Development 114, 841-852 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

An early marker of axial pattern in the chick embryo and its respecification by retinoic acid

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Summary

Chick Ghox 2.9 protein, a homeodomain-containing polypeptide, is first detected in the mid-gastrula stage embryo and its levels increase rapidly in the late gastrula. At this time, the initially narrow band of expression along the primitive streak expands laterally to form a shield-like domain that encompasses almost the entire posterior region of the embryo and extends anteriorly as far as Hensen's node. We have found that this expression domain co-localizes with a morphological feature that consists of a stratum of refractile, thickened mesoderm. Antibody-staining indicates that Ghox 2.9 protein is present in all cells of this mesodermal region. In contrast, expression within the ectoderm overlying the region of refractile mesoderm varies considerably. The highest levels of expression are found in ectoderm near the streak and surrounding Hensen's node, regions that recent fate mapping studies suggest are primarily destined to give rise to neurectoderm.

At the definitive streak stage (Hamburger and Hamilton stage 4) the chick embryo is especially sensitive to the induction of axial malformations by retinoic acid. Four hours after the treatment of definitive streak embryos with a pulse of retinoic acid the expression of Ghox 2.9 protein is greatly elevated. This ectopic expression occurs in tissues anterior to Hensen's node, including floor plate, notochord, presumptive neural plate and lateral plate mesoderm, but does not occur in the anteriormost region of the embryo. The ectopic induction of Ghox 2.9 is strongest in ectoderm, and weaker in the underlying mesoderm. Endoderm throughout the embryo is unresponsive. At stage 11, Ghox 2.9 is normally expressed at high levels within rhombomere 4 of the developing hindbrain. In retinoic-acid-treated embryos which have developed to this stage, typical rhombomere boundaries are largely absent. Nevertheless, Ghox 2.9 is still expressed as a discrete band, but one that is widened and displaced to a more anterior position.

Key words: homeobox, pattern formation, chick, gastrula, retinoic acid.

Introduction

A central question of vertebrate embryology is how distinct regions are established along the anteroposterior axis of the central nervous system. Grafting studies in amphibians (Mangold and Spemann, 1927; Nieuwkoop et al., 1985; Saxén, 1989) and birds (Waddington, 1933; Hara, 1961) suggest that the neural plate is induced and regionalized at the time of gastrulation through the interaction of mesoderm with ectoderm. By the time development reaches the neural fold stage regionalization is largely complete and cells are clonally committed to form distinct regional structures as small as single rhombomeres (Fraser et al., 1990).

Insight into the molecular basis of the regionalization of the neuraxis has been gained from studies of the vertebrate *Hox* genes (reviewed in Akam, 1989; Hunt et al., 1991), a family of transcription factors related to the *Drosophila* homeotic genes of the *Antennapedia* and *Bithorax* loci (Gehring, 1987). The *Hox* genes are expressed in staggered domains along the anteroposterior axis of the nervous system, an arrangement that suggests that they function as determinants of axial position (Gaunt et al., 1988; Duboule and Dollé, 1989; Graham et al., 1989; Murphy et al., 1989; Wilkinson et al., 1989; Sundin and Eichele, 1990; Frohman et al., 1990; Wilkinson and Krumlauf, 1991). Genetic evidence concerning *Hox* gene function comes from studies utilizing overexpression (Harvey and Melton, 1988; Wolgemuth et al., 1989; Wright et al., 1989; Kessel et al., 1990) or gene ablation (Chisaka and Capecchi, 1991; Lufkin et al., 1991). These investigations support an involvement of *Hox* genes in morphogenesis, but we are far from understanding their specific roles in the formation of the central nervous system.

Retinoic acid is a naturally occurring signalling molecule that can cause profound changes in the process of pattern formation within the developing limb bud (Tickle et al., 1982; reviewed in Eichele, 1989), in regenerating urodele limbs (Maden, 1982; Ludolph et

al., 1990) and in the central nervous system (Morriss, 1972; Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Morriss-Kay et al., 1991). In cultured teratocarcinoma cells a number of *Hox* genes are inducible upon treatment with retinoic acid (e.g. Baron et al., 1987; La Rosa and Gudas, 1988). Within the *Hox* family, the three most rapidly induced genes are the homologues of the *Drosophila labial* gene (Simeone et al., 1990; Stornaiuolo et al., 1990; Simeone et al., 1991; Papalopulu et al., 1991). These *labial* related genes are also the first genes of the *Hox* family to be activated during development (Sundin et al., 1990; Frohman et al., 1990; Murphy and Hill, 1991; Sive and Cheng, 1991). We have previously reported that the transcripts of one of these, *Ghox 2.9* (formerly *Ghox-lab*) are detected in the mid-gastrula chick embryo, prior to and during regional determination of the neurectoderm (Sundin et al., 1990). Later, during development of the hindbrain, the anterior limit of Ghox 2.9 expression coincides with the border between rhombomeres 3 and 4. Eventually, Ghox 2.9 is upregulated only in rhombomere 4 (Sundin and Eichele, 1990). In the present study, we have examined the spatial distribution of Ghox 2.9 protein during gastrulation and neurulation. The single-cell resolution afforded by the Ghox 2.9 antibody has now revealed novel features of the early expression that were not observed in the previous work based on in situ hybridization (Sundin et al., 1990; Frohman et al., 1990; Murphy and Hill, 1991). We have also studied the effect of retinoic acid on Ghox 2.9 expression during gastrulation and found a rapid induction of Ghox 2.9 in tissues whose developmental fates are subsequently affected.

Materials and methods

Embryo isolation and culture

White Leghorn chick eggs were incubated at 38.5°C, for 18 hours. Eggs were cracked open into a 10 cm bacteriological Petri dish. The embryo floating on top of the yolk was isolated by first clearing albumen away from the vitelline membrane. A dry ring of paper (7 mm internal diameter) was laid on top of the vitelline membrane so that it encircled the embryo. The ring-embryo-vitelline membrane composite was cut out with scissors, and then floated sideways into a small spoon filled with warmed Tyrode's solution (100 mM sodium chloride, 10 mM Na_2HPO_4, KH_2PO_4, 2 mM potassium chloride, 0.8 mM magnesium chloride, 0.75 mM calcium chloride, 1.5 g/l glucose, pH 7.25). The embryos were then floated dorsal side up for about 30 seconds on the surface of 32°C Tyrode's solution to remove yolk, followed by a brief rinse in Tyrode's solution at ambient temperature. At this point, they were either fixed for histochemistry or prepared for culture.

