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Expression of Homeobox-containing Genes in Common Carp *Cyprinus carpio* L.

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Jr-Gang Cheng, Ching-Fong Liao, Ya-Li Hsu and Jen-Leih Wu (1995) Expression of homeobox-containing genes in common carp *Cyprinus carpio* L. *Zoological Studies* **34**(3): 170-176. Genetic and molecular analyses of *Drosophila* development mutants led to the discovery of homeotic genes which control segmental identity in the fruit fly. All of the known homeotic genes contain a conserved protein-encoding DNA sequence of about 180 bp, named the homeobox, which is present in multiple copies in the genomes of most higher animal species. By using a *Drosophila* homeobox-containing gene from the Antennapedia (*Antp*) complex as a probe, Southern hybridization revealed that there are at least two homeobox-containing genes in carp. RNA slot hybridization showed that the *Antp*-like gene(s) expresses intensively in the carp intestine, eye, kidney, and heart, less intensively in the liver, testis, and cerebellum, but not at all in the cerebrum, pituitary gland, and muscle. Northern hybridization revealed that there are at least five transcripts present in the carp embryo and each expresses in a unique, stage-dependent pattern.

Key words: Fish, Homeotic gene, Antennapedia, Embryogenesis, Tissue-specific.

During studies of regulation of development in *Drosophila melanogaster*, a 180 bp DNA homologous sequence was defined among development-related genes by McGinnis et al. (1984c) and Scott and Weiner (1984). The DNA sequence named homeobox was found to be conserved in many genes, and it has provided a new method to understand the mechanisms of differentiation and development. By using the homeobox as probes, which are usually derived from *Drosophila* genes, one can find that the homeobox also exists in other species, such as yeast (Shepherd et al. 1984), nematodes (Costa et al. 1988), annelids (McGinnis et al. 1984a), sea urchins (Dolecki et al. 1986), molluscs (Degnan and Morse 1993), and higher metazoans such as fish (Eiken et al. 1987), frogs (Carrasco et al. 1984), chickens (Wedden et al. 1989), mice (McGinnis et al. 1984b), sheep (Choi et al. 1988), and humans (Sebastio et al. 1987). The homeobox is believed to be ubiquitous throughout the animal kingdom. The widely distributed homeobox-containing genes usually encode proteins which contain the homeodomain, translated from the homeobox. The homeodomain contains a helix-turn-helix motif which provides the ability to interact with DNA. As a nuclear protein, the function of the homeobox protein involves regulation of transcription (Bodner et al. 1988, Ingraham et al. 1988, Levine and Hoey 1988, Castrillo et al. 1989, Frain et al. 1989, Baumhueter et al. 1990, Karlsson et al. 1990). During development, the homeobox proteins are presumably involved in establishing morphological fields (Oliver et al. 1988, Wright et al. 1989, Nohno et al. 1991) and in determining the cell fates (Finney et al. 1988, Ruiz et al. 1989).

To understand certain general phenomena in higher animals, some model systems have been established in primitive species, such as yeast, for the study of eukaryotic cells, and *Drosophila* for the study of genetics and development. Fish can serve as a good model for the study of vertebrate development and differentiation. This is based on two assumptions: (1) that general control

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mechanisms of vertebrate morphological formation are evolutionarily conserved, and (2) that higher level of vertebrates, the greater is the complexity of their development. A major focus of fish development and embryogenesis has been on the zebrafish (*Brachydanio rerio*), from which engrailed-like (Fjose et al. 1988) and *Antp*-like genes have been cloned (Njølstad et al. 1988a,b).

In this preliminary report, we identify the presence of homeobox-containing genes (Hox gene) in carp (*Cyprinus carpio*), and show that the expression of these genes are dependent on the stage of embryogenesis and the type of tissue in which they occur. There are at least five different transcripts of Hox genes and these may be related to some developmental events. Extracted RNA from different tissues also shows various levels of hybridization signals which suggests that *Antp*-like Hox genes may play a role in embryonic as well as in adult tissues.

