Cloning and Expression of a Homeobox (*Hox A5*) Gene in the Mexican Axolotl (*Ambystoma mexicanum*)

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Summary

Differences along the anterior-posterior (A-P) axis in many organisms are set up by spatial and temporal expression of a common set of genes early in development. Homeobox genes are a family of regulatory genes which display very specific expression during development. Hence, they are excellent candidates to be exploited as stage-specific molecular markers during embryogenesis. We have PCR amplified a previously identified 270-bp *Ahox-1* homeobox fragment from a stage 18 axolotl embryonic cDNA library. The same library was then screened under moderately stringent conditions using the 270-bp fragment as a probe, and we have isolated six different clones and determined their partial nucleotide sequences. One of the clones, which has very high homology to human, mouse, and rat *Hox A5* (83% and 99% at the nucleotide and amino acid levels respectively in the homeodomain region), was analysed further. RT-PCR was employed to analyse the expression pattern of this gene during development. Transcript levels for *Hox A5* are low at stage 11 of embryogenesis (gastrula). The level of expression reaches maximum at stage 25 (tailbud) and then plateaus at stage 30 and 35 (heartbeat onset). Also, presence of transcripts for *Ahox-1* during embryonic development followed the previously described pattern. Tissue and organ specific expression for both *Hox A5* and *Ahox-1* was also studied by RT-PCR in the neotenous adult.

Introduction

The Mexican axolotl, *Ambystoma mexicanum*, is an excellent model system for studying vertebrate embryonic development, in part due to the availability of a number of developmental mutants (Armstrong and Malacinski, 1989), including the cardiac nonfunction mutation (gene *c*) (Lemanski et al., 1979, 1992; Smith and Armstrong, 1990). However, very little molecular biology of embryonic development in the Mexican axolotl has been done. In fact, to date no full-length developmentally relevant gene has been cloned (Busse and Séguin, 1993; Whiteley and Armstrong, 1991). Recently, an axolotl genomic fragment containing a homeobox gene, *Ahox-1*, has been cloned and partially sequenced (Whiteley and Armstrong, 1990). *Ahox-1* has high homology (79-89%) to the *Ghox-lab* sequence of the chick, the *Hox-1.6* gene in mouse, and the labia gene of *Drosophila*. When the construct, consisting of a partial cDNA of the *Ahox-1* homeobox gene placed under the control of the mouse hsp68 promoter, was microinjected into fertilized axolotl eggs, several mutant embryos were produced (Whiteley and Armstrong, 1990).

The homeotic genes involved in the development of *Drosophila* are well characterized and belong to the *Hom-c* gene complex (the antennapedia complex [ANT-C] and the bithorax complex [BX-C]) (Lewis, 1978). *Hox* genes, members of the antennapedia complex (ANT-C), contain a conserved DNA sequence of 180 bp designated as the homeobox, which encodes the homeo domain, a DNA-binding motif present in a large number of eukaryotic regulatory proteins (McGinnis et al.,1984; Scott and Weiner, 1984). *Hox* genes are known to specify pattern formation along the antero-posterior (AP) body axis of many organisms (Akam, 1989; Duncan, 1987; Kaufman et al., 1990; McGinnis and Krumlauf, 1992). About 38 *Hox* genes are known to occur in four different chromosomal complexes of the mouse and human and are most likely present in other vertebrates as well. The genes in each cluster are oriented in the same 5' to 3' direction of transcription (Gaunt et al., 1988; Duboule and Dollé, 1989; Graham et al., 1989). A variety of homeobox-containing genes have been identified in vertebrates, including frog (Cho et al., 1988), chicken (Morgan et al., 1992), newt (Belleville et al., 1992), fish (Eiken et al., 1987), axolotl (Whiteley and Armstrong, 1990), etc. as well as in many invertebrates (Bürglin et al., 1991), fungi (Schulz et al., 1990), and plants (Ruberti et al., 1991). In amphibian species (e.g., newts), the homeobox genes are organized in clusters similar to other vertebrates (Belleville et al., 1992).