Embryos were cultured on agar culture plates prepared essentially according to Schoenwolf and Watterson (1989). Briefly, liquid albumen was collected from incubated eggs and then stirred for 15 minutes at room temperature. 10% w/v glucose in water was added to create albumen containing 0.3% glucose. 123 mM NaCl containing 0.6% Difco Bacto agar was boiled, then both agar and albumen were equilibrated to 49°C, mixed in equal volumes, and poured into plates at a thickness of 0.6 cm. Plates were cooled at 4°C and used within an hour. All-*trans*-retinoic acid was added to culture agar as follows. A concentrated retinoic acid solution (in ethanol) was quickly suspended in 1 ml of water. This mixture was added at once to the albumen/agar blend. Yolk extract was prepared by mixing equal volumes of fresh yolk and Tyrode's solution. This blend was spun in a microfuge at 13000 revs/minute for 10 minutes at room temperature, and the supernatant yolk extract was collected on ice. Yolk-Tyrode (YT) was prepared by mixing 1 volume of yolk extract with 9 volumes of Tyrode's solution. Rings carrying the embryos were picked up with forceps, placed ventral side up on room temperature agar culture plates and overlaid with 20 μl of YT, with or without retinoic acid. Retinoic acid was added as described above for agar. The concentration of retinoic acid in the agar plate and in YT were the same. Plates were sealed with Parafilm and incubated between 38.5°C and 39°C. To terminate treatment, retinoic acid was removed by washing embryos for 25 minutes in 3 changes of YT at 32°C. Embryos were returned to plates free of retinoic acid. After culture and prior to antibody staining, embryos were again washed in YT at 32°C for 2 minutes followed by a wash in ice-cold Tyrode's solution for 20 minutes. This decreased the avidin-related background in the immunostains.

Immunohistochemistry

Embryos were fixed in 2% paraformaldehyde/PBS on ice for 10 minutes followed by 4% paraformaldehyde/PBS at room temperature for 30 minutes. The embryos were stored in methanol at −20°C. Prior to staining, embryos were incubated in methanol/ DMSO (1:1) for 5 minutes, followed by the addition of 1/4 volume of 10% Triton X-100 and occasional agitation for 20 minutes at ambient temperature, followed by 10 minutes in 150 mM ethanolamine-HCl containing 10 mM Tris-HCl pH 8.0 and 1% Triton X-100. Subsequent steps of whole-mount immunohistochemistry with affinity-purified polyclonal rabbit anti-Ghox 2.9 antiserum was essentially as described earlier (Sundin and Eichele, 1990) with the exception that all antibody and avidin incubations were carried out at room temperature. Whole mounts were embedded in paraffin, sectioned at 10 μm and mounted in Canada balsam mixed with methyl salicylate for visualizing Hoechst staining (Sundin et al., 1990) or in xylene/Canada balsam (2:1) for bright-field photography.

Results

Development of the Ghox 2.9 pattern: from definitive streak to four somites

Cells expressing significant levels of Ghox 2.9 protein are first detected in the definitive streak gastrula (Hamburger and Hamilton stage 4, Hamburger and Hamilton, 1951). The prominent feature of embryos at this point in development is the primitive streak, the avian and mammalian equivalent of the amphibian blastopore (Fig. 1A). At stage 4, the streak has completed its elongation towards the anterior end of the embryo, but formation of notochord has not yet begun. Cells expressing Ghox 2.9 protein revealed by immunoperoxidase staining of whole-mount embryos (Fig. 1A), are detected throughout the primitive streak except for the anterior 250 μm. This non-staining area includes Hensen's node, the amniote equivalent of the amphibian blastopore lip (Waddington, 1933, 1952). In