MATERIALS AND METHODS

Plasmid pAT153 containing an inserted homeobox-containing fragment excised from the *Drosophila Antp* gene was transformed to *E. coli* (JM101). Bacteria were incubated in LB broth with or without chloramphenicol and selected under 50 μg/ml ampicillin. A large amount of plasmid DNA was purified by using the alkaline lysis and CsCl gradient methods and was identified by restriction endonuclease of *Bam* HI, *Pvu* II, *Kpn* I and *Xba* I. The portions of *Bam* HI/*Kpn* I DNA fragments which included most of the homeobox sequence were used as hybridization probes labelled with ^{32}P-dCTP by nick translation (Sambrook et al. 1989).

The genomic DNA of *Drosophila* was purified by the proteinase K/Sacrosyl method which was modified by Dr. McGinnis et al. (1984c). The fish DNA for Southern transfer was extracted by use of PBS/guanidine hydrochloride buffer (6M guanidine HCl and 0.1 M NaOAc in PBS pH 7.3) at 37°C after tissue frozen with liquid nitrogen was powdered. This solution was extracted with phenol/chloroform twice. After ethanol precipitation, the DNA was dissolved in TE buffer in a 68°C bath. In addition to Southern hybridization, carp testis powder was incubated in 0.5% SDS and 100 μg/μl proteinase K at 50°C for 2 hr to generate genomic DNA and to construct a library.

An aliquot of 10 μg DNA, extracted from fish and *Drosophila*, was digested by *Eco* RI and *Bam* HI, separated by 0.8% agarose, and then these DNA fragments were transferred to nitrocellulose (NC) paper. Hybridization conditions were the same as in the Dr. McGinnis' procedures (36 hr, 37°C, 43% formamide). After hybridization, the NC paper was washed under low stringency conditions (2 × SSC, 0.1% SDS) three times at 37°C for 5 min each, twice at 50°C for 15 min each, and then exposed to X-ray film.

The developmental stages of carp embryos were identified according to the illustrations of Iwayi (1985). Total RNA was extracted by the illustrations of Iwayi (1985). Total RNA was extracted by the phenol method from different carp tissues and eggs at various embryogenic stages. Samples were homogenized by use of a polytron with 30 ml RNase-free extraction buffer (100 mM NaOAc, pH 5.0; 25 mM NaCl; 35 mM $MgCl_2$, 25 mM EGTA plus 20% SDS (0.5 ml); 25 μg/ml polyvinyl sulfate (20 μl); 35 mg/ml spermine (20 μl); 200 mM vanidyl ribonucleoside complex (0.5 ml) and β-mercaptoethanol (0.5 ml) with a final volume of 30 ml). An aliquot of 10 ml phenol was added after homogenization, and the solution was shaken vigorously for 10 min. Chloroform was then used to extract the reaction mixture until the solution clarified from the original dark green color, after which RNA was precipitated by the ethanol method. For RNA slot blot, 2 μg of RNA samples from different organs or 6 μg of RNA samples from different embryonic stages were transferred to nitrocellulose paper under vacuum. For northern transfer, 15 μg of RNA samples from each different embryonic stage were separated with 1% formaldehyde agarose gel. Before loading on the gel, the RNA samples were air dried, dissolved in 20 μl loading buffer and then heated to 95°C for 2 min. After running RNA in 1 × MOPS at 75 V for 4 hr, the RNA was transferred to nitrocellulose paper and hybridized under the same conditions as for Southern blotting.

RESULTS

Several plasmids containing *Drosophila* genes were a gift from Dr. W. McGinnis. Only the plasmid with *Antp* could generate the nearly 180 bp *Kpn* I/*Bam* HI fragment which includes most of the homeobox as predicted from the restriction enzyme map (Fig. 1). In addition to the *Kpn* I site located on the homeobox of the *Antp* gene, the *Xba* I site, which is close to the *Kpn* I site, also can be identified in this plasmid. The *Kpn* I/*Bam* HI-excised

DNA fragment was used as the probe for homeobox identification.

