A homeo domain protein reveals the metameric nature of the developing chick hindbrain

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The segmented embryonic hindbrain of vertebrates develops by sequential constriction of the neural tube into eight metameric units known as rhombomeres. The cellular and molecular basis of this segmentation process is largely unknown. Using an antibody, we analyzed the expression pattern of the chick homeo domain-containing protein Ghox-lab in the developing chick hindbrain. At the neural plate stage, prior to the appearance of rhombomeres, Ghox-lab is expressed within a single domain that extends anteriorly up to the site where rhombomere 4 will later form. After rhombomere 4 has appeared and as hindbrain segmentation progresses, the level of Ghox-lab protein increases significantly within the fourth rhombomere. This intensification, accompanied by the elimination of Ghox-lab protein in rhombomeres 5 and 6, eventually results in the formation of a distinct island of expression in rhombomere 4. All cells in the newly formed rhombomere 4 express Ghox-lab, except for the cells of the floor plate. In addition, neural crest cells migrating from the fourth rhombomere are also Ghox-lab-positive. These data raise the possibility that Ghox-lab protein might be one of the factors involved in the specification of the metameric pattern of the vertebrate hindbrain.

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It has long been proposed that vertebrates are segmented in the manner of annelids and arthropods. The earliest observations of segmentation in the vertebrate central nervous system come from the classical studies by von Baer (1828), who described periodic bulges of the neural tube in the early chick embryo. These bulges were later designated as neuromeres (Orr 1887). The metameric nature of the neural tube is especially pronounced in the rhombencephalon (hindbrain), where up to eight rhombomeres transiently form and then disappear during embryogenesis (Vaage 1969). Although the lack of overt segmentation in the adult hindbrain calls into question the significance of these transient embryonic segments, their presence throughout the vertebrates indicates a crucial role in development. One imprint that this embryonic segmentation leaves on the adult hindbrain is a highly conserved morphological relationship in which individual rhombomeres give rise to particular cranial nerves (Orr 1887; Vaage 1969; Lumsden and Keynes 1989).

There has been renewed interest in the metameric organization of the vertebrate hindbrain (Keynes and Lumsden 1990), particularly in the context of insect development. In insects, the identity of segments along the anteroposterior axis is determined largely by the expression pattern of a set of homeotic selector genes clustered in the Antennapedia and Bithorax complexes (ANT-C and BX-C; see Lewis 1978; Duncan 1987; Mahaffey and

Kaufman 1989). These genes encode transcription factors (Struhl et al. 1989; Winslow et al. 1989) that contain a homeo domain, an evolutionarily conserved DNA-binding motif (McGinnis et al. 1984; Gehring 1987; Qian et al. 1989). Genes of the ANT-C and BX-C have cognates in mammalian genomes that are also arranged in clusters (*Hox-1*, *-2*, *-3*, *-4*; for review, see Boncinelli et al. 1989). Interestingly, the organization of the Hox clusters resembles that of *Drosophila* ANT-C and BX-C (Duboule and Dollé 1989; Graham et al. 1989; for review, see Akam 1989).

Although the expression patterns of the *Drosophila* ANT-C and BX-C genes are restricted to parasegmental units (Martinez-Arias and Lawrence 1985; for review, see Akam et al. 1988), most vertebrate cognates are expressed in broad and overlapping domains of the CNS, usually devoid of any recognizable metameric pattern (e.g., Holland and Hogan 1988). However, examination of mouse embryos with well-developed rhombomeres reveals that when the anterior limit of expression of a Hox gene falls within the hindbrain, this limit consistently coincides with a rhombomere border (see Wilkinson et al. 1989b). Of particular interest to this report is the observation that in the hindbrain *Hox-2.9* mRNA is localized specifically in rhombomere 4 (Murphy et al. 1989; Wilkinson et al. 1989b).

In an earlier study (Sundin et al. 1990), we characterized Ghox-lab, a chicken *Hox* gene related to *labial*, a

Drosophila homeotic selector gene (Diederich et al. 1989). Using in situ hybridization, we found that in primitive streak and head process-stage chick embryos, *Ghox-lab* expression defines two regions: Ectoderm, mesoderm, and endoderm in the prospective trunk express *Ghox-lab*, whereas tissues fated to become head lack *Ghox-lab* transcripts. The border between *Ghox-lab*-positive and *Ghox-lab*-negative cells lies in a region fated to form hindbrain (Spratt 1952). In the work reported here, we use an antiserum directed against the protein encoded by *Ghox-lab* to describe how a single domain of expression evolves into an intricate pattern that reflects the metameric nature of the hindbrain and its associated neural crest.

Results

In situ hybridization and antiserum detect high levels of Ghox-lab expression in rhombomere 4

In situ hybridization of serial sagittal sections through Hamburger–Hamilton stage 15 embryos revealed the presence of *Ghox-lab* mRNA along the entire length of the spinal cord (Sundin et al. 1990; and unpubl.). Sections passing through the hind- and midbrain displayed a well-demarcated region of neuroepithelium that was *Ghox-lab*-positive (Fig. 1A). The silver grain distribution had a clear anterior and posterior boundary, suggesting that the *Ghox-lab*-positive tissue corresponds to one of the rhombomeres. In an effort to determine which rhombomere was expressing *Ghox-lab*, sections parallel to that illustrated in Figure 1A were reconstructed serially (not shown). It was straightforward to identify rhombomeres 5 and 6 as they are proximal to the otic vesicle, a distinct landmark in the vertebrate hindbrain. Because the *Ghox-lab*-positive neuroectoderm is situated immediately anterior to rhombomere 5, it must be the neuroectoderm of rhombomere 4.