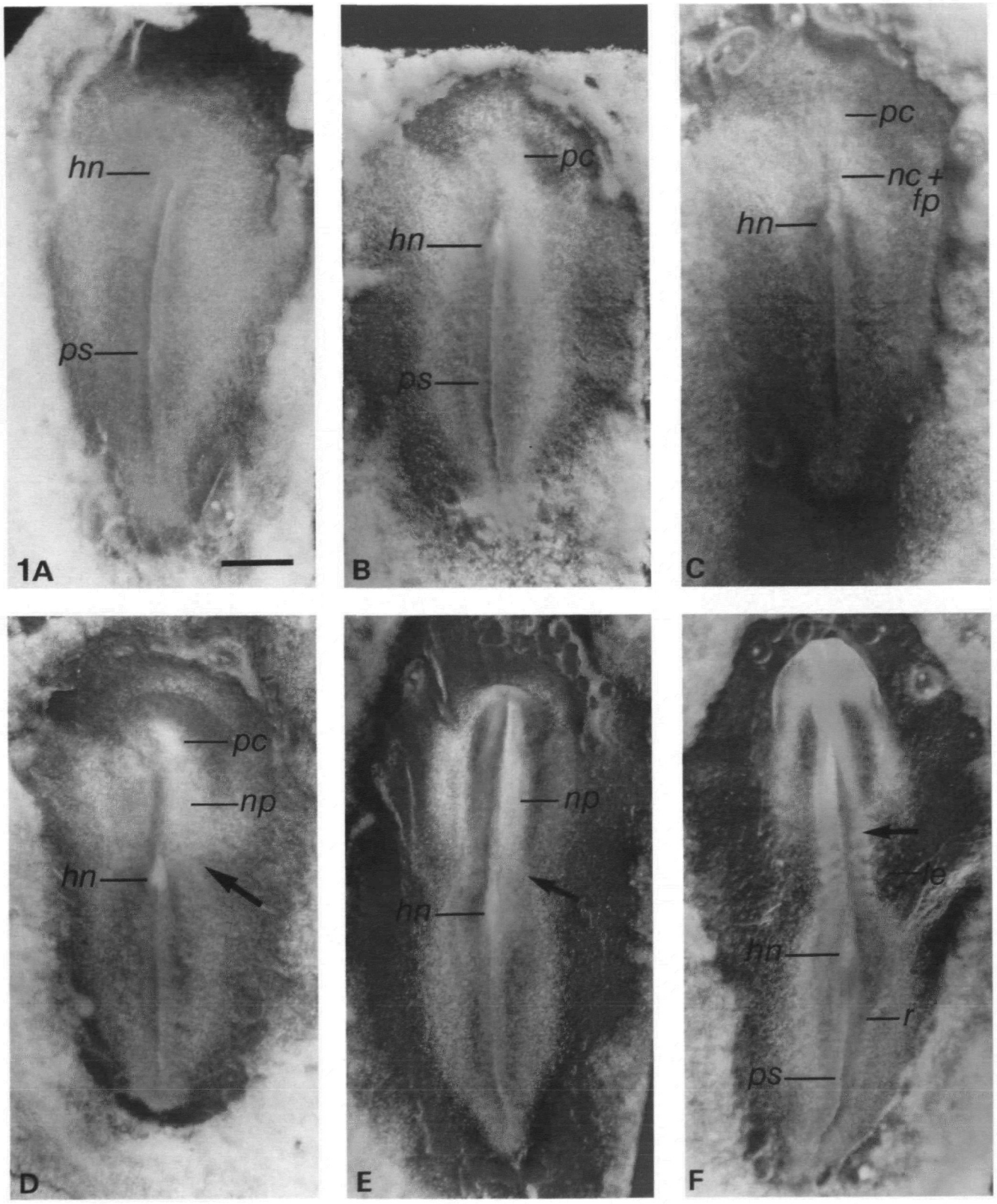


Fig. 1. Ghox 2.9 expression from the definitive streak to the four-somite embryo. Embryos from stages 4 through 8 were stained for Ghox 2.9 protein by whole-mount immunohistochemistry. Embryos were photographed uncleared, in methanol. (A) Stage-4, definitive streak embryo. (B) Early stage-5 embryo. (C) Stage-5 embryo. (D) Early stage-6 embryo, neural plate stage. Arrow indicates position of the the early presomitic cleft. (E) Stage-7, one-somite neural plate stage. Arrow points to anterior boundary of first somite, which is the anterior border of Ghox 2.9 expression in the mesoderm. Dark area in anterior left region of neural plate is not Ghox 2.9 signal. (F) Stage-8, four-somite embryo. Arrow indicates anterior boundary of first somite. hn, Hensen's node; le, lateral ectoderm; nc+fp, notochord and floor plate; np, neural plate; pc, prechordal plate; ps, primitive streak; r, ridge lateral to primitive streak. Bar, 400 μm (A through F).

the stage 4 embryo shown in Fig. 1A, the signal observed within the primitive streak is contributed by Ghox 2.9-positive cells located in both ectoderm and mesoderm. The faint signal observed in regions lateral to the streak, results from Ghox 2.9 expression exclusively in mesodermal cells, with no contribution from the overlying ectoderm.

While the embryo develops from stage 4 to stage 5, it begins to form notochord and floor plate (see Fig. 1B, C). As part of this process, Hensen's node moves posteriorly. During this regression, cells located in the center of the node generate notochord and floor plate (Bellairs, 1986; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991). At stage 5, there is a considerable increase in the expression level of Ghox 2.9 both within the primitive streak as well as in the adjacent lateral tissue (Fig. 1B, C). Within the shield-like domain of Ghox 2.9 expression, the primitive streak and the region around the node exhibit the most intense staining, while the center of the node does not stain (Fig. 1C). Structures anterior to the node such as prechordal plate, notochord, floor plate, lateral plate mesoderm and associated ectoderm show no evidence of Ghox 2.9 expression.

In the early stage-6 embryo illustrated in Fig. 1D, the neural plate is clearly visible and exhibits Ghox 2.9 expression in the area lateral to the node and 120 μm anteriad to this level. The raised surface of the neural plate becomes even more obvious in the stage-7 embryo (Fig. 1E, one-somite). Again there is considerable expression in the posterior portion of the neural plate. In the stage-8 embryo (four-somite, Fig. 1F), the presumptive forebrain-midbrain region of the anterior neural plate folds together and the outlines of the future head are clearly visible. At this stage Ghox 2.9 protein is found at high levels within the posterior region of the neural plate and in the underlying somites, but at decreased levels in the lateral ectoderm (Fig. 1F). A major Ghox 2.9-positive feature at this stage is the pair of plateau-like ridges located lateral to the streak. These ridges are continuous with the neural plate and extend to the posterior end of the embryo.

The anterior boundary of Ghox 2.9 expression

In general terms the domain of Ghox 2.9 expression encompasses the posterior two thirds of the early embryo. Close examination of whole mount stained embryos (Fig. 1B through F) reveals that the anterior boundary of this domain is relatively complicated and changes with time. In stage-5 embryos the center of Hensen's node essentially does not express the gene while lateral tissue at the level of the node is positive for Ghox 2.9 protein (Fig. 1B,C). The negative zone located in the center of the node, also seen in the transverse section in Fig. 5D, corresponds to the ectodermal and mesodermal layers of the 'chorda center' described by Spratt (1955). Cell lineage studies have shown that this region gives rise to floor plate and notochord, respectively (Selleck and Stern, 1991). At later stages of regression (Fig. 1F) the entire node expresses Ghox 2.9 protein. Those portions of the notochord and floor plate that emerge from the node prior to stage 7 lack Ghox 2.9 signal (white region along the midline, anterior to Hensen's node, Fig. 1E), while notochord and floorplate produced after stage 7 express the gene. In later embryos, Ghox 2.9 ceases to be expressed in the notochord, but is maintained in the floor plate (Sundin and Eichele, 1990).

Another aspect of the anterior boundary of Ghox 2.9 expression is the alignment between neural plate and its underlying mesoderm. In the early stage-6 embryo, we observe high levels of Ghox 2.9 protein in both mesoderm and ectoderm, and the anterior expression boundaries in these layers are in close alignment and coincide with the presomitic cleft (Fig. 1D). In the stage-7 embryo, however, Ghox 2.9 signal in the neural plate extends about 110 μm anterior to the limit of Ghox 2.9 expression in the mesoderm (Fig. 1E). It remains to be determined whether this dislocation is a consequence of differential movement of the tissue layers or results from a turning on of Ghox 2.9 in previously non-expressing neuroectoderm.

A morphological feature marks the domain of Ghox 2.9 expression

In the stage-5 chick embryo we noticed a subtle morphological structure that coincides with the shield-like domain of Ghox 2.9 expression. This feature is a zone of refractile, thickened mesoderm visible in living as well as in fixed embryos (Fig. 2A, B). To determine more precisely the relationship between the anterior boundary of this thickened mesodermal layer and Ghox 2.9-expressing cells, a stage-5 whole mount was sectioned and counterstained with Hoechst dye. In a Hoechst-stained section parasagittal to Hensen's node (Fig. 2C), the anterior boundary of Ghox 2.9 expression coincides with the transition (vertical line) from thick posterior mesoderm to a thinner layer of anterior mesoderm. It should be noted that the presomitic cleft of the first somite will appear in this transition zone during stage 6.

Ghox 2.9 expression occurs throughout the refractile mesoderm. In the overlying ectoderm, however, expression varies considerably. Along the ridges of the primitive streak and within the region around the node, all ectodermal nuclei express the gene in a uniform manner, but more posterior and lateral regions of ectoderm contain nuclei that lack expression. A section lateral to the mid-portion of the primitive streak (Fig. 2D) reveals expression of Ghox 2.9 in the lateral mesoderm but not in the overlying ectoderm. A transverse section in the mid-streak region (Fig. 2E) shows expression in both ectoderm and mesoderm. However, in ectoderm lateral to the streak there is a mixture of Ghox 2.9-positive and negative nuclei. The positive nuclei are located near the basal lamina (upward arrow), while the negative ones tend to be placed towards the apical surface (downward arrow). It is possible that these positive and negative cells have different fates.