In order to test whether the *Antp* gene can recognize any hybridization signal from fish genomic DNA, 10 μg of genomic DNA from *Drosophila* (used as positive control), carp or zebrafish were digested with *Eco* RI or *Bam* HI and subjected to Southern hybridization according to the procedures and conditions described by McGinnis et al. (1984c). The results show that the *Eco* RI-digested genomic DNA of *Drosophila* has three major bands whereas the carp genomic DNA after *Eco* RI or *Bam* HI digestion has three major bands (two larger than 12 kb and one approximately 4 kb); the zebrafish genomic DNA has one major band at about 4 kb after *Bam* HI digestion (Fig. 2). The results indicate that the *Antp* DNA probe from *Drosophila* can be used for preliminary characterization of homeobox-containing fish genes.

Tissue-dependent expression was determined by use of RNA slot hybridization under low stringency conditions. As shown in Fig. 3, yeast tRNA (F1 in Fig. 3) and *E. coli* rRNA (D1) did not cross-hybridize with the probe; however, samples of the same amount of RNA from carp organs showed different levels of hybridization signals. The homeobox gene transcripts were highly expressed in intestine (H3), eye (F2), kidney (H2), and heart (G2); less in liver (D3) and testis (G3), and were little expressed or not detectable in cerebrum (D2), cerebellum (E2), muscle (E3), and pituitary gland (F3).

RNA slot hybridization was also used to detect transcripts during embryogenesis. However, hybridization with 2 μg of loaded RNA showed no hybridization signal (data not shown). After the amount of total RNA was increased to 6 μg (Fig. 4), stronger signals could be detected at the stage of eye formation (E2 in Fig. 4), ear vesicle formation (F1), heart-beat and brain differentiation (G1), just before hatching (H1), and in fingerlings (H2), whereas weaker signals were present in the unfertilized egg (C1), the fertilized egg (C2), blastula and gastrula stage (D2), the stage of 3-somites formation in embryo (E1), and pigment cell formation (G2).

For further characterization of the size of transcripts of homeobox-containing genes, 15 μg

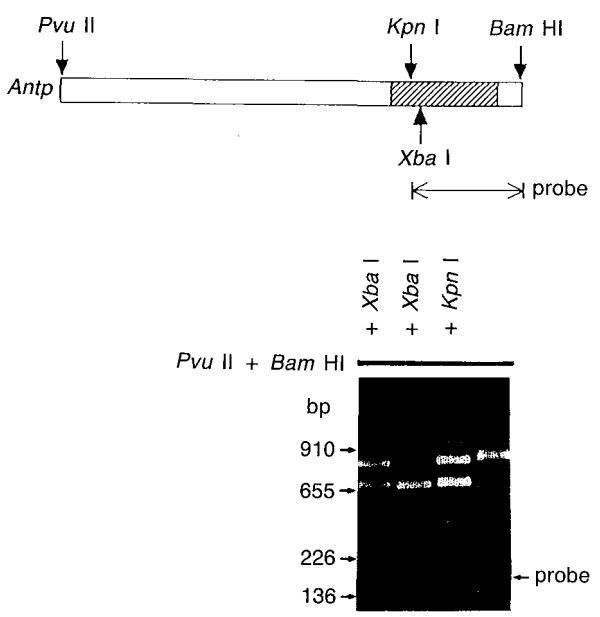

Fig. 1. Mapping and characterization of *Drosophila Antp* probe. Plasmid containing *Drosophila Antp* homeodomain (striped region in the upper panel) was characterized by endonuclease digestion to give the correct size of fragments (lower panel). Lane one is an incomplete *Xba* I digestion. The probe was purified from agarose gel after *Kpn* I and *Bam* HI digestion. Plasmid pBR322 digested with *Alu* I was used as molecular weight marker as shown on the left.