These in situ hybridization studies have two limitations: First, it remains unknown whether *Ghox-lab* transcripts are translated; second, assuming that this gene is involved in the determination of cell fate in the hindbrain, it becomes critical to find out whether or not all cells in rhombomere 4 express *Ghox-lab*. Such cellular resolution requires an antibody. Therefore, we prepared a rabbit antiserum to an *Escherichia coli trpE* fusion protein that contained protein encoded by the upstream, nonhomeo box exon of *Ghox-lab* (for sequence data and gene structure, see Sundin et al. 1990). The serum was affinity-purified with a fusion protein consisting of LacZ and the first 94 amino acids of *Ghox-lab* protein. It should be noted that this portion of the protein shows no homology to mouse Hox-1.6 or Hox-2.9, with the exception of a short sequence extending from the amino terminus (Sundin et al. 1990; Frohman et al. 1990). In addition, it was observed by immunohistochemistry that the antibody yields no detectable signal in the nuclei of mouse embryo sections and gives only a weak signal with quail, a bird closely related to the chicken (data not shown). The fact that the antiserum

exhibits such a high degree of species specificity argues that it should not cross-react to any significant extent with proteins encoded by other homeo box genes of the chick.

The regions of the embryo in which nuclear signal was detected were those that also contain *Ghox-lab* mRNA (for in situ hybridization data, see Sundin et al. 1990). Specifically, antibody signal was present in spinal cord (Figs. 2B–J), in somites (e.g., Fig. 2B), in posterior flank mesoderm (not shown), in gut-associated splanchnic mesoderm (not shown), and in foregut endoderm (Fig. 1A; e.g., Fig. 2E). In contrast, signal was completely absent in all neural and mesenchymal tissue anterior to the hindbrain. Examination of a frontal section of a stage 17 chick embryo (Fig. 1C) illustrates the distribution pattern in the hindbrain. The highest level of signal is found in the neuroectoderm of rhombomere 4. There is no signal in the anterior rhombomeres 1–3 or in rhombomeres 5 and 6; however, *Ghox-lab* is expressed at modest levels in rhombomeres 7 and 8 (Fig. 1C), as well as along the entire length of the spinal cord (not shown). We conclude that in situ hybridization (Fig. 1A) and immunohistochemistry (Fig. 1C) consistently detect expression of *Ghox-lab* in rhombomere 4.

All cells in the neuroectoderm of rhombomere 4 express Ghox-lab protein but not cells of the floor plate

To determine whether all cells in the domain of rhombomere 4 express *Ghox-lab*, we examined sagittal and transverse sections through rhombomere 4. Because *Ghox-lab* protein is localized tightly to the nucleus (Fig. 1B), the precipitated immunoperoxidase reaction product effectively quenched the fluorescence emitted by the DNA-bound Hoechst dye. This quenching allows one to readily recognize *Ghox-lab*-negative nuclei, as these will stand out against the background. Figure 1, C and D, shows that in the main body of the fourth rhombomere, all cells express *Ghox-lab*. An apparent exception concerns the few intensely fluorescent features near the ventricular surface (Fig. 1D). Inspection of these features at high magnification revealed that they are mitotic chromosomes (not shown). It has been observed previously that *caudal*, a *Drosophila* homeo domain-containing protein, is found normally in the nucleus but is not bound to the chromosomes during mitosis (Macdonald and Struhl 1986).

The ventral midline of the neural tube constitutes a special structure known as the floor plate. The floor plate has an embryonic origin separate from lateral neuroectoderm (Schoenwolf et al. 1989) and appears to perform important functions such as axon guidance within the developing central nervous system (Jessell et al. 1989). Floor plate cells in the spinal cord contain *Ghox-lab* protein (not shown), but the floor plate of rhombomere 4 shows no immunoreactivity (see Fig. 1D). At stage 11⁺, shortly after the fourth rhombomere has formed (for a summary of hindbrain morphogenesis, see Fig. 4), an optical section passing through the floor plate