Cells of the thin endodermal layer also fluoresce brightly with the Hoechst counterstain, demonstrating

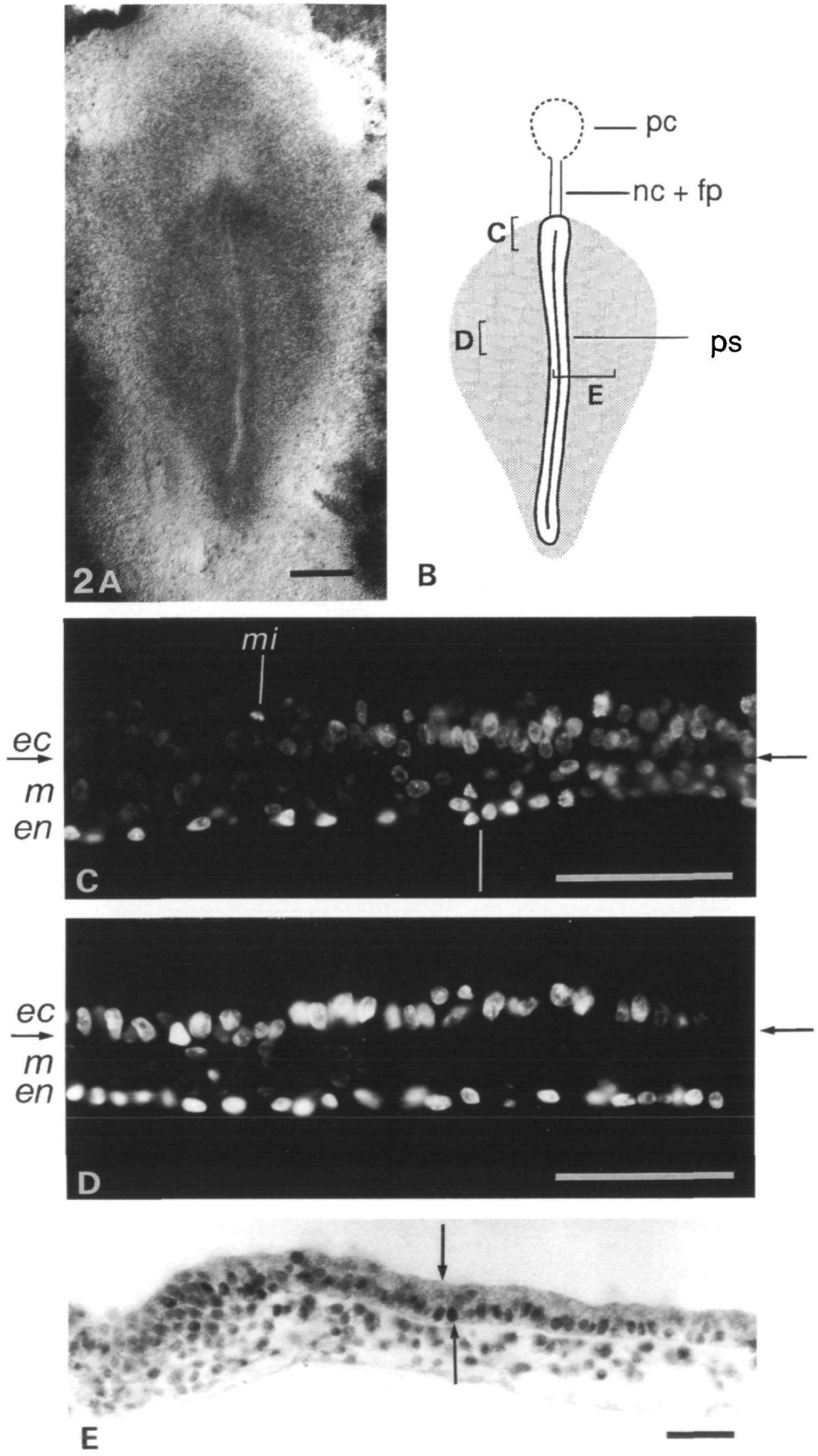


Fig. 2. Domain of refractile mesoderm and cellular distribution of Ghox 2.9 protein. (A) An early stage-5 embryo was fixed in 4% paraformaldehyde and then photographed under bright-field illumination in 17% w/v sucrose-saline. Note: this embryo was not stained with anti-Ghox 2.9 antibody. Bar, 400 μm (A and B) (B) Diagram explaining features in A and positions of sections shown in panels C, D and E. Grey area represents Ghox 2.9-expressing refractile mesoderm. (C) Section of early stage-5 embryo stained with anti-Ghox 2.9 antibody. Section parasagittal to the node, spanning the anterior boundary of the refractile mesoderm. Anterior to right, dorsal up. Ghox 2.9 peroxidase stain present in interphase nuclei quenches the Hoechst counterstain and nuclei appear darker. Unstained nuclei stain more brightly. Mitotic chromosomes of Ghox 2.9 positive cells do not quench the fluorescence. Horizontal arrows indicate the basal lamina separating ectoderm and mesoderm. Bar, 50 μm. Vertical line indicates transition from thick posterior mesoderm to anterior mesoderm. (D) Section of the same embryo as in C, with same orientation and magnification, but taken far lateral to the mid-streak. Shows expression of Ghox 2.9 in mesodermal layer, but little in ectoderm and endoderm. Horizontal arrows indicate position of basal lamina. Bar, 50 μm. (E) Transverse section of mid-streak region. Bright-field photograph. Vertical arrow pointing down indicates position of an unstained nucleus near the dorsal surface of the ectoderm. Arrow pointing up indicates Ghox 2.9-positive ectodermal nucleus positioned near basal lamina. Bar, 50 μm. ec, ectoderm; en, endoderm; m, mesoderm; mi, mitotic chromosomes; nc+fp, notochord plus floor plate; pc, pre-chordal plate; ps, primitive streak.

that they do not express Ghox 2.9 (Fig. 2C and D). This fact was not appreciated by earlier studies in chick and mouse due to the lower resolution of the in situ hybridization procedure employed (Sundin et al., 1990; Frohman et al., 1990). However, a distinct region of the foregut expresses Ghox 2.9 during later development (Sundin et al., 1990; Frohman et al., 1990; Sundin and Eichele, 1990; Murphy and Hill, 1991). It is presently not known whether these foregut cells derive from the Ghox 2.9-negative early endoderm, or from other precursors located in Ghox 2.9-positive mesoderm or in primitive streak.