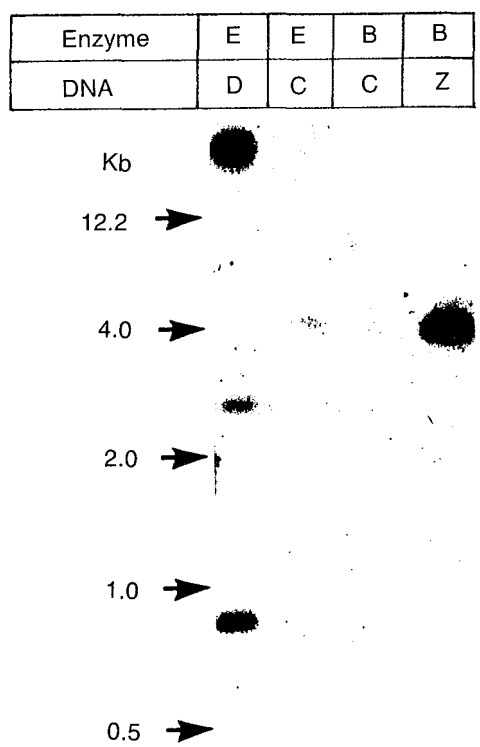

Fig. 2. Autoradiogram of Southern hybridization of genomic DNA digested with restriction endonucleases and hybridized with *Antp* probe. For Southern hybridization, 10 μg of genomic DNA from *Drosophila* (D), carp (C), and zebrafish (Z) were digested with *Eco* RI (E) and *Bam* HI (B). Probes were labelled with ^{32}P-dCTP by nick translation as described in Materials and Methods.

of total RNA from different embryo stages were loaded on formaldehyde agarose gels to perform electrophoresis, Northern transfer and hybridization. After exposure for 24 hr (Fig. 5A), the 4.5 transcript (1st arrow in Fig. 5A) was expressed at the stage of tail formation (lane 5), and the 2.1 kb transcript (2nd arrow in Fig. 5A) was expressed at the stages of optic vesicle formation (lane 4), tail formation (lane 5), and heart-beat and brain differentiation (lane 6). In addition to these two major bands, there were three smaller transcripts (3rd, 4th, and 5th arrows in Fig. 5A) which were co-expressed at the same stages. After long exposure (6 days), the stage-dependent expression pattern showed a very complicated distribution (Fig. 5B). Just after egg fertilization (morula stage, lane 1), a 6.4 kb transcript began to express through the stage of tail formation (lane 5). The 4.5 kb transcript began to express at the blastula and gastrula stages (lane 2) and continued to express through the time just before hatching (lane 8). Another 2.6 kb transcript was also present at the morula stage (lane 1) but vanished at the stage of 3-somites formation in the embryo (lane 3). The expression of the smaller transcripts ($\leq$ 1.6 kb) shown in Fig. 5A were also present at other stages but primarily at the stages of optic vesicle formation (lane 4), tail formation (lane 5), and heart-beat and brain differentiation (lane 6) in Fig. 5B.

DISCUSSION

To our knowledge, this is the first demonstration of the presence of Hox genes in the carp. Previous studies of Hox genes in fish have focused on zebrafish, because of its small size and its short developmental period. However, the small size limits its usefulness in some physiological experiments that require manipulations, such as bleeding, cannulation, etc. Accordingly a larger species, such as the carp, which belongs to the

