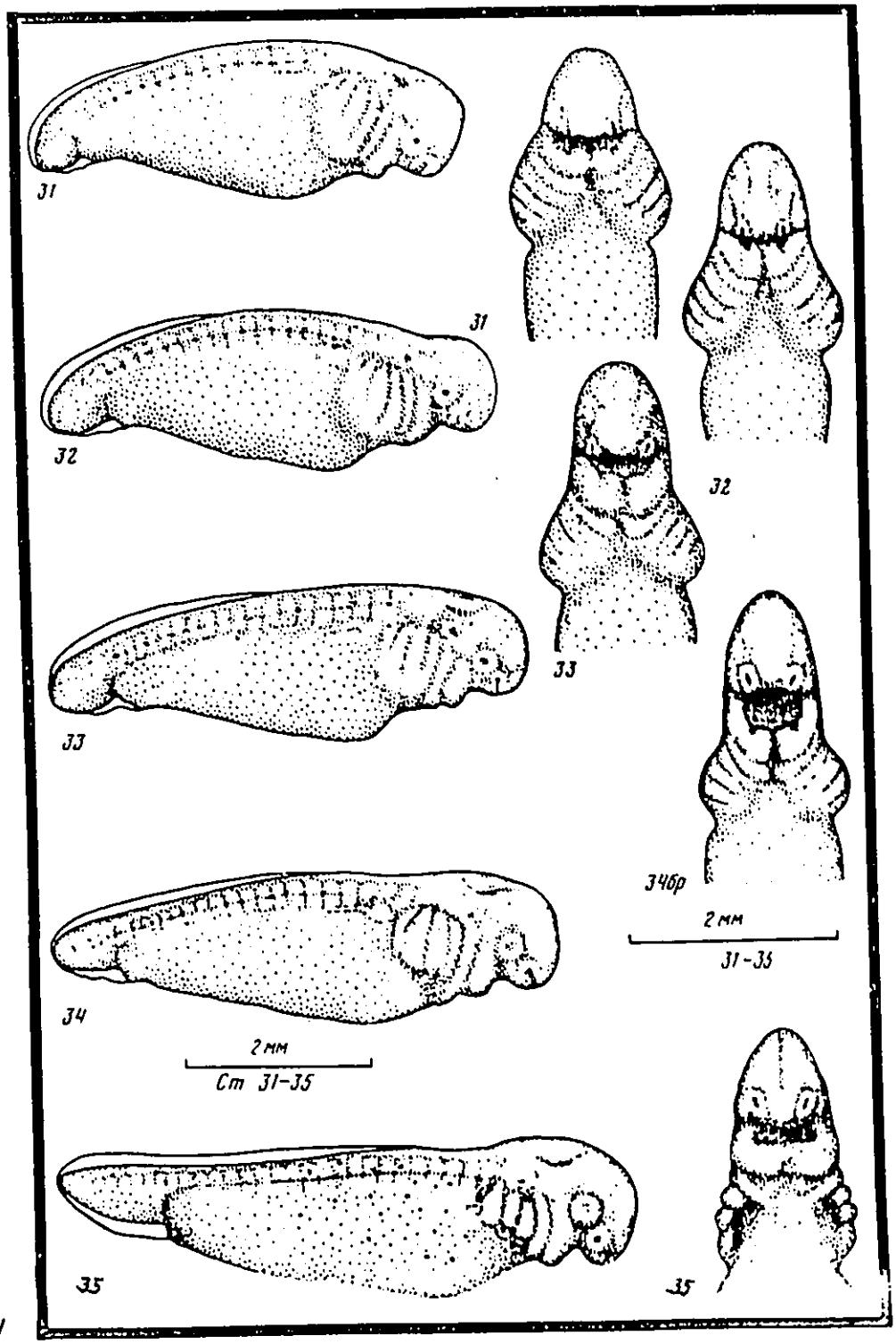

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