Retinoic acid alters morphology of the developing brain

Retinoic acid treatment of primitive streak chick embryos induces pronounced morphological changes in several tissues. Embryos are significantly smaller, there is less mesodermal tissue, they lack a properly developed heart, and most strikingly, there are notable changes in the segmental organization of the central nervous system (Fig. 3C,D). The anteriormost central nervous system was less affected; telencephalon, diencephalon and eye primordia were readily recognizable. However, one does not observe a normal midbrain and hindbrain. Instead, posteriad of the border of the diencephalon, there is a relatively short portion of neural tube followed by broad hindbrain-like neurectoderm (region between dashed lines in Fig. 3D). The precise nature of this neurectoderm is obscured by the absence of clear rhombomeric constrictions. However, there is a morphological marker allowing us to identify the location of rhombomere 4. This marker is the massive cluster of neural crest cells attached to the anteriormost section of the hindbrain-like neurectoderm (Fig. 3C and D). This neural crest cell cluster has the characteristic morphology of the 7th/8th ganglion primordium derived from rhombomere 4 (Fig. 3A, B). We therefore suggest that the neurectoderm next to the 7th/8th ganglion primordium is rhombomere 4 or at least rhombomere 4-like. This interpretation is also supported by the observation that this portion of neurectoderm expresses the rhombomere-4-specific Ghox 2.9 marker (see below). One interpretation of the RA- induced morphological changes is that midbrain, and the entire anterior hindbrain (rhombomeres 1 through 3) have either been lost or been compressed into the small segment situated between the forebrain and rhombomere 4.

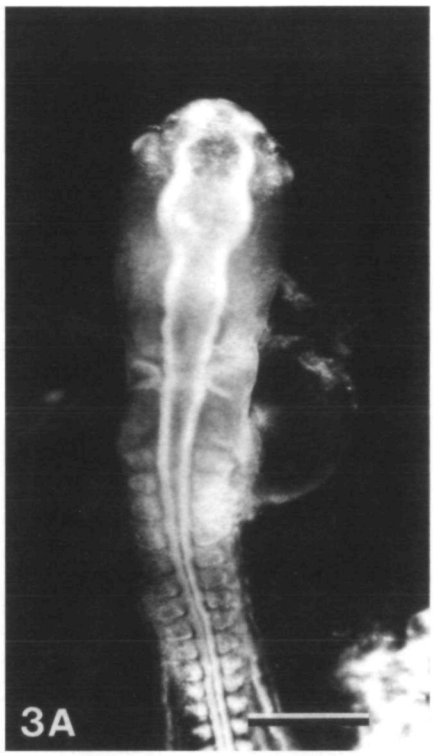
The severity of these morphological changes depends on the dose, duration and stage of the retinoic acid treatment. We have found that treatment of embryos at stage-4, just prior to and during early streak regression, is the most efficient way of inducing dysmorphogenesis of the central nervous system. Stage-4 embryos that are treated with 6 μM retinoic acid for 4 hours develop during treatment to a readily recognizable late stage-4 or early stage-5. Of those that survive to stage 12 and have an interpretable morphology (about 40% of the treated specimens), all display a significant decrease in the size of midbrain and anterior hindbrain. Variations of this basic protocol were also examined, and the results are summarized as follows. Stage-4 embryos, treated for 4 hours with 0.6 μM retinoic acid, show no significant differences from to the controls. 2 μM retinoic acid causes a significant decrease in the size of the midbrain, and a slight decrease in the anterior hindbrain. 10 μM retinoic acid gives results similar to 6 μM, but the embryos are smaller and most show some failure of neural tube closure. Treating stage-4 embryos for 2.5 hours with 6 μM retinoic acid results in generally milder effects, similar to those seen after a 4-hour treatment with 2 μM retinoic acid. If late stage-4 through stage-5 embryos are given this treatment the outcome is more variable, and one generally observes a lesser reduction in the size of midbrain and anterior hindbrain. The treatment of stage-6 or of more advanced embryos, however, rarely results in substantial alterations of the proportions of forebrain, midbrain and hindbrain, indicating that the sensitive period ends by the onset of neurulation. The beginning of the sensitive period is not as clear. When stage-3 or earlier embryos are treated with retinoic acid, very few survive to stage 11 or 12. Therefore, we cannot draw conclusions from these few cases.

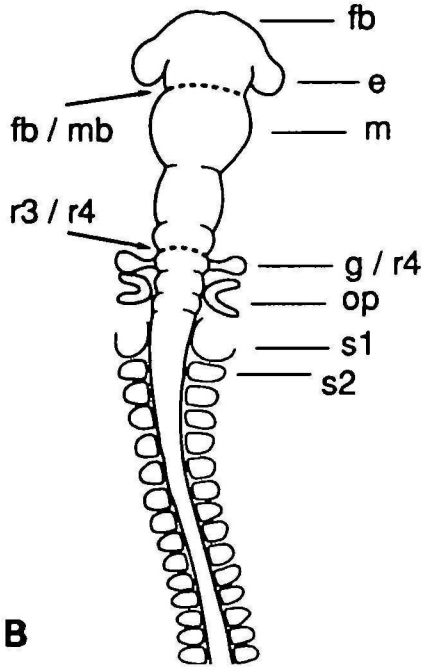
Ghox 2.9 expression is changed by retinoic acid

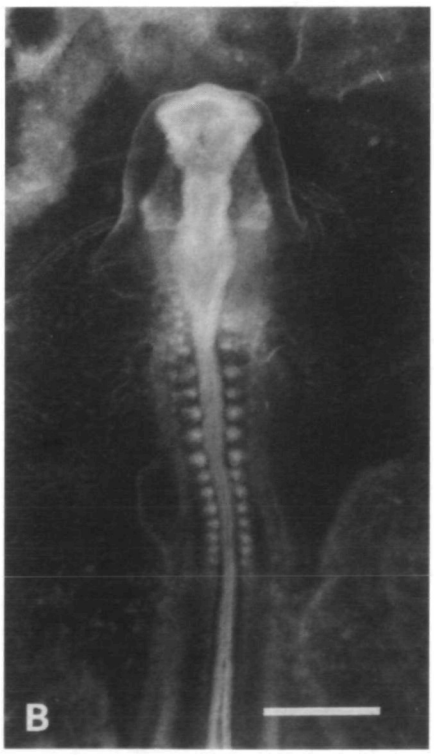
The overt morphological changes caused by retinoic acid treatment are not evident until the stage when the neural tube becomes partitioned into segments (stages 9 through 11), yet the period of greatest sensitivity is much earlier. This raises the question of what changes occur in the embryos at these early stages and how these produce the malformations observed later on. In an effort to examine this issue at molecular and cellular levels, we have investigated how Ghox 2.9 expression is affected by RA treatment. In essence, we found that exposing embryos to RA causes a rapid and complex series of changes in the pattern of Ghox 2.9 expression.