We have amplified the homeo domain region of *Ahox-1* from a Stage 18 embryonic axo-

lotl cDNA library in lgt11 using a primer-pair designed from the published *Ahox-1* genomic DNA sequence (Whiteley and Armstrong, 1991) and confirmed the amplified DNA as *Ahox-1* by subsequent cloning and sequencing the PCR-amplified DNA. We used the [^{32}P]-labeled PCR product (270 bp) to screen the same cDNA library under moderately stringent conditions and isolated several positive clones. After sequence analyses, most of these clones were found to have homology with different homeobox genes. In this paper, we now report that one 1.15 kb cDNA clone (#2a.1) containing the entire homeo domain is homologous to the murine *Hox A5* gene. Also, we have compared the level of expression of *Hox A5* and *Ahox-1* using reverse transcription polymerase chain reaction (RT-PCR) of the RNA isolated from various stages of embryonic development of the Mexican axolotl.

Experimental and Discussion

(a) Cloning the axolotl homeobox genes. The homeo domain region of *Ahox-1* was amplified by the polymerase chain reaction (PCR) using the primer-pairs designed on the basis of the published sequence from a stage 18

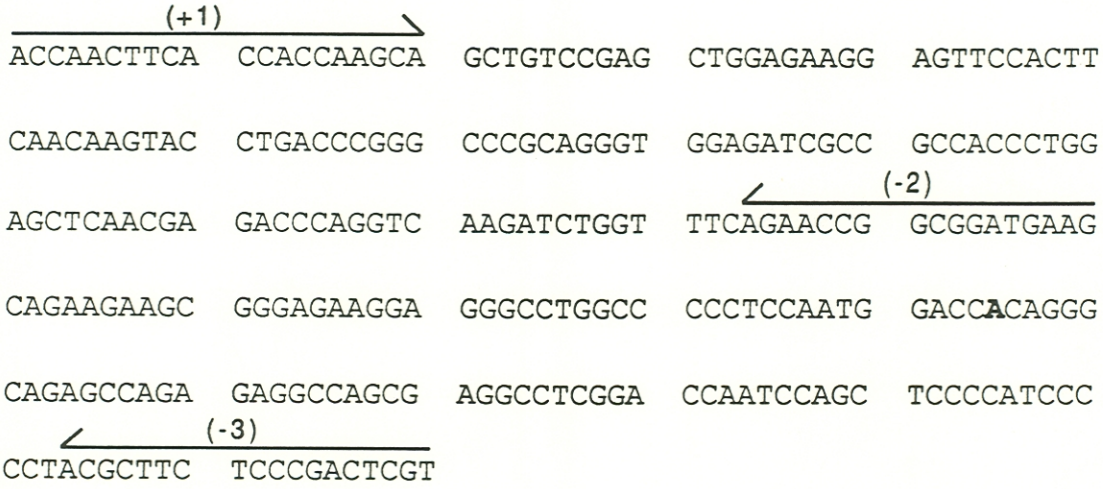

Figure 1. Sequence of *Ahox-1* PCR-amplified DNA. A stage 18 cDNA library, lAM18 (generous gift from Dr. Carl Séguin, Centre de recherche, Quebec, G1R 2J6), was screened by polymerase chain reaction (PCR) using primer-pairs designed on the basis of the published genomic *Ahox-1* sequence. The sequences of the oligonucleotides used as the primer-pair and the detector are marked in the figure. The sequences of the forward (+1) primer and backward (-3) primer are 5'-ACCAACTTCACCCACCAAGCA-3' and 5'-ACGAGTCGGGAGAAGCGT-3' respectively. The sequence of the detector oligonucleotide flanked by the primer-pair is 5'-CTTCATCCGCGGTTCT-3' designated as (-2) in the figure. Prior to amplification by PCR with the primer-pair, an aliquot of the cDNA library was heated in a boiling water bath or five minutes and the amplification was carried out following the published procedure using the Amplitaq PCR kit with Taq polymerase from the Perkin-Elmer/Cetus Corporation. Amplification was accomplished in a total volume of 100 µl containing 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 270 mM of each of dATP, dGTP, dCTP and dTTP, 3 units of Taq DNA polymerase, and 20 pmol of each of two primer-pairs. The amplification was carried out for either 45 or 60 cycles in a thermocycler. If necessary, the extension time was increased up to 2 minutes at 78°C. All pre- and post-PCR analyses were done in separate rooms with different sets of equipment (Dube et al., 1993). The amplified DNA was subjected to southern blot hybridization after agarose (1.5%) gel electrophoresis followed by the alkaline capillary transfer of the amplified DNA. The detector oligonucleotide (-2) was end-labeled with [g-^{32}P]ATP (DuPont-NEN) using T4 polynucleotide kinase (Gibco-BRL) and was subsequently used as the probe for the hybridization which was carried out in 6 X SSC/0.5% SDS at 42°C. The filters were washed four times at 42°C with 0.2% SSC/0.5% SDS and were subsequently autoradiographed at -70°C with an intensifying screen using Kodak X-OMAT AR film. One µl of PCR amplified DNA was ligated to a PCR™ 1000 TA vector (Invitrogen) using T4 DNA ligase following the manufacturer's specifications. Subsequent transformations, colony hybridization to pick up the desired clones and preparation of DNA templates were carried out as described earlier (Saksena et al., 1992; Luque et al., 1994). DNA sequencing was carried with double-stranded DNA using di-deoxy chain termination method (Sanger et al., 1977) with the Sequenase version 2.0 kit (USB). "**A**" denotes a change in sequence from the published sequence (Whiteley and Armstrong, 1991).