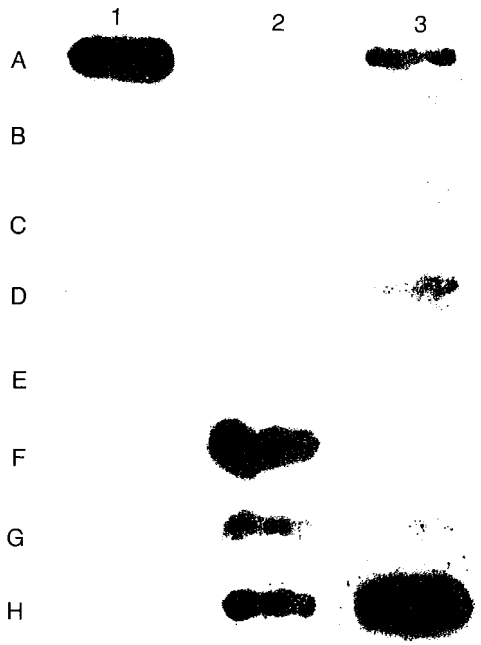

Fig. 3. Autoradiogram of slot hybridization of carp total RNA isolated from various organs and hybridized with *Antp* probe. Each slot was loaded with 2 μg of RNA samples isolated from the carp organs as indicated.
A1: *Antp* plasmid; A3: carp genomic DNA; D1: *E. coli* rRNA; D2: cerebrum; D3: liver; E2: cerebellum; E3: muscle; F1: yeast tRNA; F2: eye; F3: pituitary gland; G2: heart; G3: testis; H2: kidney; H3: intestine.

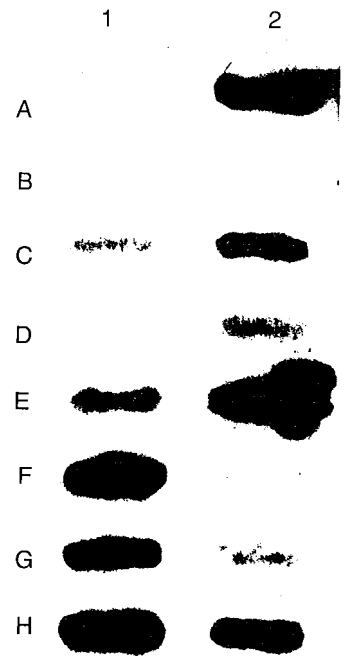

Fig. 4. Autoradiogram of slot hybridization of carp total RNA isolated from embryos at different developmental stages with *Antp* probe. Each slot was loaded with 6 μg of RNA samples from different carp embryonic stages as indicated.
A2: carp genomic DNA; C1: egg; C2: fertilized egg; D1: morula; D2: blastula & gastrula; E1: 3-somites formation in embryo; E2: optic vesicle formation; F1: ear vesicle formation; F2: tail formation; G1: heart-beat; brain differentiation; G2: pigment cell formation; H1: just before hatching; H2: fingerling.

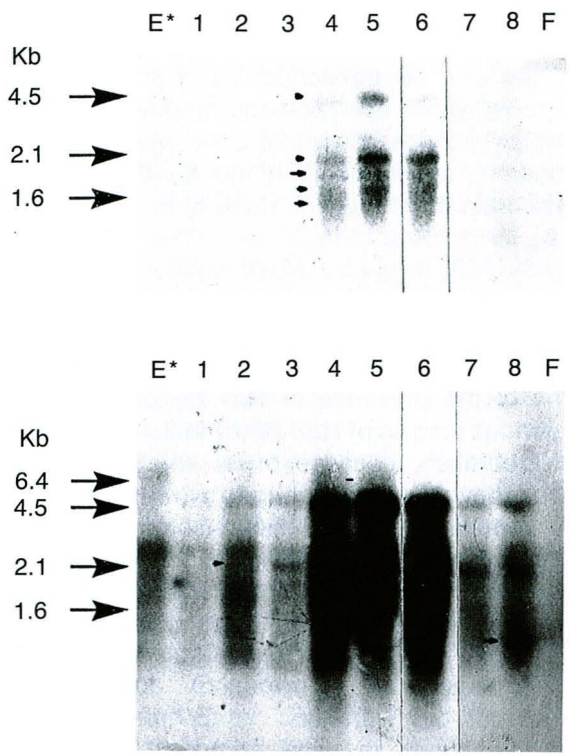

Fig. 5. Autoradiogram of northern hybridization of carp total RNA isolated from embryos of different developmental stages with *Antp* probe. Each lane was loaded with 15 μg of RNA samples from different carp embryonic stages as indicated. Upper panel: 1-day exposure; Lower panel: 6-day exposure of the same blot.
E*: fertilized egg; 1: morula; 2: blastula & gastrula; 3: 3-somites formation in embryo; 4: optic vesicle formation; 5: tail formation; 6: heart-beat; brain differentiation; 7: pigment cell formation; 8: just before hatching; F: fingerling.