Figure 1. (A) Rhombomere 4-specific expression of Ghox-lab revealed by in situ hybridization. This parasagittal section of a stage 15 chick embryo passes through the medial face of the otic vesicle, the lateral walls of rhombomeres 3–5, and the lumen of the anterior hindbrain and midbrain. The embryo is visualized by the fluorescence of Hoechst-stained nuclei (blue), whereas the ^{35}S -labeled hybridization signal appears as silver grains (red). Orientation: Anterior is to *right* and dorsal is *up*. Bar, 100 μm . (B) Distribution of Ghox-lab protein at cellular levels. A stage 7⁺ embryo was immunoperoxidase-stained in whole mount with Ghox-lab antibody. It was then embedded in paraffin and sectioned transversely at the level of the first somite. The reddish-brown diaminobenzidine reaction product is viewed by bright-field illumination. Note the nuclear localization of the signal. Orientation: Dorsal *up*; lateral to *left*. Bar, 20 μm . (C) Expression of Ghox-lab in rhombomere 4. A 20- μm frozen frontal section through the hindbrain region of a stage 17 embryo was stained with Ghox-lab antibody. Bright-field illumination reveals the peroxidase reaction product (brown) in labeled nuclei, and Hoechst fluorescence (blue) reveals the unlabeled nuclei. This specimen exhibits high levels of Ghox-lab protein in rhombomere 4 and significantly lower levels in rhombomeres 7 and 8. Mesenchyme, otic vesicle, the seventh/eighth cranial nerve and ganglion, and rhombomeres 2, 3, 5, and 6 are all Ghox-lab-negative. Orientation: Anterior *up*. Bar, 100 μm . (D) Transverse section of a stage 15 embryo passing through rhombomere 4 (photographed in the same manner as in Fig. 1C). All cells in rhombomere 4 express Ghox-lab, except for cells in the floor plate and roof plate. Examination at higher magnification (not shown) reveals that the Hoechst fluorescent features at the inner surface of the tube are mitotic chromosomes, and the cytoplasm of these mitotic cells stains with the Ghox-lab antibody. Orientation: Dorsal *up*. Bar, 100 μm . (E) A stage 11 embryo whole-mount immunoperoxidase stained for Ghox-lab. Optical section through the floor plate of the hindbrain, viewed from above in bright-field illumination. Notice how the Ghox-lab-positive neuroepithelial cells in rhombomere 4 closely abut the negative floor plate. Orientation: Anterior *up*. Bar, 100 μm . Abbreviations: (ect) ectoderm; (em) endo-mesoderm; (fg) foregut endoderm; (fp) floor plate; (mb) midbrain; (ng) seventh/eighth nerve root and ganglion; (np) neural plate; (nc) notochord; (opr) optic vesicle; (ov) otic vesicle; (r2–r8) rhombomeres 2–8; (rp) roof plate.

of a whole mount (Fig. 1E) reveals that *Ghox-lab*-positive neuroectodermal cells in rhombomere 4 are crowded up to the edge of the entirely negative floor plate. The expression pattern of *Ghox-lab* in the floor plate of earlier embryos is schematized in Figure 4. Another region in the neural tube of the stage 15 embryo devoid of

Ghox-lab protein is the thin roof plate (Fig. 1D). Earlier, in stage 12 embryos, when the roof plate is thicker, its cells express *Ghox-lab* protein (not shown). The loss of expression in the roof plate occurs long after the appearance of rhombomere 4 and at a time when the levels of *Ghox-lab* mRNA are already in decline.

Figure 2. (See facing page for legend.)

Development of the expression pattern in rhombomere 4

In situ hybridization to transcripts of *Ghox-lab* (see Fig. 5 in Sundin et al. 1990) and localization of its protein by immunohistochemistry (Fig. 2A) show that at the neural plate stages, it is expressed throughout the presumptive trunk up into the presumptive hindbrain. In contrast, presumptive midbrain and forebrain do not express *Ghox-lab*. At stage 15, when all rhombomeres have formed and neurons begin to differentiate, a similar simple pattern is still recognizable. A continuous stretch of *Ghox-lab* expression is seen in rhombomeres 7 and 8 (Fig. 1C) and extends posteriorly along the entire length of the spinal cord. Anterior to this uninterrupted stretch of expression is the insular region of expression in rhombomere 4 (Fig. 1C). Does this "island" of expression arise by de novo activation of the *Ghox-lab* gene in a previously negative region of anterior neuroectoderm? Alternatively, does it arise in a domain of the hindbrain that already expresses the gene?

To distinguish between these two possibilities, we examined a developmental series of embryos stained with *Ghox-lab* antiserum. A representative selection of these embryo whole mounts is reproduced at identical magnification in Figure 2. The embryos range from stages 7 to 12 and cover the period of hindbrain segmentation (for a time frame of rhombomere formation, see Fig. 4). Figure 2A shows the flat neural plate of a stage 7 embryo. Granular, that is, nuclear, signal of the homeo domain protein is seen in the neural plate, as well as in mesodermal tissues that are below the neural plate (see also Fig. 1B). The anterior boundary of expression does not coincide with any distinct morphological landmarks but is situated substantially anterior to the first somite. It should be noted that the overall level of *Ghox-lab* expression at its anterior boundary is lower than in tissue nearer the somite (Fig. 2A). This is due to the interspersions of *Ghox-lab*-negative and *Ghox-lab*-positive cells

and not to a uniform decrease in the level of expression. By stages 8⁺ and 9 (Fig. 2B,C) a "filling-in" process has taken place, and essentially all cells near this anterior boundary express *Ghox-lab*. Furthermore, the boundary appears to have a fixed position along the anteroposterior axis relative to the somites and the head end (cf. Fig. 2A–D). Another feature of this boundary, one that persists through later stages of tube closure and rhombomere formation, is the slight tendency of positive cells to sweep anteriorly along the midline and the lateral edges of the plate (see Fig. 2A and arrow in Fig. 2C).