axolotl embryonic cDNA library. The amplified DNA was subcloned into a T/A cloning vector, and the nucleotide sequence was determined (Figure 1). The nucleotide sequence of *Ahox-1* cDNA is in good agreement with the published genomic sequence. We have observed a single base change (T, A) from the earlier published sequence as specified in the legend for Figure 1. However, this does not alter the amino acid sequence. This discrepancy could be due to either axolotl strain variation or to misincorporation by reverse transcriptase during cDNA synthesis or else by misincorporation by Taq DNA polymerase during PCR amplification. The sequence of the primer-pair and the detector oligonucleotide is marked in the figure. The same primer-pair was employed to amplify the [^{32}P-dCMP]-labeled DNA which was subsequently used as a probe for screening the stage 18-specific cDNA library with moderate stringency (hybridization at 55°C and the washing at 55°C with 2xSSC containing 0.5% SDS). A total of 500 thousand plaques were screened, and six positive clones were isolated ranging in size from 450 bp to 1.9 kb. Each of these clones was partially sequenced from both ends. The sequences were then compared with known sequences in the Gen/Embl data bank using the GCG program. All of these sequences were found to have significant sequence homology with a number of homeobox genes. One of the clones (#2a.1) of 1.2 kb, which was found to be homologous to murine *Hox A5* (formerly *Hox 1.3*), was sequenced and characterized further. Sequencing was performed by subcloning into the TA vector PCR™1000 and also subcloning into M13mp18 at the *Eco*RI site. This clone was found to be 1157 nucleotides long and to contain a complete homeobox sequence along with a stop codon and 686-nucleotide long 3'-untranslated region (3'-UTR). It has a polyadenylation signal 663 nucleotides downstream from the stop codon. The nucleotide, as well as the deduced amino acid sequence, gives an ORF of 471 nucleotides (Figure 2). Both the nucleotide and the amino acid sequences were subjected to FASTA and TFASTA analysis using the GCG program. This clone was found to have the highest homology with the murine *Hox 1.3* gene, which is known currently as *Hox A5* (Bürglin, 1994). *Hox A5* has been identified and isolated from a variety of organisms, such as human, mouse, rat and *Xenopus*, and is known to possess fairly conserved sequences both at the nucleotide and amino acid levels. This partial clone accounts for about 60% of the coding sequence of the *Hox 1.3* gene from man or rat (Tournier-Lasserve et al., 1989). Sequences of the murine and human *Hox A5* genes were analyzed extensively, and the degree of similarity between the two genes is 98% in the 250 nucleotides upstream from the start codon, 94% in the coding region, 72% in the intron, and 90% in the 3'UTR. The high degree of similarity between the human and murine *Hox A5* cognates in the 3'UTR drops to 35% 30 nucleotides downstream of the polyadenylation signal, suggesting functional importance for the region between the translation termination and the polyadenylation signal. The proteins encoded by the murine and human genes differ only in 7 of 270 residues (Tournier-Lasserve et al., 1989). The axolotl *Hox A5* shares 79% and 85% homology at the nucleic acid and protein level, respectively, with its murine counterpart. The 3'UTR also shares 72% homology (Figure 2).