As for the embryo in Fig. 3C, stage-4 embryos were treated for 4 hours, and during that time they developed to early stage-5. There was a general increase in the intensity of Ghox 2.9 signal within the regions of the embryo that normally express the protein (compare Fig. 4A, B). More importantly, we found significant ectopic expression of Ghox 2.9 protein within and anterior to Hensen's node (Fig. 4B) where the gene is normally not active (Fig. 4A). Note, however, Ghox 2.9 was not induced in the anteriormost part of the embryo, the region containing the prechordal plate (Fig. 4B). Likewise, we did not see Ghox 2.9 protein induction in the normally Ghox 2.9-negative endodermal cell layer (not shown). Hence, Ghox 2.9 is not induced indiscriminately, but appears in specific regions and cell types.

Hensen's node and midline structures extending up to the prechordal plate showed a striking increase in anti-Ghox 2.9 antibody labelling (Fig. 4B). There were also Ghox 2.9-positive cells in a region lateral to the notochord and floor plate. This region reaches about halfway between Hensen's node and the prechordal plate. Transverse sections of treated embryos cut at







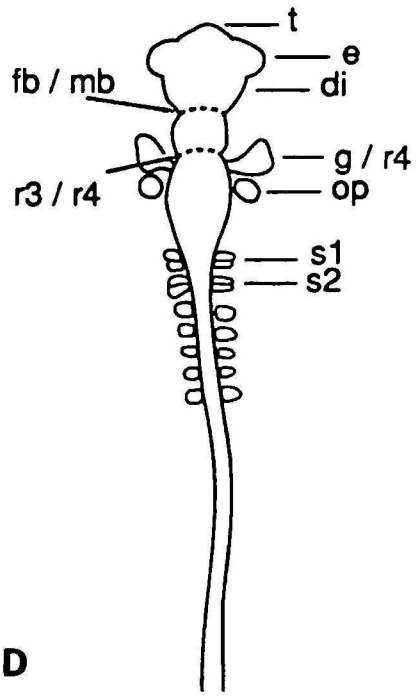


Fig. 3. Effect of early retinoic acid treatment on the morphology of stage-12 embryos. (A) Control embryo grown in culture from stage 4 to stage 12, fixed, photographed in DMSO/ methanol 1:1 with incident illumination. Dorsal view. (B) Interpretive drawing of A. Dashed lines indicate the forebrain/midbrain boundary and the anterior boundary of rhombomere 4, respectively. (C) Experimental embryo, treated with retinoic acid as described in text. (D) Interpretive drawing of C. Dashed lines are as in B. di, diencephalon; e, eye; fb, forebrain; fb/mb, forebrain/midbrain boundary; g/r4, primordium of acousticofacial ganglion derived from rhombomere 4 neural crest; m, midbrain; op, otic placode; r3/r4, anterior boundary of rhombomere 4; s1, the somite which first appears at stage-7; s2, second somite; t, telencephalon. Bars, 400 μm.

different axial levels reveal that the degree of Ghox 2.9 induction by RA in ectodermal and mesodermal structures is different for different axial levels (see Fig. 5A to D). The cross-section situated just posterior to the prechordal plate (Fig. 5A), displays much higher Ghox 2.9 expression in the floor plate than in the underlying notochord. Lateral ectoderm, which will contribute to the lateral neural plate, contains few nuclei expressing Ghox 2.9 protein. At a more posterior level in the embryo, both floor plate and presumptive lateral neurectoderm express very high levels of Ghox 2.9 (Fig. 5B).The underlying mesodermal structures, notochord and lateral plate mesoderm, show some response to RA but the degree of Ghox 2.9 induction is

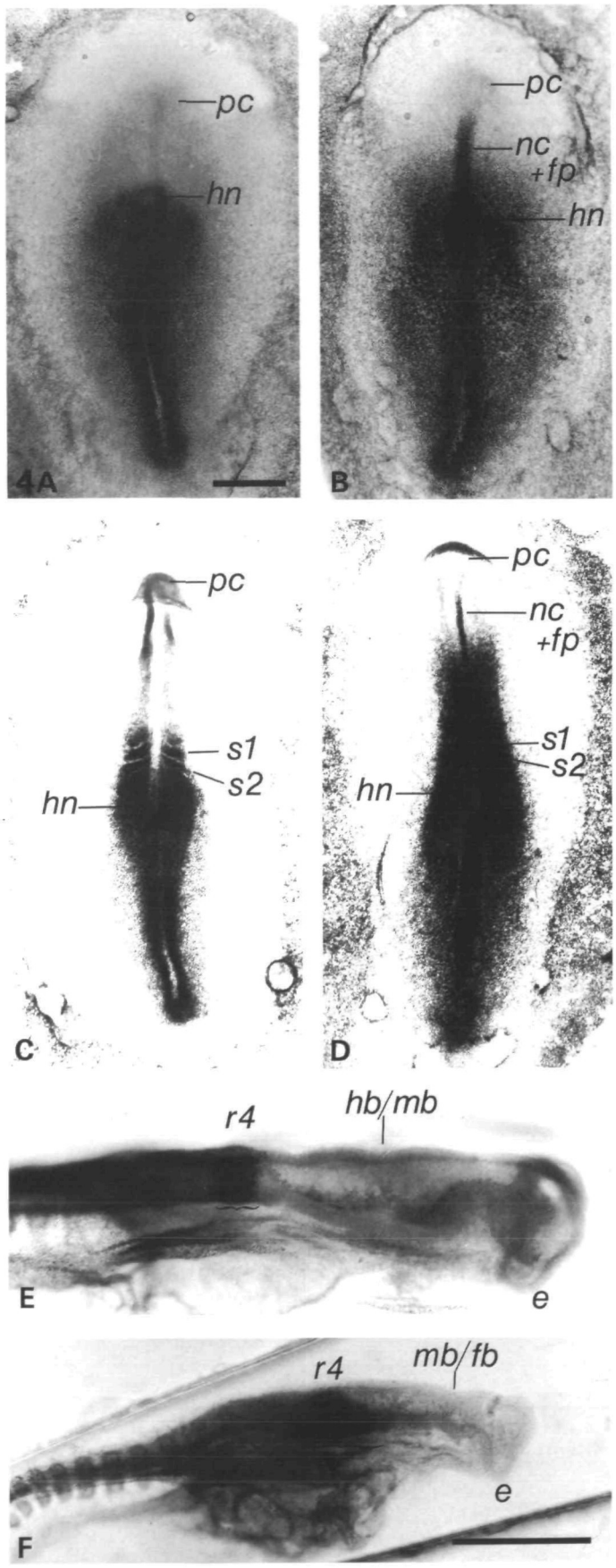


Fig. 4. Effect of retinoic acid on territory of Ghox 2.9 expression. Embryos grown in culture (see text) were fixed at different times after retinoic acid treatment and whole-mount stained for Ghox 2.9 protein. Embryos were fully cleared in benzyl alcohol/benzyl benzoate to distinguish individual stained nuclei. (A) Control embryo, stage 5. (B) Retinoic-acid-treated embryo, stage 5. (C) Control embryo, late stage 7, with two somites and neural plate. (D) Retinoic acid treated, late stage 7, with two somites and neural plate. (E) Control embryo, late stage 11, lateral aspect, anterior to right. (F) Retinoic-acid-treated embryo, stage 11, lateral aspect, anterior to right. In E and F, brackets indicate anteroposterior extent of rhombomere-4-specific Ghox 2.9 signal. e, eye; hb/mb, hindbrain-midbrain boundary; hn, Hensen's node; mb/fb, midbrain-forebrain boundary; nc+fp, notochord plus floor plate; pc, prechordal plate; r4, rhombomere 4; s1, first somite; s2 second somite. Bar in 4A, 400 μm (A through D). Bar in 4F, 400 mm (E and F).