The stage 9⁺ embryo depicted in Figure 2D is especially important as it shows two closely spaced constrictions (arrows) that have just become visible in the hindbrain. These constrictions mark the anterior and posterior boundaries of rhombomere 3 according to the scheme of Vaage (1969). The more posterior of these constrictions also defines the anterior limit of rhombomere 4. Close examination reveals that the pattern of *Ghox-lab* expression does not follow precisely the newly formed rhombomeric constrictions. At this early stage, many positive cells are found scattered randomly within the boundaries of rhombomere 3 (Fig. 2D–F). At stage 10 (Fig. 2E), the posterior limit of rhombomere 4 is established, but at this point, there is no selective increase in *Ghox-lab* expression within the newly formed rhombomere. It is not until stages 10⁺ and 10²⁺ (11 and 11.5 somites; Fig. 2F,G) that one observes an increase in *Ghox-lab* expression within rhombomere 4. However, in more posterior regions of the hindbrain and in the spinal cord, expression remains uniform. Less than an hour later, by stage 11[–] (Fig. 2H), the intensity of signal in rhombomere 4 is greatly increased. By stage 11⁺, the level of *Ghox-lab* antigen has decreased significantly in the posterior rhombomeres and spinal cord. By stage 12[–] (Fig. 2I), rhombomere 5 is devoid of *Ghox-lab*-positive nuclei, and by stage 17 (Fig. 1C), signal has also been lost from rhombomere 6.

In summary, the developmental series depicted in

Figure 2. Developmental profile of *Ghox-lab* protein distribution in the chick hindbrain (stages 7–12). Embryos stained in whole mount with *Ghox-lab* antibody. Dorsal views of the anterior portion of the embryo, including anterior spinal cord, hind-, mid- and forebrain. All frames are at identical magnification, with the bar in 4F representing 500 μ m. Somites are useful reference points along the anteroposterior axis, and their locations (s1, s2) are indicated by horizontal brackets. They are also useful for marking time, as one set is added roughly each hour (Hamburger and Hamilton 1951). (A) A stage 7 embryo. Neural plate stage with one somite. (B) A stage 8⁺ embryo. Neural-fold stage, with five somites. Somites express *Ghox-lab*. (C) A stage 9 embryo, with seven somites. The neural tube is largely closed. Arrow indicates stray cells anterior to the main expression domain. (D) A stage 9⁺ embryo with eight somites. Two constrictions appear (arrows), defining rhombomere 3. (E) A stage 10 embryo with 10 somites. The constrictions defining rhombomere 4 and 5 appear (arrows). Note stray *Ghox-lab*-positive cells in rhombomere 3. (F) A stage 10⁺ embryo with 11 somites. A slight increase in *Ghox-lab* expression becomes visible within rhombomere 4. (G) A stage 10²⁺ embryo, with 11 somites. Further intensification of *Ghox-lab* signal is visible in rhombomere 4. The slight lateral spreading seen best in the right half of rhombomere 4 (thin arrow) is the neural crest emerging from the dorsal midline of the neuroepithelium. (H) A stage 11[–] embryo, with 12 somites. The *Ghox-lab*-positive neural crest (arrow) migrates laterally, and intensification of expression with rhombomere 4 reaches a maximum. (I) A stage 11⁺ embryo, with 14 somites. Rhombomere 4 neural crest (arrow) migrates farther outward. There is now significantly less signal in posterior rhombomeres. (J) A stage 12[–] embryo, with 15 somites. *Ghox-lab*-positive neural crest (arrow) coalesces into ganglia. Rhombomere 5 has lost signal completely. The identity of *Ghox-lab*-positive cells in the body wall lateral to rhombomeres 6–8 is not yet clear, but we suspect that these are probably also neural crest cells. The underlying arch-like immunopositive feature seen most clearly in E is foregut endoderm. *Note:* Background and stain-trapping artifacts visible in the anterior regions of the head (e.g., G, I) do not exhibit the characteristic nuclear localization of true *Ghox-lab* signal. Orientation: Anterior to right. Abbreviations: (fb) forebrain; (fg) foregut endoderm; (hb) hindbrain; (hn) Hensen's node; (mb) midbrain; (S1 or S2) somite position; (nc) neural crest; (r3–r5) rhombomeres 3–5. Selected rhombomere constrictions are marked by heavy arrows.

Figure 2 and schematized in Figure 4 suggests that right from the beginning of hindbrain formation and before the appearance of morphologically detectable constrictions, *Ghox-lab* is expressed all the way to the presumptive boundary of rhombomere 4. After the morphological delineations of rhombomere 4 appear, rhombomere 4-specific expression intensifies. Subsequently, *Ghox-lab* protein levels decline in rhombomeres 5 and 6; hence the island of *Ghox-lab* expression in rhombomere 4 is sculpted from an initially uniform expression pattern and is not the result of induction of *Ghox-lab* in a previously nonexpressing region of the neuroectoderm.

