Int. J. Dev. Biol. 34: 453-456 (1990)

Short Contribution

The *Xenopus laevis* Hox 2.1 homeodomain protein is expressed in a narrow band of the hindbrain

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ABSTRACT The expression pattern of the *Xenopus* homeodomain protein Hox 2.1 during development was determined using an affinity-purified antibody directed against a carboxy-terminal peptide. Nuclear staining was detected in a very narrow band of the hindbrain. This pattern was compared to that of the previously described *Xenopus* gene XlHbox 1 in serial sections and found to be more anterior than the XlHbox 1 long protein expression but overlapping with that of the short protein. *Xenopus* Hox 2.1 protein expression is restricted to a much narrower antero-posterior band than that reported for mouse Hox 2.1 RNA expression by *in situ* hybridization.

KEY WORDS: *Xenopus laevis, Hox 2.1, XlHbox 4, hindbrain*

The homeobox codes for a conserved sixty amino acid DNA-binding protein domain. In *Drosophila*, most homeoboxes are found in homeotic genes, *i.e.* genes that when mutated, transform one or more segments of the fly into other regions of the body (Gehring, 1987; Akam, 1989).

Similar genes exist in vertebrates, such as frog, mouse, and human (reviewed by Holland and Hogan, 1988b; De Robertis *et al.*, 1990). The regions of expression of many of these genes have been analyzed during embryogenesis by *in situ* hybridization and by antibody staining, and several important conclusions derived from these studies: 1) the early embryo is divided into regions of homeobox gene activity along the antero-posterior (A-P) axis (first shown by Awgulewitsch *et al.*, 1986) long before specific tissues and organs are formed. 2) Homeobox genes, which are clustered in the genome, are expressed so that genes at the 5' end of the Hox complex are expressed in posterior regions of the body, and those in the 3' direction are expressed in progressively more anterior regions. 3) This gene organization and A-P order of expression is also found in the fruit fly (Lewis, 1978; Akam 1989), leading to the remarkable conclusion that the genes controlling A-P polarity of the body first appeared in evolution in a common ancestor of insects and mammals. Perhaps the mechanism for determining head, trunk and tail arose only once in the evolution of metazoans.

Most studies on the expression of vertebrate homeobox genes have analyzed the distribution of transcripts by *in situ* hybridization, rather than protein distribution. However, the localization of proteins need not be identical to that of transcripts. For example, in vertebrates there is evidence suggesting translational control (Burglin *et al.*, 1987) or of having multiple overlapping transcripts from the same gene (Wright *et al.*, 1987; Cho *et al.*, 1988; Oudejans *et al.*, 1990), which could potentially complicate the interpretation of localization studies by *in situ* hybridization.

The expression of the mouse gene Hox 2.1 has been analyzed in considerable detail by *in situ* hybridization (Krumlauf *et al.*, 1987; Holland and Hogan, 1988a). Its anterior border of expression is found in the eighth (last) rhombomere of the hindbrain, and expression extends all the way to the caudal end of the spinal cord (Holland and Hogan, 1988b; Wilkinson *et al.*, 1989). At later stages this pattern of expression remains, but the spinal cord expression is weak. In the mesoderm, there is expression in the lung, stomach, mesonephros and metanephros (Holland and Hogan, 1988a).

In this paper, we report the expression of a *Xenopus* gene, which, on the basis of sequence comparisons, is considered to be the homolog of mouse gene Hox 2.1 (also known as Xhox1B, Harvey *et al.*, 1986, and XlHbox 4, Fritz and De Robertis, 1988). We prepared antibodies against a synthetic peptide corresponding to the carboxy-terminal region, and found expression of this protein in a narrow band of the hindbrain.

To determine the expression pattern of *Xenopus* Hox 2.1, an antibody was prepared against a peptide corresponding to the carboxy terminal 11 amino acids of *Xenopus* Hox 2.1, which are just downstream of the conserved homeodomain (Fig. 1). This region was chosen because it has 9 out of 11 amino acid identity with mouse Hox 2.1 but only 5 out of 11 amino acids with the closely related mouse gene Hox 1.3. This was the region of the two proteins

Abbreviations used in this paper: A-P, anteroposterior; Hox, Homeobox; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline.