(b) Amino acid alignment of the homeo domain. The most conserved region of the homeodomain shares amino acid sequence and structural similarities with DNA-binding regulatory proteins from both yeast and bacteria (Laughon and Scott, 1984; Shepherd et al., 1984). It has been proposed that the homeodomain proteins function by binding to *cis*-acting regulatory elements and modulating the transcriptional activity of specific genes. This proposal has been reinforced by *in vitro* DNA binding studies (Desplan et al., 1985; Cho et al., 1988). The murine *Hox A5* homeodomain protein is a nuclear phosphoprotein capable of binding specific DNA sequences (Odenwald et al., 1989). Both phosphorylated and nonphosphorylated forms bind DNA directly in a sequence-specific manner. The alignment of the peptide sequence of homeodomains (data not shown) from different animals reveals that the axolotl *Hox A5* gene is 100% homologous to human and mouse *Hox A5* and about 98% with the rat counterpart. However, there is only about 90% homology with that from *Xenopus*. Thus, the axolotl *Hox A5* is closer to the mammalian homologue. It is interesting to note that the amino acid homology within the homeobox was 100% with a *Hox 1.3* paralogue, *Hox 2.1* and 90% with *Hox 3.4*, another paralogue (Krumlauf et al., 1987; Simeone et al., 1988). Axolotl *Hox A5* also has a conserved hexapeptide upstream of the homeobox, which is a characteristic of this class of Antennapedia-type homeobox genes