Neural crest cells derived from rhombomere 4 express Ghox-lab

A novel feature revealed by the *Ghox-lab* antiserum not seen by *in situ* hybridization is that neural crest cells emerging from rhombomere 4 also express the gene, although at a level lower than in the rhombomere itself. The stage 12⁻ whole-mount embryo shown in Figure 2J revealed *Ghox-lab* signal in two dumbbell-like structures extending distally from rhombomere 4 (arrow). An embryo stained for the HNK-1 antigen (Fig. 3, left), a marker found on migratory neural crest cells (Vincent and Thiery 1984), showed that these structures conform to the shape and position of genuine neural crest. The origin of these cells can be traced in the embryo series depicted in Figure 2G–J. When *Ghox-lab* expression begins to intensify in rhombomere 4 at stage 10²⁺, one can observe a rounded front of cells pointing laterally away from the neural tube (see thin arrow in Fig. 2G). By stage 11⁻, cells in the center of this mass move distally, lending the migrating crest a triangular appearance (Fig. 2H). By stage 11⁺, the population has spread out into a

Figure 3. Neural crest cells and *Ghox-lab*-positive cells of the hindbrain. Stage 12⁻ embryos stained in whole mount with either *Ghox-lab* or leu 7 antibody (HNK-1) and photographed in bright field with high-contrast film. (Left) HNK-1 staining reveals the distinct shape of the neural crest cell population derived from the fourth rhombomere and crest in anterior regions of the hindbrain. (Right) Another embryo stained with *Ghox-lab* antibody reveals signal in rhombomere 4 crest and possibly more posterior crest cells (dark region under nc4 label). Bar, 100 μ m. Abbreviations: (nc) neural crest; (nc4) neural crest derived from rhombomere 4; (ov) otic vesicle; (r3, r4) rhombomeres 3 and 4.

tongue-like configuration (Fig. 2I). At stage 12⁻, the cells begin to coalesce distally, and the stalk becomes thinner (Figs. 2J and 3, right). These condensed cells are fated to form the glial anlage of the seventh and eighth cranial nerves and their associated sensory ganglia (De Lièvre and Le Douarin 1982; D'Amico-Martel and Noden 1983). The eventual function of these ganglia is to relay sensory information from the face and ear to the brain. Expression of the protein in the crest cells begins to decline by stage 12 and has completely vanished by stage 15 (data not shown). Hence, within the time frame of gangliogenesis, the period of *Ghox-lab* expression is relatively brief, as ganglia continue to develop well past stage 24.

Discussion

Rhombomere unit

We have studied the distribution of *Ghox-lab* protein at the cellular level and can now draw a detailed picture of its pattern of expression in relation to the metameric organization of the hindbrain. It is an intricate relationship that changes during development and culminates in a pattern where *Ghox-lab* protein is largely restricted to rhombomere 4 (Fig. 4). In the mouse, such a rhombomere-restricted pattern is exhibited by *Hox-2.9* (Murphy et al. 1989; Wilkinson et al. 1989b; Frohman et al. 1990) but not by any of the other *Hox* genes studied to date. The conservation of this pattern between birds and mammals indicates that *Ghox-lab* and *Hox-2.9* each play an important role in rhombomere 4 development. The similarity of their homeo boxes also suggests that they are functionally homologous. A central question left unanswered by the recent *in situ* hybridization studies with *Hox-2.9* (Murphy et al. 1989; Wilkinson et al. 1989b) and the related *Ghox-lab* gene (Fig. 1A) is whether all or only a fraction of cells within the fourth rhombomere express this homeo box gene. In the case of *Ghox-lab*, we find that there is a domain in rhombomere 4 within which all cells contain *Ghox-lab* protein (Fig. 1C,D). Because of this all-inclusive expression pattern, it is possible that *lab* is involved in determining the segmental identity of this *Ghox*-rhombomere and could therefore be the vertebrate equivalent of a *Drosophila* homeotic selector gene (for review, see Akam et al. 1988; Peifer et al. 1987). *Ghox-lab* has the opportunity of time and place to influence neuronal fates, because it is expressed at high levels within the rhombomere prior to and during the birth of these motoneurons (Kuratani et al. 1988; Covell and Noden 1989; Lumsden and Keynes 1989). If *Ghox-lab* behaves like a selector gene, it could also program the neural crest of rhombomere 4 to form the very distinctive glial anlage of the facial and acoustic ganglia (Vaage 1969; Le Lièvre and Le Douarin 1982; D'Amico-Martel and Noden 1983). Experimental evidence demonstrates early imprinting in the case of the premigratory cranial neural crest (Noden 1983). Presumptive mandibular arch crest cells grafted into a region near the second arch were found to migrate into

this arch and generate an additional jaw, complete with skeleton and muscles.

Another novel feature revealed by the antibody is that the cells of the floor plate of rhombomere 4 do not express Ghox-lab. The floor plate has an embryonic origin distinct from that of the lateral neuroectoderm (Schoenwolf et al. 1989) and functions in axonal guidance (Jessel et al. 1989). As can be seen in Figure 4, floor plate cells adjacent to presumptive rhombomere 4 do not express Ghox-lab, even at early stages. However, more posterior floor plate cells contain Ghox-lab protein, but the pattern shows no segmental features. This suggests that the floor plate does not constitute part of the segmental unit, an idea supported by its lack of any morphological segmentation (Lumsden and Keynes 1989). The rhombomere thus appears as a curious segmental unit that consists of two separated halves, quite different from the contiguous parasegments of the *Drosophila* blastoderm (Martinez-Arias and Lawrence 1985; Lawrence 1988).