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0214-6282/90/$02.00

Printed in Spain

PEPTIDE USED FOR IMMUNIZATION

Xenopus Hox 2.1	Homeobox	LysLeuLysSerMetSerLeuAlaThrGlySerSerAlaPheGlnPro
Mouse Hox 2.1	Homeobox	LysLeuLysSerMetSerLeuAlaThrAlaGlySerAlaPheGlnPro
Mouse Hox 1.3	Homeobox	LysLeuLysSerMetSerMetAlaAlaAlaGlyGlyAlaPheArgPro

Fig. 1. Comparison of the sequences of *Xenopus* Hox 2.1 with mouse Hox 2.1 and Hox 1.3, a member of the same gene subfamily located in the Hox 1 complex. *The* Xenopus *Hox 2.1 carboxy terminal end is compared with mouse Hox 2.1 and Hox 1.3 (Fritz and De Robertis, 1988). The region chosen for the synthetic peptide is the most divergent between Hox 2.1 and 1.3. The resulting antibodies did not crossreact with mouse embryos.*

- both highly related to each other - that was most likely to result in antibodies specific for Hox 2.1 (for sequence comparison of these proteins, see Fritz and De Robertis, 1988). The antibodies were affinity-purified and used to stain sections of *Xenopus* embryos. They did not cross-react with mouse embryos (not shown).

Sagittal sections (Fig. 2A) show that this antibody stains the hindbrain of stage 46 *Xenopus* embryos (Nieuwkoop and Faber, 1969). Transverse sections stained with *Xenopus* Hox 2.1 antibody (Fig. 3A) show that expression is limited to a few nuclei in the dorsal hindbrain. The staining is clearly nuclear as can easily be seen by staining the sections with the nucleus-specific dye Hoechst 33258. Staining was abolished by addition of the peptide used as antigen, suggesting that the antibody staining is entirely specific (data not shown). We do not show the RNA distribution since *in situ* hybridizations in *Xenopus* embryos have generally not been successful with rare transcripts, although there are some promising results with more abundant messages (Kintner and Melton, 1987; Hopwood *et al.*, 1989).

One of the best studied vertebrate genes at the protein level is *Xenopus* XlHbox 1. The gene encodes a short and a long protein from two different promoters (Cho *et al.*, 1988). The proteins share