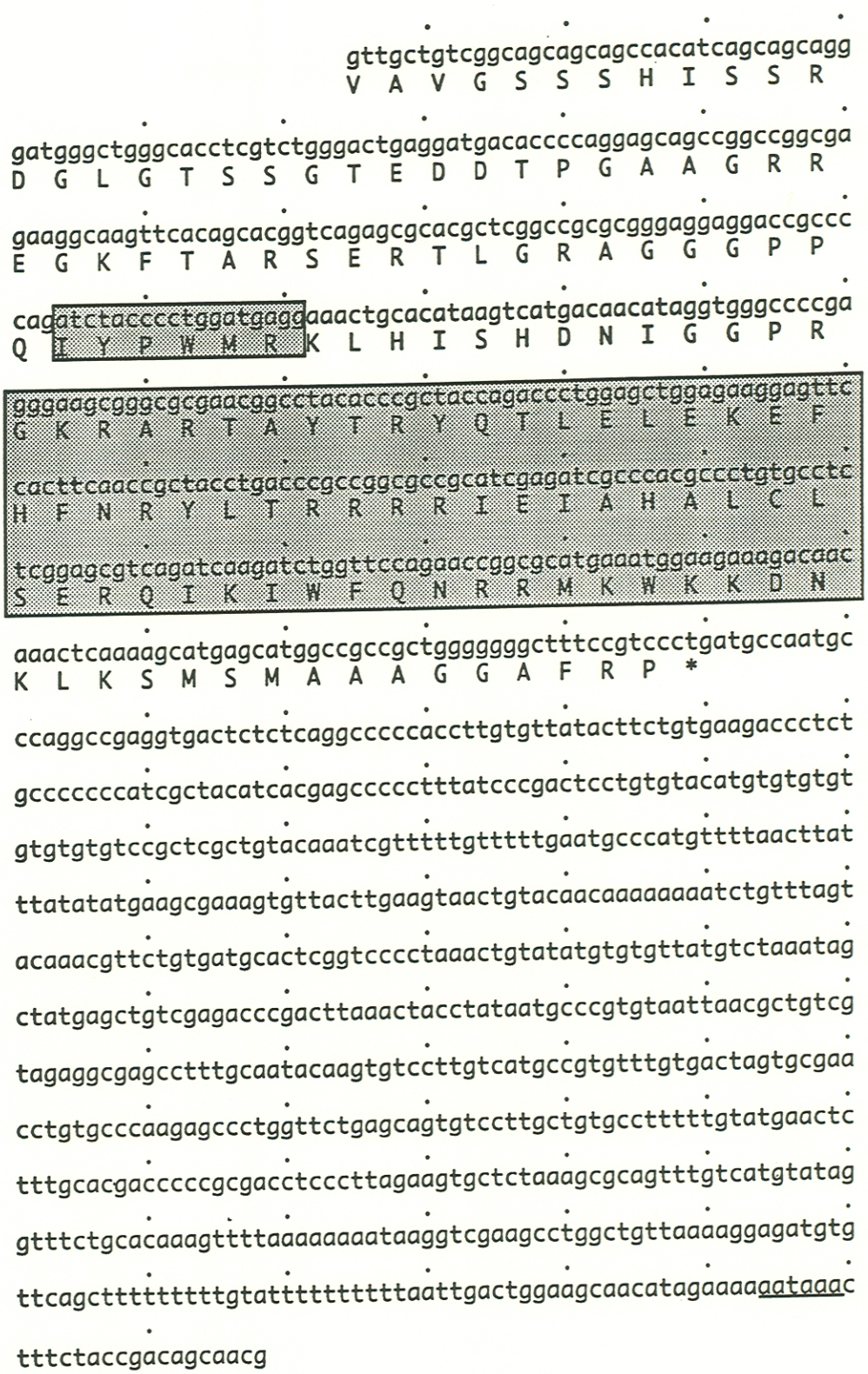

Figure 2. Nucleotide and the deduced amino acid sequences of the *Hox A5* cDNA clone from the lAM18 library constructed from the RNA of stage 18 embryonic axolotl. *E. coli* Y1090 cells were used as the host to plate out the lAM18 cDNA library (Busse and Séguin, 1993) at a density of 50,000 pfu per 150 mm petri dish. After an overnight incubation at 37°C, filter lifts were taken from the plates using Schleicher and Schuell maximum strength Nytran plus nylon membranes. The prehybridization as well as hybridization were carried out at 55°C in 6 x SSC, 0.02% Ficoll 400, 0.02% polyvinyl pyrollidone, 1mM EDTA, 0.5% SDS, 0.02% bovine serum albumin and 10% (w/v) dextran sulfate (MW 500,000). Positive plaques were purified for four rounds until all of the plaques in the plate were positive. A pure plaque was taken for PCR amplification using primers complimentary to the lambda sequences flanking the inserts. PCR-amplified DNA was subcloned into the PCRII™ 1000 TA vector (Invitrogen). Another aliquot of PCR amplified DNA was digested with EcoR1 and ligated to M13mp18 cut with the same restriction endonuclease following standard protocols (Sambrook et al., 1989). Sequencing was performed in both directions using either double-stranded or single-stranded DNA templates following Sanger's di-deoxy chain termination method. In the figure, the conserved hexapeptide and homeo domain are boxed. The sequence for the polyadenylation signal is underlined. 'H' denotes the stop codon.