Developing pattern of Ghox-lab expression

In the chick and other vertebrates, rhombomeres are formed in a sequential manner, beginning with rhombomere 3 (Vaage 1969). The successive appearance of rhombomeres and the chief features of Ghox-lab expression are schematized in Figure 4. In essence, the relatively simple early phase of Ghox-lab expression (stages 7–10, Fig. 4) evolves into the rhombomere-restricted pattern seen in later embryos (stages 10+ to 17, Fig. 4). Early, at the neural plate stage (stage 7, Figs. 2A and 4), Ghox-lab protein is expressed throughout the neuroectoderm up to a region ~250 μ m anterior to the first somite. The lack of appropriate fate maps makes it difficult to determine precisely the future position of cells in

this boundary region. However, a simple side-by-side comparison of Figure 2A–D reveals that at the neural plate stage the anterior extent of Ghox-lab protein expression falls within the region in which the anterior border of rhombomere 4 will later form. Such a direct comparison seems warranted for two reasons: (1) The region is quite close to a visible marker, the first somite; and (2) during the period considered, there seems to be no significant extension of the neuroectoderm along the anteroposterior axis between the first somite and the front end of the embryo. Hence, it appears that within the limits of our observations the anterior border of expression of Ghox-lab represents a molecular prepattern visible well before the morphological appearance of the anterior border of the fourth rhombomere.

Further development of Ghox-lab expression in the hindbrain yields a rhombomere-specific pattern through a “sculpting” of the simple “block” of early expression. Initially, the anterior limit of Ghox-lab-expressing cells comprises a mixture of Ghox-lab-positive and Ghox-lab-negative cells (Figs. 2A and 4, stage 7). As the neural plate folds and closes, this boundary becomes more distinct. However, even when the constriction separating rhombomeres 3 and 4 first appears (stage 9+; see Figs. 2D and 4), there is some variability in the anterior boundary of Ghox-lab expression between embryos. Furthermore, we consistently observe Ghox-lab-positive stray cells in rhombomere 3. Somehow, these will eventually vanish. One possible explanation is that they eventually migrate into rhombomere 4. An alternate explanation is that these stray cells remain within rhombomere 3 but that they cease to express Ghox-lab once they assume a rhombomere 3 identity. A very recent study of single cell lineages in the chick hindbrain (Fraser et al. 1990)

Figure 4. Summary: Expression of Ghox-lab during hindbrain development. The scheme shows a sequence of icons that illustrate the morphological development of the embryonic chick hindbrain. Included in these icons depicting the ventral aspect of the neural plate and tube are the floor plate, the segmental units of the neuroepithelium (initially proneuromeres A–C and, subsequently, rhombomeres 1–8), and the neural crest cell population originating in rhombomere 4. The series begins with the one somite neural plate embryo (stage 7) and ends at stage 17, when all rhombomeres are formed and neurons differentiate within the hindbrain. Superimposed on these icons is the pattern and level of expression of Ghox-lab protein, as revealed by antibody histochemistry of whole-mount embryos and sections. Density of shading reflects the level of expression of Ghox-lab. Abbreviations: (fp) floor plate; (gVII/VIII) seventh/eighth cranial ganglion and associated nerve root; (hn) Hensen's node; (nc) neural crest; (A–C) proneuromeres; (r1–r8) rhombomeres 1–8; (S1) first somite; (S2) second somite.

suggests that rhombomere boundaries are barriers to cell migration. If this suggestion is true, it would lend weight to the second explanation, but the issue of how this boundary sharpening process takes place is far from resolved.

While the anterior boundary is being refined, the level of Ghox-lab protein greatly increases in rhombomere 4. Shortly afterward, a noticeable decline in signal intensity begins in rhombomere 5, which ends with a complete shutoff of the gene by stage 12 (Figs. 2J and 4). By stage 17, expression has also been lost in rhombomere 6 (Figs. 1C and 4). It remains to be determined which cell-autonomous regulatory genes and/or external signals carry out this sculpting process.

Relationship of Ghox-lab to other vertebrate labial cognates

In *Drosophila*, there is a single gene, *labial*, which can be identified as the prototype of the mouse genes *Hox-1.6* (Baron et al. 1987; La Rosa and Gudas 1988) and *Hox-2.9* (Murphy et al. 1989; sequence courtesy of Frohman et al. 1990), human *Hox 2I* (Boncinelli et al. 1989), and *Ghox-lab* (Sundin et al. 1990). This relationship is inferred from similarities in their homeo box sequences and their chromosomal locations within homeo box gene clusters (Boncinelli et al. 1989). In principle, the chromosomal duplications that led to four vertebrate *Hox* clusters allow for up to four labial cognates (Duboule and Dollé 1989; Graham et al. 1989; for review, see Boncinelli et al. 1989). Until all of these genes have been characterized fully, the question of homology cannot be resolved; however, a preliminary comparison of the expression patterns of Ghox-lab with that of *Hox-1.6* and *Hox-2.9* sheds some light on this issue.