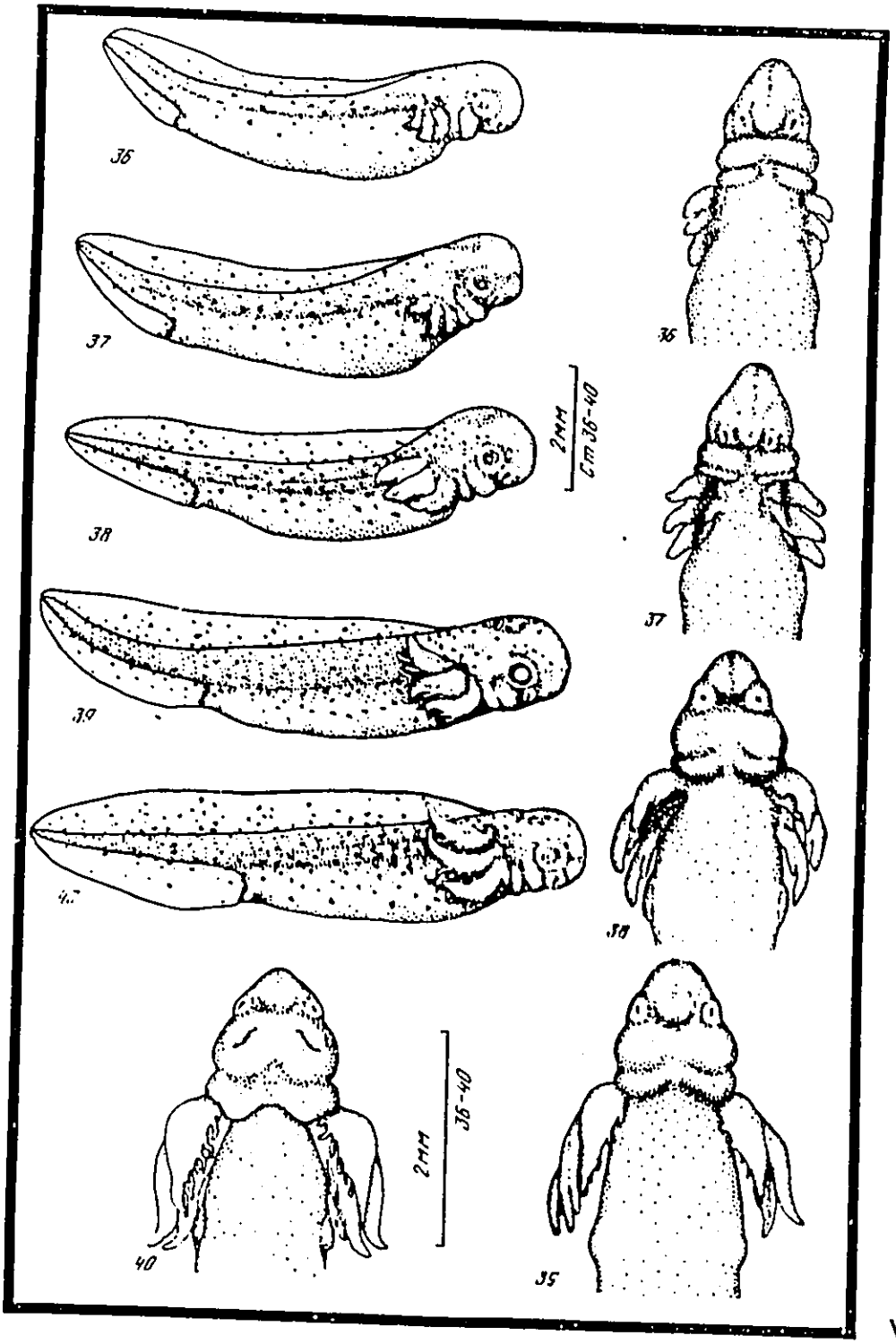
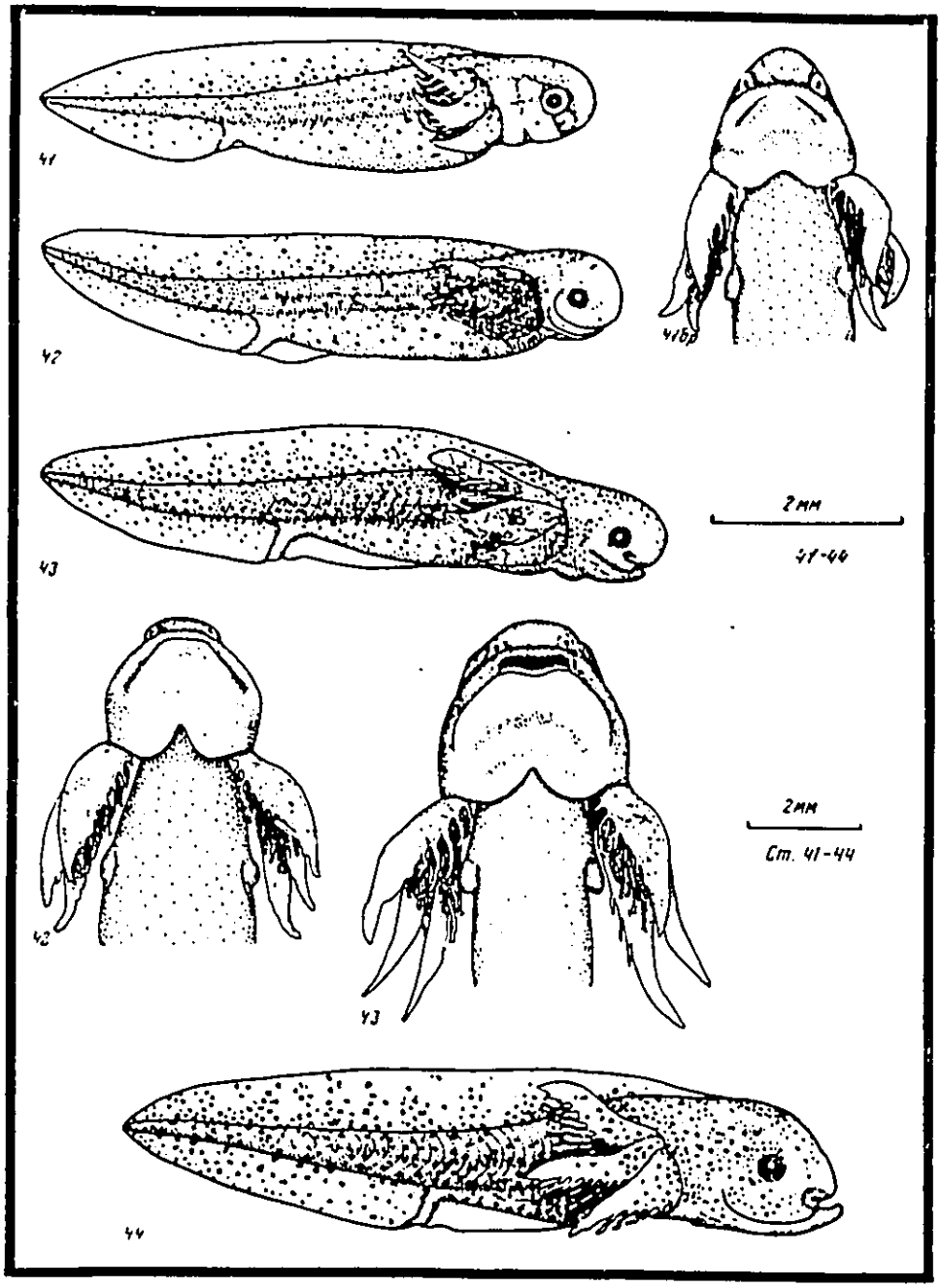