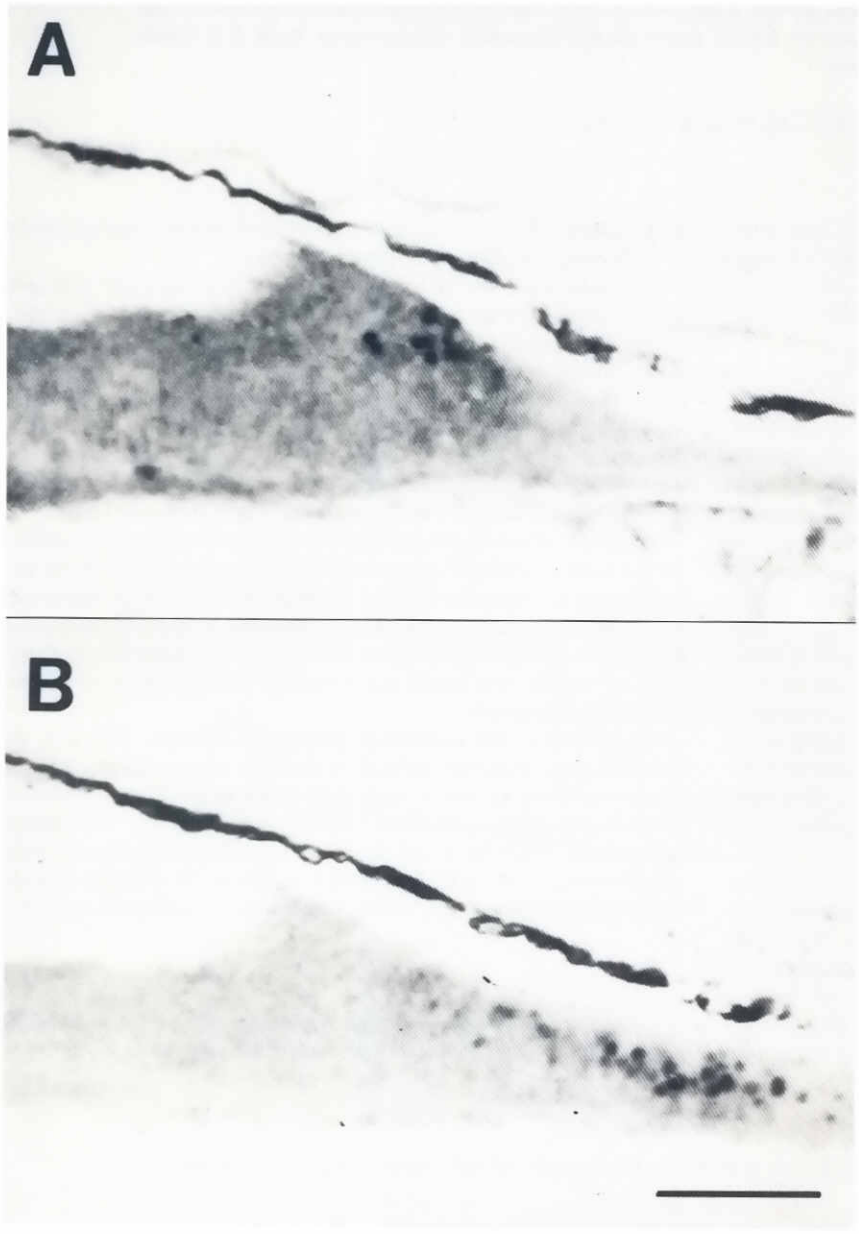

Fig. 2. Sagittal sections of *X. laevis* stage 46 tadpoles through the hindbrain and anterior spinal cord were stained with Hox 2.1 (A) and with XlHbox 1 long protein antibody (B). *Note that Hox 2.1 is expressed in a narrow band of hindbrain neurons. Bar=100μm.*

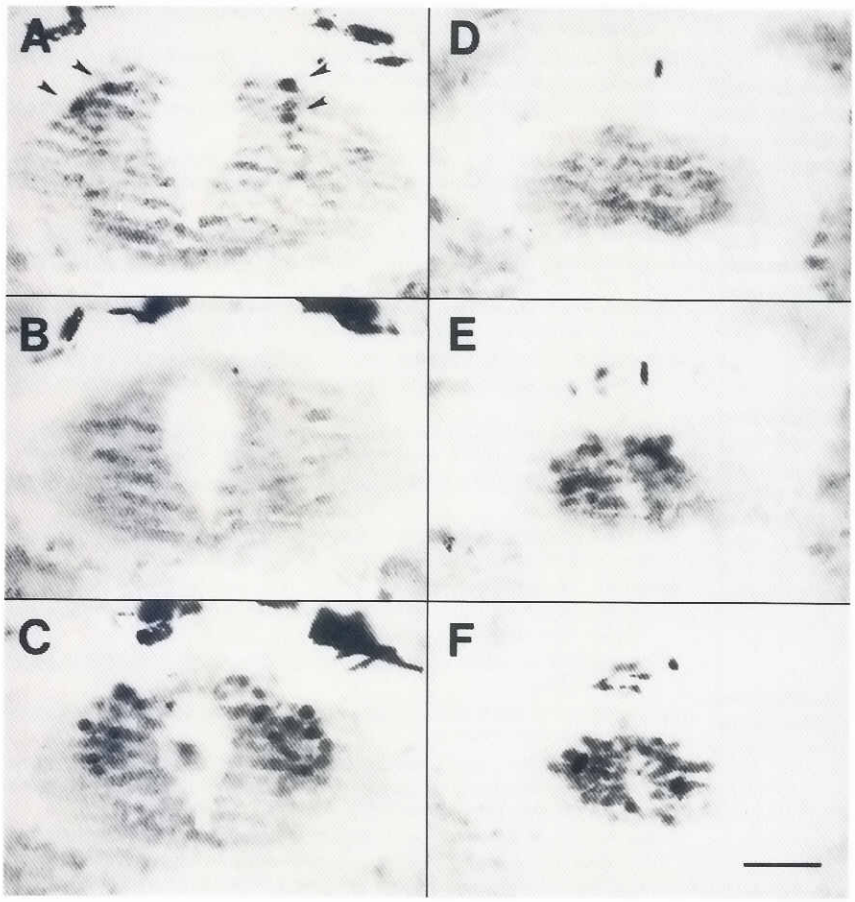

Fig. 3. Transverse sections of *X. laevis* stage 46 tadpoles were stained with antibodies. *Serial sections at the level of the hindbrain* **(A-C)**; *at the level of anterior spinal cord* **(D-F)**. *(A) and (D) show staining with* X. laevis *Hox 2.1 antibodies, (B) and (E) with XlHbox 1 long protein-specific antibodies, and (C) and (F) with XlHbox 1 common antibodies (Oliver* et al., *1988). Bar=50μm.*

carboxy terminal sequences including the homeodomain, but the long protein has an additional 82 amino acids at the amino terminus. Using different affinity-purified polyclonal antibodies, it was shown that the long protein is expressed in a band at the level of the anterior spinal cord, but the short protein is expressed more anteriorly into the hindbrain (Oliver *et al.*, 1988). When the region of *Xenopus* Hox 2.1 expression was compared to that of XlHbox 1 long protein in sagittal sections, *Xenopus* Hox 2.1 (Fig. 2A) and XlHbox 1 long protein (Fig. 2B) were found in discrete regions along the A-P axis, with XlHbox 1 long protein expression being more posterior than *Xenopus* Hox 2.1.