substantially lower than in the overlying ectoderm. The section through Hensen's node (Fig. 5C) reveals that at this more posterior level all tissues, including the mesoderm, exhibit considerable induction of Ghox 2.9 protein. This is in reference to an untreated embryo (Fig. 5D) where the degree of antibody staining is lower than in the treated embryo. Finally, at axial levels posterior to the node where both mesoderm and ectoderm normally express Ghox 2.9, retinoic acid also causes superinduction in both germ layers (data not shown).

To examine Ghox 2.9 expression in later stages, embryos treated with RA were allowed to develop until Hamburger-Hamilton stage 7. On the whole, such embryos developed with a normal timetable, i.e. kinetics of notochord elongation, of node regression and of somite formation were the same as in control specimens. Ectoderm and mesoderm of treated embryos are slightly thinner, but key landmarks appear in their normal location and display normal spacing relative to each other. Nevertheless, the pattern of Ghox 2.9 expression is still markedly different from that of untreated embryos (compare Fig. 4C and D). As at stage 5, Hensen's node and midline structures of treated embryos express high levels of Ghox 2.9 protein (Figs 4D, 5E,F). In the normal embryo, Ghox 2.9 expression terminates a short distance anterior to the first somite (Fig. 4C). In contrast, Fig. 4D makes it very clear that in a treated embryo the Ghox 2.9 domain extends far anterior to somite 1. In this domain, the ectopic Ghox 2.9 signal is concentrated almost entirely within the neuroectoderm, with only modest expression within the mesoderm (Fig. 5F). At the position of the first somite and more posteriorly, high levels persist in both germ layers (Fig. 5G and control, Fig. 5H).

When embryos were grown to stage 11 and stained with Ghox 2.9 antibody, there is a band of particularly intense Ghox 2.9 expression (Fig. 4F) in the anterior portion of the hindbrain-like region (see also Fig. 3C, D). This band is sometimes marked by rhombomeric constrictions, is considerably longer than in the control (Fig. 4E) and is connected to neural crest cells