In mouse embryos, after the formation of rhombomere 4, *Hox-2.9* mRNA is found only in this rhombomere (Murphy et al. 1989; Wilkinson et al. 1989b), and this constitutes a striking similarity with the Ghox-lab pattern. *Hox-1.6* lacks the distinctive island of expression in rhombomere 4 (Duboule and Dollé 1989) and therefore seems less likely to be the mouse homolog of Ghox-lab. In the case of *Hox-2.9*, it should be noted that there appear to be substantial differences in the pattern of expression within the early, prerhombomere neuroepithelium. Unlike the early chick, which shows uniform levels of expression within the posterior two-thirds of the embryo (see Fig. 5C in Sundin et al. 1990), in the equivalent mouse embryo (day 8) the highest levels of expression are at the caudal end of the embryo, with a considerable decline toward more anterior regions (see Fig. 3H in Wilkinson et al. 1989b). The observations made by Wilkinson and co-workers (1989b) led them to conclude that *Hox-2.9* is not expressed at an appropriate location within the hindbrain at a time when Krox 20 reveals the definition of rhombomere boundaries and suggested that this argues against a role for *Hox-2.9* in segment formation. It should be noted that in contrast to this, Frohman et al. (1990) have found *Hox-2.9* mRNA in the presumptive hindbrain of the

early neural plate stage embryo, and this suggests that its domain of expression may be equivalent to that of Ghox-lab. Therefore, the early prerhombomere phases of *Hox-2.9* and Ghox-lab expression could be similar in their essential features and perform homologous functions.

In the chick, we have shown that for embryos ranging from 1 to 7 somites (Hamburger–Hamilton stages 7–9), the domain of Ghox-lab expression extends from the presumptive mid-hindbrain region to the posterior end of the embryo. The recent single cell lineage work of Fraser and collaborators (1990) indicates that the clonal restriction of cells to either side of the rhombomere 3/4 border happens when this morphological boundary first appears, at about stage 9. Taken together, these observations in the chick raise the possibility that this early pattern of Ghox-lab expression prefigures at least an approximate location of the fourth rhombomere.

Methods

In situ hybridization

In situ hybridization was carried out with 7- μ m paraffin sections exactly as described in Sundin et al. (1990). Antisense riboprobe labeled with 35 S was prepared from a 293-bp stretch of DNA within the 3' half of the upstream exon (nucleotides 280–573, as defined in Sundin et al. 1990). This sequence is unique to Ghox-lab and does not cross-react with other homeo box genes, even at low stringency.

Preparation of antibodies

A fragment of the Ghox-lab genomic clone was generated by trimming with exonuclease III and cloning into a Bluescript KS+ plasmid. The trimmed clone begins 12 nucleotides upstream of the amino terminus, spans the 204 amino acid-coding region of the upstream exon, and ends at an *Sst*I (*Sac*I) site located within the adjoining intron. A key feature of this fragment is that it does not encode the homeo box and has only small regions of homology to the mouse *Hox-1.6* gene that was used to isolate the clone (Sundin et al. 1990). The fragment was excised with the Bluescript polylinker sites *Bam*HI and *Hind*III and cloned into the TrpE expression vector PATH 1 (Dieckman and Tzagoloff 1985). The fusion protein was expressed at high levels in *E. coli* strain XL1-blue (Stratagene). The bacteria were grown in M9 medium with 0.5% casamino acids, with 50 μ g/ml 3-indolepropionic acid added as an inducer (Morse et al. 1969), followed by growth for 6 hr at 37°C. Inclusion bodies containing the fusion protein were prepared by sonication of cells in TE [10 mM Tris (pH 7.4), 1 mM sodium EDTA] containing 0.2 mM PMSF at 0°C until complete lysis, followed by two successive washes in TE containing 1% (vol/vol) Triton and 2 M urea. The final pellet was resuspended in TE containing 6 M urea, incubated at 25°C for 10 min, and centrifuged at 45,000 rpm for 30 min at 4°C. Fusion protein in the supernatant extract was purified further by electrophoresis in SDS/10% acrylamide gels. Bands were located by a conventional staining of the gels for 1 min in Coomassie Blue, followed by rinsing in water for 30 min. Gels were frozen, ground into fine particles, mixed with Freund's adjuvant, and used to immunize three rabbits. Immunizations were performed by the Berkeley Antibody Company (Richmond, CA). The initial injection contained ~500 μ g of protein, whereas the booster injections (with incomplete Freund's adjuvant) each contained half this amount.

Affinity purification of antibodies

A second fusion protein, this time consisting of LacZ and the first 94 amino acids of the predicted Ghox-lab protein, was prepared by cloning a *Bam*HI–*Pst*I fragment of the trimmed Ghox-lab clone into the expression vector PUR 292 (Rüther and Müller-Hill 1983). The approach uses a two-fusion-protein method to select positively only antibodies specific for protein encoded by the cloned chick DNA (Gaul et al. 1987). Nitrocellulose-bound protein serves as the immobile affinity matrix in an affinity purification procedure similar to that of Olmstead (1981).