(Mavillio et al., 1986). The hexapeptide in *Xenopus Hox A5* is about 80% homologous to that of axolotl. On the other hand, the hexapeptide is identical in the mouse, rat, and human *Hox A5* (Bürglin, 1994). In *Xenopus*, one Tyr has been changed to Phe. Currently, the significance of this hexapeptide present in this class of homeobox protein is not known. When comparing the nucleotide sequences of the homeoboxes between man and axolotl *Hox A5*, 27 codons are different. Twenty-one changes are synonymous; that is, there are no changes in the amino acids. However, the remaining six changes are non-synonymous. It is noteworthy that the coding sequence of the homeo domain of axolotl *Hox A5* has only 72% homology at the nucleic acid level and 71% homology at the amino acid level with those from A*hox-1* (Whiteley and Armstrong, 1991).

(c) Expression of *Hox A5* and *Ahox-1* at various stages of embryonic development. The expression of the homeobox containing genes in vertebrates are known to be restricted both spatially and temporally during development (Condie and Hardland, 1987). The expression of *Ahox-1* from northern blot analyses was found to be developmentally regulated. We have also studied the expression of *Hox A5* and *Ahox-1* at different stages of the embryonic development in the Mexican axolotl using reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from axolotl embryos at stages 11, 17, 25, 30, and 35 of development (Bordzilovskaya et al., 1989). The total RNA was treated with RNase-free DNase prior to the RT-PCR analyses to ensure that the RNA was free of any DNA contamination (Erginel-Unaltuna et al., 1994). First strand cDNA was synthesized from one microgram of the DNase-treated total RNA from each of the developmental stages mentioned after annealing with oligo dT using superscript II reverse transcriptase (Gibco/BRL). Next, gene-specific primer-pairs (as given in the figure legend) were employed to perform PCR amplification for *Hox A5, Ahox-1,* and alpha tropomyosin (as control). A detector [^{32}P]-labeled oligonucleotide, specific for each gene flanked by the respective primer-pairs used in PCR amplification, was employed as a probe for southern blot analyses of the ampli-

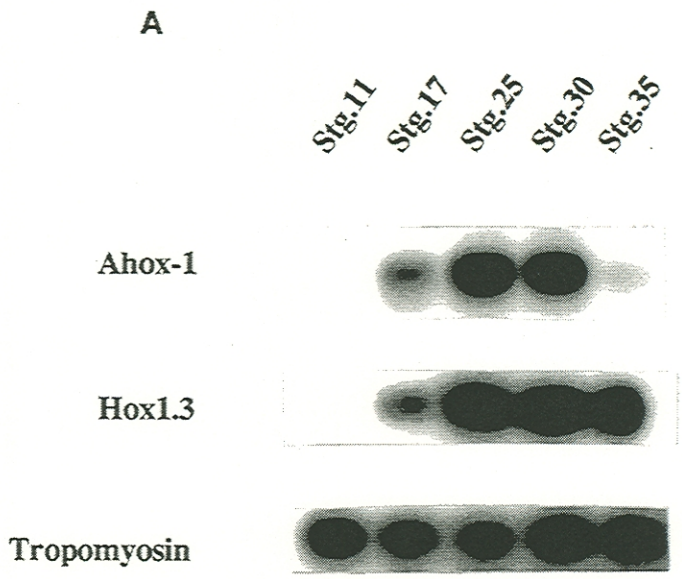

Figure 3. RT-PCR analyses for expression of *Hox A5* and *Ahox-1* at different embryonic stages of axolotl. Total RNA was extracted from 500 mg of embryonic tissues at each of the developmental stages indicated in the figure, using the RNagents™ total RNA isolation kit from Promega and following the manufacturer's instructions. The quality of RNA was checked on formaldehyde denaturing agarose (1.5%) gels as well as by determining the absorptions at 260 nm and 280 nm in a Beckman DU64 spectrophotometer. The reverse transcription was performed using the Superscript II™ -H with oligo dT hooked to a universal adapter primer (UAP) supplied by the manufacturer in the kit. We followed the manufacturer's instructions for the entire operation. The RNA samples were treated with DNase-I for 20 minutes at room temperature and the DNase-I was subsequently inactivated with 20 mM EDTA. An aliquot of the RNA-DNA hybrid was used for PCR amplification using specific primer-pairs as indicated in the text. The PCR amplified DNA was subjected to Southern blotting using [^{32}P]-labeled specific oligonucleotide probe for subsequent autoradiography. Upper panel: A*hox-1*; Middle panel: *Hox A5* and the bottom panel: alpha tropomyosin.