This was examined in more detail by staining serial transverse sections. *Xenopus* Hox 2.1 antibody stains in the hindbrain (Fig. 3A) but not in the anterior spinal cord (Fig. 3D), whereas XlHbox 1 long protein does not appear in the hindbrain (Fig. 3B) but is seen in anterior spinal cord (Fig. 3E). Antibody against the common part of the long and short protein, on the other hand, stains both hindbrain (Fig. 3C) and anterior spinal cord (Fig. 3F). Thus our results show that *Xenopus* Hox 2.1 protein is expressed in dorsal hindbrain nuclei in a domain anterior to that of XlHbox 1 long protein, subdividing the central nervous system into domains along the anteroposterior axis.

Many homeobox genes are organized in clusters (reviewed by Kessel and Gruss, 1990). XlHbox 1 is related to mammalian Hox 3.3, a gene that is located more 5' in the Hox gene clusters than Hox 2.1. The more posterior expression of XlHbox 1 long protein compared to *Xenopus* Hox 2.1 is consistent with the observation that 5' located genes tend to be expressed starting more posteriorly than 3' genes in the Hox clusters.

The mouse Hox 2.1 gene is expressed in the hindbrain and throughout the spinal cord as seen by *in situ* hybridization (Krumlauf *et al.*, 1987; Holland and Hogan, 1988a). In later stages, the central nervous system expression becomes strongest in the hindbrain, but the spinal cord continues to express at a low level. In *Xenopus*, we see expression in the hindbrain but not in the rest of the nervous system with our peptide antibody. The results suggest that Hox 2.1 protein expression in the *Xenopus* embryo may be more restricted than RNA expression in the mouse. Other possible explanations include species differences and differences in sensitivity. The more limited expression in *Xenopus* could also be explained by the delayed differentiation of mesodermal structures such as prevertebrae when compared to the mouse embryo.

We conclude that the expression of *Xenopus* Hox 2.1 protein is in a narrow region of the *Xenopus* hindbrain, in a domain anterior to XlHbox 1 long protein. The comparison with mouse Hox 2.1 *in situ* hybridization to RNA, which extends over a much wider region of the central nervous system, highlights the importance of analyzing protein products in addition to RNA. The antibody reported here may provide a useful marker for the development of the *Xenopus* hindbrain.

Experimental Procedures

Peptides and coupling

A peptide corresponding to the region least conserved between Hox 2.1 and Hox 1.3 was synthesized and coupled to keyhole limpet hemocyanin (Sigma) with glutaraldehyde in a procedure based on a previously described method (Kagan and Glick, 1979). Briefly, 15 micromoles of peptide was mixed with 15mg KLH in 4.5ml 0.2M sodium phosphate, pH 7.5. At room temperature, 1ml 50mM glutaraldehyde (Polysciences) was added dropwise over 5 minutes with stirring. Stirring was continued for 30 minutes. Next, 0.55ml 1M glycine was added, and the solution was stirred for 30 minutes and then dialyzed against PBS at 4°C. In order to provide an epsilon-amino group for efficient crosslinking by glutaraldehyde, a lysine residue was added to the amino-terminus of the synthetic peptide.

Antibody preparation and staining

Rabbits were immunized with 2 mg of antigen per injection as described (Oliver *et al.*, 1988). Antibodies were affinity-purified using a column of peptide coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions, as described previously for fusion protein antibodies (Oliver *et al.*, 1988). 10mg of peptide was coupled per gram of dry matrix. Bouin-fixed and paraffin-embedded sections were stained using 1/50 or 1/100 dilutions of primary antibody (Oliver *et al.*, 1988). Competition experiments were done by including 200μg/ml peptide during the incubation with primary antibody.

Acknowledgments

We thank Christopher Wright for useful advice and Bruce Blumberg and Ken Cho for critical reading of the manuscript. This work was supported by NRSA Medical Scientist Training Program Grant GM 8042-08 (B.G.J.) and by NIH grant HD21502-05.

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Accepted for publication: October 1990