same family (Cyprinidae) as zebrafish, was chosen for these studies of the Hox genes.

Although the probe used for this study was derived from the *Drosophila Antp* gene, the hybridization conditions revealed only three major hybridization signals from the carp, and only one from the zebrafish genomic DNA (Fig. 2). The number of DNA hybridization signals was smaller than the number of expected endogenous Hox gene clusters. The signals resulted from use of RNA slot and Northern hybridization procedures were dependent on tissue and developmental stage (Figs. 3, 4, 5), which strengthens our tentative estimation that the signals were real. On the other hand, because of the limitations of the methodology, Southern analysis can reveal only part of the Hox genes (i.e., *Antp*-related) in fish. Comparing the results of Southern hybridization from fish with other animals, it is possible that there are fewer Hox genes in fish than in higher vertebrates.

The results of RNA slot blot of different organs suggest that Hox genes express in the embryonic as well as adult organs such as kidney, intestine, eye, etc. This is consistent with features of Hox genes in other animals. For example, the mouse Hox genes also express in adult tissues, e.g., the *Hox-3.1* gene expresses in spinal cord and kidney (Breier et al. 1988) and the *Hox-2.3* in testis and spinal cord (Meijlink et al. 1987). The function of the homeodomain protein in those organs might be related to maintaining tissue specificity or continuous differentiation of special cell types. For example, the human *Hox*-2.3 gene is expressed in mucous cells of the intestine and is related to epithelial cell differentiation (Sebastio et al. 1987). The Hox gene Pit-1, involved in pituitary gland differentiation, is a tissue-specific transcription factor (Ingraham et al. 1988). However, the pituitary gland of carp shows no hybridization signal. Possibly the expression of pituitary Hox genes is transient, or the fly *Antp* probe can not detect these groups of Hox genes. Another significant finding was that the carp eye showed a very strong hybridization signal. Since the formation of the eye is a very dramatic event during embryogenesis of fish, it will be worthwhile to study this in more detail in the future.

At present, we do not know whether the co-expression of several transcripts (Fig. 5) are from a single gene or different genes. To obtain conclusive evidence, it is necessary to clone and sequence the carp Hox genes.

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鯉魚同位序列基因之表現

成 之 綱[1] 廖 欽 峰[1] 徐 亞 莉[1] 吳 金 洌[1]

分子遺傳學家在研究果蠅體節突變機制的過程，發現了控制體節發生位置的基因，稱之爲同位基因(homeotic gene)。所有同位基因皆包含由60個保留性氨基酸組成之同位胜肽區(homeodomain)，其180個鹼基核甘酸對序列稱爲同位序列(homeo box)。利用果蠅觸角足(Antennapedia)基因(*Antp*)同位序列當探針，南氏轉移雜合法(Southern hybridization)證明鯉魚至少有兩種*Antp*型同位基因存在，RNA slot雜合法證明鯉魚*Antp*型同位基因主要表現於腸、眼、腎、心等器官，在肝、睪丸、小腦亦有少量表現，而在大腦、腦垂體、肌肉則不表現。利用北方轉移雜合法(Northern hybridization)證明，鯉魚胚胎發生時期，至少有五種*Antp*型同位基因轉錄本(transcript)存在，而其表現之強弱視胚胎發生階段而定。

關鍵詞：魚類, 同位基因, 觸角足, 胚胎發生, 組織。

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