V



VI

Stage Number	Time from First Cleavage: In Hours and Minutes	t_n/t_0	Size (mm)	Features
I			1.85–2.0	Freshly laid fertilized egg in jellycoat.
1¼			2.0	Activated egg: a broad perivitelline space has formed.
2–	0.0	0	2.0	First appearance of the first cleavage furrow on the animal pole; timing begins.
2	0.65	0.72	2.0	2 cells.
3	2.40	1.7	2.0	4 cells.
4	4.12	2.7	2.0	8 cells.
5	5.22	3.5	2.0	16 cells.
6	6.45	4.5	2.0	32 cells.
7	8.26	5.5	2.0	64 cells.
8	16.06	10.5	2.0	Early blastula (fall of mitotic index in animal blastomeres: Rott and Beritashvili, 1975).
9	21.28	14	2.0	Late (epithelial) blastula; surface is smooth.
9½	24.32	16	2.0	Morphogenetic function of nuclei begins (Ignatieva, 1972).
10	26.00	17	2.0	Early gastrula I: First signs of dorsal blastopore lip are visible.
10½	32.10	21	2.0	Early gastrula II: Invagination continues; blastopore is a nearly horizontal slit in one quadrant.
10¾	37.00	24	2.0	Middle gastrula I: Dorsal lip forms a semicircle.
11	38.30	25–26	2.0	Middle gastrula II: Blastopore covers three quadrants. Lateral lips are formed; ventral lip is marked only by pigment accumulation. Yolk plug reaches its maximum diameter (1.2 mm).
11¼	40–42	27–28	2.0	Late gastrula I: Blastopore is circular; invagination continues; yolk plug diameter, 0.6 mm.
12	47.30	31	2.0	Late gastrula II: Blastopore has an oval or circular shape; size of yolk plug, 0.40 × 0.45 mm.