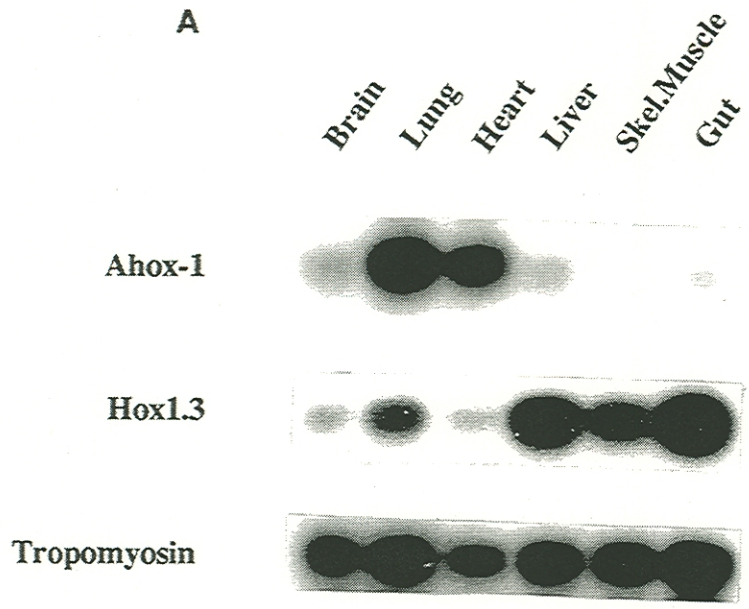

Figure 4. The organ/tissue specific expression of *Ahox*-1, *Hox* A5 and Alpha tropomyosin in juvenile axolotl using RT-PCR. Eight-month-old juvenile axolotls were sacrificed and the relevant tissues were dissected and frozen immediately in liquid nitrogen. Isolation of total RNA from various tissues and subsequent RT-PCR analyses were performed using the methods as described in the legends under Figure 3. One microgram of total RNA was used in every reverse transcription reaction.

fied DNA. Such analyses ensure the specificity of RT-PCR. The results of southern blot analyses as given in Figure 3 reveal that the expression of *Hox A5* is very low at stage 11 (gastrula). The level of expression appears to increase at stage 17 (neurula) and beyond. In fact, it reaches a plateau at stage 30 (tail bud) and continues until reaching prehatching stage 35. Although the expression of *Ahox-1* was found to start at stage 11 and to reach a maximum at stage 25. At stage 30, the expression continues but it declines at stage 35. These results are in good agreement with those of already published northern blot analyses (Whiteley and Armstrong, 1990). RT-PCR of tropomyosin was performed as a control.

(d) Organ/tissue-specific expression of *Hox A5* and *Ahox-1*. The various organs as described in Figure 4 were dissected from eight-month-old juvenile axolotls, and total RNA was extracted for RT-PCR analyses as stated above. The expression of *Hox A5* was found to be lower in heart tissue than *Ahox-1*. However, the expression of *Ahox-1* is significantly lower than *Hox A5* in skeletal muscle (dorsalis trunci). Expression of *Hox* A5 in the liver and lung is found to be higher than that in the brain or gut. The message levels for *Ahox-1* are higher in lung than in liver and brain. Very little expression was noted for *Ahox-1* in the gut. Odenwald et al. (1987) also studied the expression of murine *Hox A5* by northern blot analyses of 16-week postnatal adult tissues. The major transcripts were present at low levels in liver, kidney, ovary, testis, spinal cord and brain with the greatest abundance observed in the spinal cord. *In situ* hybridization for the analyses of tissue-specific expression of *Hox A5* and *Ahox-1* is currently in progress in our laboratory. The preliminary results (data not shown) show that *Hox A5* transcripts are expressed in the ventricular myocardium of the embryonic heart at stage 41.

Acknowledgments

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