Preparation of affinity matrix The 6 M urea inclusion body extract containing the LacZ fusion protein was prepared exactly as described for the TrpE protein. Electrophoresis of the LacZ fusion protein was carried out in 7.5% acrylamide gels, which were then transferred electrophoretically (Towbin et al. 1979) to nitrocellulose filters. The filters were stained with Ponceau S to visualize the fusion protein and air-dried, after which strips containing the immobilized fusion protein were excised and stored at -20°C .

Affinity purification About 40 cm² of these strips were blocked in three changes of 5% milk TST [5% (wt/vol) Carnation nonfat dry milk, 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Tween 20]. Four milliliters of anti-fusion protein rabbit serum was diluted with 16 ml of 5% milk TST, and the strips were incubated with this mixture overnight at 4°C , with gentle agitation. After five 5-min washes in 1% milk TST, a 1-hr wash in 1% milk TST at $\sim 23^{\circ}\text{C}$ and, finally, two 1-min washes in 150 mM NaCl, bound antibodies were eluted in 10 ml of 150 mM glycine (pH 2.7) at $\sim 23^{\circ}\text{C}$ for 10 min, followed by immediate neutralization of the solution by the addition of 1 ml of 2 M Tris (pH 8.2). Antibodies were stabilized by the addition of an equal volume of 10% TST, frozen quickly on dry ice, and stored in small aliquots at -80°C .

Immunohistochemistry

Fixation of embryos Embryos were incubated and staged according to Hamburger and Hamilton (1951), dissected, and collected in ice-cold PBS. They were then fixed in 4% paraformaldehyde–PBS for 15 min at $\sim 23^{\circ}\text{C}$, rinsed briefly in ice-cold PBS, transferred to methanol at $\sim 23^{\circ}\text{C}$ for 30 min, and stored at -20°C .

Frozen sections Embryos in methanol were rehydrated stepwise into TS [10 mM Tris (pH 7.4), 150 mM NaCl] containing 0.5% sucrose. The embryos were warmed to 37°C , incubated in TS containing 0.5 M sucrose and 7.5% (wt/vol) gelatin (Sigma 300 Bloom) for 1 hr at 37°C and, finally, chilled on ice for 1 hr. Blocks were trimmed at 4°C and frozen by immersion in *n*-pentane (dry-ice chilled) for 90 sec. Sections (10 μm) were cut at -29°C and transferred to gelatinized slides, air-dried for 30 min at $\sim 23^{\circ}\text{C}$, and stored at -80°C . Gelatin was removed by incubation in TS at 42°C for 10 min, followed by blocking with centrifugation-clarified 5% milk TST for 1 hr. The first antibody was diluted into 5% milk TST, spin-clarified in a microfuge at 12,000 rpm at 4°C for 20 min, applied to the sections, and incubated for 30 min in a humidified chamber at $\sim 23^{\circ}\text{C}$. Sections were then washed five times in 1% milk TST, 2 min per wash. Biotinylated second antibody (goat anti-rabbit IgG or horse anti-mouse IgM; Vector Laboratories) was diluted 1 : 100 in 5% milk TST. Incubations and washes were as for the first antibody. Avidin–HRP (avidin–horseradish peroxidase; Vector) was applied to the sections at 1 : 100 dilution and incubated exactly as for the second antibody. After two final 10-min

washes with TST, a solution of TS containing 300 $\mu\text{g}/\text{ml}$ diaminobenzidine and 0.03% H_2O_2 was applied to the sections for 15 min at $\sim 23^{\circ}\text{C}$, after which they were rinsed in water, air-dried, and mounted in methyl salicylate/Canada balsam as described previously (Sundin et al. 1990).

Embryo whole mounts Fixed embryos (stages 8–12) were rehydrated stepwise from methanol to TST, and blocked in clarified 5% milk TST at 4°C for 2 hr. The first antibody [either a rabbit anti-Ghox-lab or mouse monoclonal anti-leu 7 (Becton Dickinson)], was diluted in 5% milk TST and spin-clarified. The blocked embryos were incubated in this solution for 10–16 hr at 4°C , with gentle agitation on a rocker platform. The embryos were then washed in 1% milk TST, with five changes of solution over a period of 4 hr at $\sim 23^{\circ}\text{C}$. They were then incubated in biotinylated goat anti-rabbit (or horse anti-mouse) IgG, heavy- and light-chain-specific (Vector), diluted 1 : 200 in clarified 5% milk TST at $\sim 23^{\circ}\text{C}$ for 4 hr, followed by 4 hr of washes in 1% milk TST. The final incubation was in a 1 : 500 dilution of avidin–HRP in clarified 5% milk TST for 10–16 hr at $\sim 23^{\circ}\text{C}$, followed by 4 hr of washes in 1% milk TST. The peroxidase reaction was carried out by incubation in TS containing 300 $\mu\text{g}/\text{ml}$ diaminobenzidine for 10 min, followed by addition of H_2O_2 to a final concentration of 0.03%, and incubated for 15 min at $\sim 23^{\circ}\text{C}$. Embryos were washed with TS, and dehydrated stepwise with ethanol. They were cleared and viewed in 2 : 1 benzyl benzoate and benzyl alcohol.

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