Stage Number	Time from First Cleavage: In Hours and Minutes	Time from First Cleavage: t_n/t_0	Size (mm)	Features
12½	49–51	32–34	2.1	Late gastrula III: Oval blastopore is closing; size of yolk plug, 0.15 × 0.20 mm.
13–	50.30–54.00	33–35	2.1	Stage with slitlike blastopore; boundaries of neural plate are not distinct.
13	55.00–56.30	36–37	2.1	Early neurula I: Blastopore is a narrow vertical slit; groove in the midline of the neural plate; boundaries of neural plate are outlined, but neural folds are not yet raised above surface of embryo; dorsal side is slightly flattened.
14	58.15	38	L = 2.2[b] B = 2.0	Early neurula II: Neural plate is broad. Neural folds are outlined and begin to rise above the surface in the head region. Embryo is slightly elongated.
15	59.50	39	L = 2.25 B = 2.1	Early neurula III: Neural plate is shield shaped. Neural folds are raised and bound all regions of the neural plate.
16	63.0	41	L = 2.25 B = 2.1	Middle neurula: Neural folds become higher, the spinal region of neural plate narrows, and the neural plate becomes sunken.
17	64.30	42	L = 2.35 B = 2.0	Late neurula I: Neural folds are higher, especially in the head region; further narrowing and deepening of the neural plate occur both in the head and in the spinal regions. Hyomandibular furrow limiting the mandibular area is slightly outlined. The segmentation of the mesodermal material begins. There are two pairs of somites.
18	66.0–67.30	43–44	L = 2.4 B = 1.9	Late neurula II: Neural plate is deeply sunken. Neural folds are closing and are especially high in the head region where three slight bulges corresponding to fore-, mid-, and hindbrain vesicles are outlined. The neural folds of the spinal region are almost in contact. Hyomandibular furrow is more marked. There are two pairs of somites.

Stage Number	Time from First Cleavage: In Hours and Minutes	Time from First Cleavage: t_n/t_0	Size (mm)	Features
19	69.00	45	L = 2.7 B = 1.7 H = 2.1	Late neurula III: Neural folds are in contact throughout, but are not yet fused. Brain curvature is quite distinct in profile; fore-, mid-, and hindbrain vesicles are also distinct. The swelling of optic vesicles is outlined. Hyomandibular furrow is deeper. There are three pairs of somites.
20	70.30	46	L = 2.7 B = 1.5 H = 1.7	Late neurula IV: Neural folds are fused in spinal region; in brain region, they are only in contact. Optic vesicles are distinct and becoming larger. Grooves in ectoderm appear at the level of the hindbrain. A very slight swelling marks the future gill region. Mandibular arch becomes prominent and four pairs of somites are present.
21	72.0	47	L = 2.7 B = 1.5 H = 1.8	Neural folds are completely fused. The posterior boundary of the gill region is more distinct. Pronephros is visible as a very slight swelling, and four pairs of somites are present. The ventral side of the embryo is slightly concave; the head region curves downward from the mandibular arch. The dorsal side forms a semicircle; occipital and parietal brain curvatures are apparent.
22	73.00	48.0	L = 2.8 B = 1.4 H = 2.3	The gill region and the pronephros are now more distinct. The tailbud is very slightly outlined, and five to six pairs of somites are present. The ventral side of the embryo becomes more concave as the downward curvature of the head increases.
23	~74.00	~48.5	L = 3.5 B = 1.35	The primordium of the ear is outlined as a shallow depression in the ectoderm in the region above the future hyoid arch. In the dorsal region of the gill swelling, the hyobranchial furrow appears, outlining the boundary between the hyoid arch and the first branchial arch.

Stage Number	Time from First Cleavage: In Hours and Minutes	t_n/t_0	Size (mm)	Features
24	80.00	52.0	L = 3.0 B = 1.35 H = 1.85	Ear pit is more distinct. The hyobranchial furrow continues to lengthen ventrally. The pronephric swelling is clearly outlined, and both the pronephros itself and the beginning of the pronephric duct are clearly visible. Eight or nine pairs of somites are present.
25	83.00	54	L = 3.25 B = 1.45 H = 2.0	The gill swelling continues, and the hyobranchial furrow begins to lengthen. The first branchial furrow appears faintly outlined in the dorsal region of gill swelling. The tailbud is still small; nine pairs of somites are present. The body of the embryo continues to lengthen; the ventral side is more concave; downward curvature of the head increases.
26	84.30	55	L = 3.25 B = 1.6 H = 1.9	Ear pit is quite distinct. The first branchial furrow is longer and more marked. The gill region is a large, distinctly outlined swelling, the height of which is accentuated by a deep groove formed between it and the developing pronephros. The pronephric duct is visible along six somites. The primordium of the olfactory organ appears as a tubercle on the anterior part of the head. The tailbud is gradually enlarging. The body of the embryo is stretched; the head is curved downward. There are 10–11 pairs of somites.
27	86.00	56 (54–59)	L = 3.35 B = 1.6 H = 1.85	The second branchial furrow appears faintly in the dorsal part of the gill swelling; 12 pairs of somites are present.
28	92.30	60 (57–63)	L = 3.55 B = 1.6 H = 1.75	Further lengthening of the body of the embryo occurs, and maximum downward curvature of the head is reached. The olfactory pit is distinctly outlined in front of the eye, and 14 pairs of somites are present.
29	97	63.5–64.0	L = 4.2 H = 1.75	The body of the embryo begins to straighten, visible only by the lesser curvature of the head. The tailbud enlarges; 16 pairs of

Stage Number	Time from First Cleavage: In Hours and Minutes	Time from First Cleavage: t_n/t_0	Size (mm)	Features
30	102	67 (65–68)	L = 4.5 H = 1.65	The head continues to straighten and the dorsal curvature is reduced; the body elongates and the tailbud enlarges. The finfold appears for the first time. The dorsal finfold begins at somite 14.
31*	109	71 (69–74)	L = 4.7 H = 1.7	There are 19 pairs of somites. A groove appears in the region of the lens primordium. The third branchial furrow is apparent in the dorsal part of the gill region. The dorsal finfold begins at somite 12.
32	113	74 (73–75)	L = 5.0 H = 1.7	There are 20 pairs of somites. The dorsal fin begins at somite 10.
33*	113	74	L = 5.25 H = 1.6	There are 21–22 pairs of somites. The dorsal fin begins at somite 8.
34	115	75	L = 5.5 H = 1.6	There are 24–25 pairs of somites. The dorsal fin begins at somite 7.
35	122	80	L = 6.25 H = 1.6	From this stage on, the body axis from the hindbrain to the tail base is quite straight. Three external gills show as nodules on the surface of the gill swelling. The lateral line reaches to the sixth somite. The dorsal fin begins at the fifth somite. The first chromatophores appear; heart pulsation begins. The somites are now difficult to count.
36	130	85–98	L = 7.1 H = 1.7	External gills are short sprouts directed to the side from the gill swelling.
37*	177	115	L = 7.5 H = 1.7	Gills elongate and push ventroposteriorly. No limb buds are yet visible.
38*	178	114–132	L = 7.9 H = 1.8	Filament sprouts appear as two nodules on each gill. The primordium of the operculum is visible as a fold upon the hyoid arch. Neither of the two rudiments of the operculum reaches the midline. The limb buds are slightly outlined.
39	220	144	L = 9.0 H = 1.9	The first gill has two pairs of filament sprouts; the second and third have three pairs each. The gills cover the limb buds. Both rudiments of the operculum approach the midline. The angles of the mouth begin to show.

Stage Number	Time from First Cleavage: In Hours and Minutes	Time from First Cleavage: t_n/t_0	Size (mm)	Features
40	240	157	L = 9.3 H = 2.1	The gills are longer, and the number of filaments increases. On the first gill are four pairs; on the second and third are six or seven pairs. The rudiments of the operculum join at the midline. The angles of the mouth are marked more distinctly. The limb buds protrude slightly.
41	265	173–177	L = 10.0 H = 2.2	The gills continue to elongate. The number of filaments increases, and they become longer. The mouth is distinctly outlined. The second lateral line runs along the flank toward the limb bud and bypasses it on the ventral side. The forelimb buds are still small. Hatching begins.
42	296	193	L = 10.5 H = 2.1	The gills extend far beyond the forelimb buds. The mouth is completely outlined, but is not broken through.
43	342	223	L = 11.3 H = 2.3	The breaking through of the mouth occurs, or the mouth is already open.

[a]Stage numbers in table correspond to those in Plates I–VI. Plate I (stages 1–13); Plate II (stages 14–22); Plate III (stages 23–30); Plate IV (stages 31–35); Plate V (stages 36–40); Plate VI (stages 41–44).

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