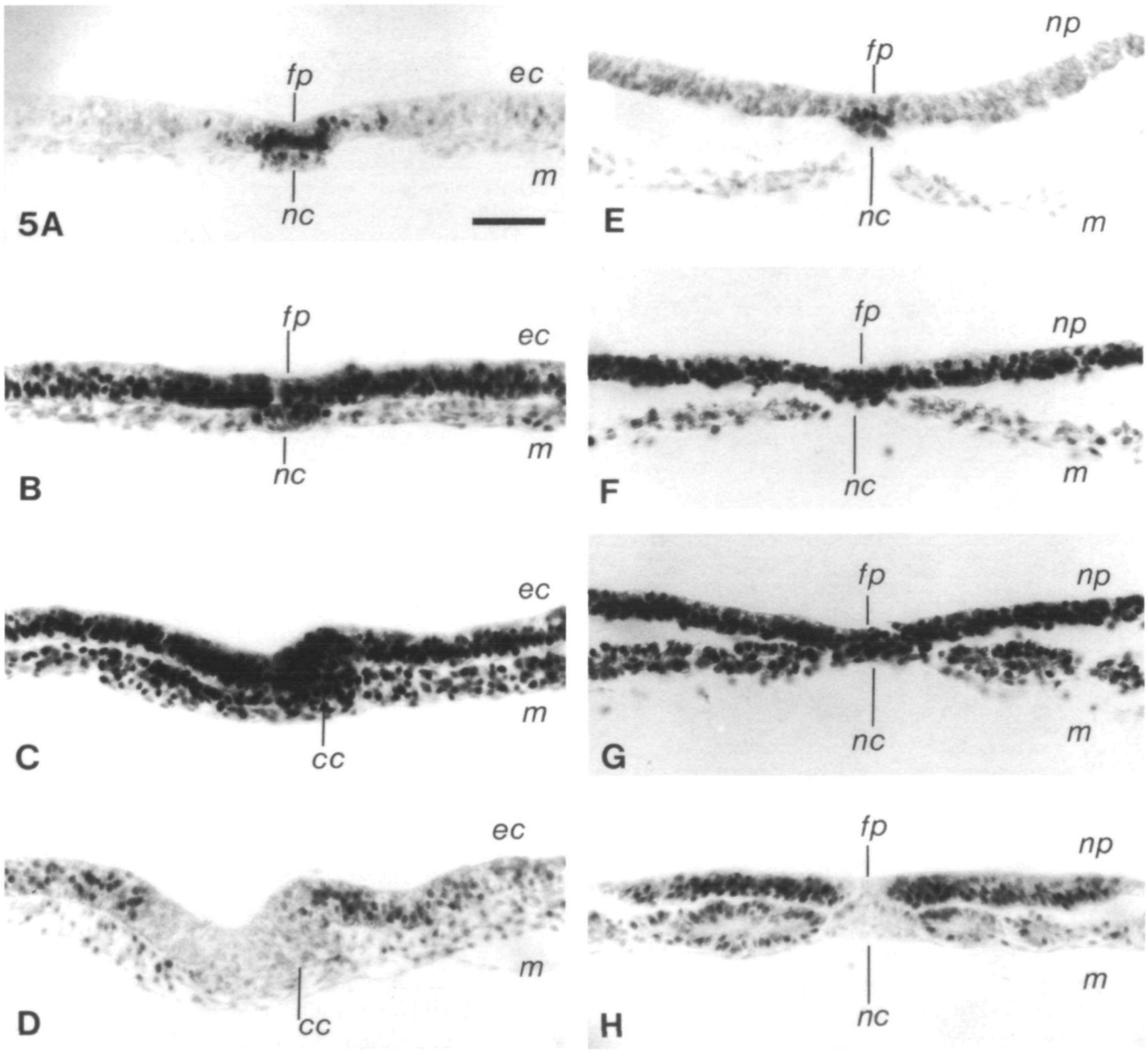


Fig. 5. Tissue-specific induction of Ghox 2.9 by retinoic acid. Embryos corresponding to those in Fig. 4A through D were sectioned transversely (10 μm), and sections were photographed in bright field. Dorsal is up. (A, B, C) Retinoic-acid-treated, fixed at stage 5. (A) Anterior section, near pre-chordal plate. (B) Section anterior to Hensen's node. (C) Section through Hensen's node. Chorda center is asymmetric, mesodermal component shifted to the right. (D) Control embryo cultured without retinoic acid, section passes through the node. Chorda center mesoderm shifted to the right. (E, F, G) Retinoic acid treated, fixed at late stage 7. (E) Anterior section, near pre-chordal plate. Note: stain in the lateral neural plate is largely background, with some weakly positive nuclei. (F) Section midway between first somite and prechordal plate. (G) Section at level of first somite. (H) Control embryo, cultured without retinoic acid, fixed at late stage 7, and sectioned at level of first somite. cc, chorda center; ec, ectoderm; fp, floor plate; m, lateral plate mesoderm; nc, notochord; np, neural plate. Bar, 50 μm (for entire figure).

characteristic of rhombomere 4. On the basis of this evidence, we suggest that this band corresponds to an enlarged rhombomere 4. We have not yet examined other hindbrain markers, and it remains to be seen whether more posterior rhombomeres are similarly enlarged.

Discussion

A broad body of data strongly suggests a regulatory role for homeobox-containing genes at all stages of vertebrate morphogenesis (reviewed in Davidson, 1991; Hunt and Krumlauf, 1991). These data are drawn from

the analysis of expression patterns, ectopic expression studies and gene disruption experiments. In this study, we have examined the spatial distribution of Ghox 2.9 protein (formerly Ghox-lab) during early chick development. Furthermore, we have manipulated the expression of Ghox 2.9 by exposing mid-gastrula embryos to retinoic acid. This resulted in ectopic expression of Ghox 2.9 in more anterior ectoderm of the late gastrula, followed by dysmorphogenesis of midbrain and hindbrain.

Ghox 2.9 is one of several vertebrate homeobox-containing genes known to be expressed in the gastrula (for other examples see Rosa, 1989; Ruiz i Altaba and Melton, 1989; Blumberg et al., 1991; Frumkin et al., 1991). Its protein is first detected in the primitive streak at the mid gastrula stage (Fig. 1A). During late gastrulation, it is expressed at high levels in a shield-like domain (Fig. 1B). In such late gastrula embryos, we have observed a region of refractile, thickened mesoderm (Fig. 2A,B) that coincides with the zone of Ghox 2.9 expression. Therefore, the expression domain of Ghox 2.9 in the gastrula stage embryo is not a prepattern in the strict sense, but is a molecular manifestation of a subtle structural feature.

Relationship between the Ghox 2.9-expression domain and fate maps of the early chick embryo

A key issue in understanding the role of genes involved in establishing the body plan is to determine how their domains of expression relate to the fate map of the organism. In analogy to *Drosophila*, where homeotic selector genes specify segment identity (Akam, 1989; Lawrence, 1988) it is possible that expression of a gene like *Ghox 2.9* is tightly correlated to a specific cell fate. However, the situation in the late chick gastrula is apparently different. Recent cell lineage studies of Schoenwolf and Sheard (1990) suggest that precursor cells of the forebrain, midbrain, hindbrain and spinal cord are not yet fully segregated into separate territories within the embryo. The midbrain, for example, is derived from ectodermal cells that are widely scattered along the anteroposterior axis of the late gastrula embryo. When ectodermal cells are dye-labelled in an entirely Ghox 2.9-positive region located lateral and slightly posterior to the node, progeny of these cells can later be found in the midbrain (see Fig. 2C, left side). Since the midbrain consists entirely of Ghox 2.9-negative cells (Sundin and Eichele, 1990), some cells that initially express Ghox 2.9 must later turn off the gene. It therefore seems that the activation of *Ghox 2.9* in the late gastrula ectoderm is neither irreversible nor is it a rigid determinant of an individual cell's fate.

The anterior boundary of the Ghox 2.9 expression domain eventually coincides with the anterior delineation of the fourth rhombomere (Sundin and Eichele, 1990). On further development of the hindbrain, *Ghox 2.9* is strongly upregulated in only this rhombomere (Sundin and Eichele, 1990). Moreover, all cells in the neurectoderm of rhombomere 4 express Ghox 2.9. On the basis of this pattern of expression, which is conserved between chick and mouse, it has been suggested that Ghox 2.9 determines the position of rhombomere 4, and later its segmental identity (Murphy et al., 1989; Wilkinson et al., 1989; Sundin and Eichele, 1990; Frohman et al., 1990; Murphy and Hill, 1991). In contrast to Ghox 2.9 expression at early stages, the rhombomere-4-specific expression domain exists within a very different cellular context. It is known that at this later stage neurectodermal cells are clonally restricted to individual rhombomeres (Fraser et al., 1990).

Ghox 2.9 expression pattern and retinoic acid

We were able to change the pattern of expression of Ghox 2.9 by treatment of stage-4 chick embryos with a pulse of retinoic acid. In such treated embryos, the anterior boundary of expression of Ghox 2.9 shifts forward by several hundred μm (compare Fig. 4C,D). Eventually, treated embryos exhibit striking morphological changes in the central nervous system (Fig. 3). One of the main features observed is that neurectoderm displaying the hallmarks of rhombomere 4 (i.e. high levels of Ghox 2.9 protein and a characteristic neural crest) is larger and is located more anteriad (Fig. 4F). In contrast to the neurectoderm, key mesodermal landmarks such as the refractile mesoderm of the stage-5 embryo and the first somite of the stage-7 embryo are not repositioned as a result of treatment with retinoic acid (see Fig. 4A-D). This would suggest that positional values in the mesoderm are not altered by retinoic acid, or that their alteration would require treatment at a stage earlier than the definitive streak. It should be noted that while retinoic acid induces Ghox 2.9 expression in both anterior mesoderm and ectoderm, induction in the ectoderm is substantially higher (see Fig. 5B,F).

Studies of retinoic acid treated *Xenopus* and mouse embryos have also revealed an anterior shift of axial markers in the central nervous system. Sive and Cheng (1991) examined the expression of two labial type genes, *Xhox.lab.1* and *Xhox.lab.2* by means of dissection and RNAse protection assay. They found that retinoic acid caused rapid induction of these genes in anterior tissues. Similar studies with *XlHbox-6* were reported by Sharpe (1991). Unlike the labial type genes, this is normally turned on later in development, and in a more caudal region of the embryo. In this case retinoic acid also causes an anterior extension of its domain of expression, with a higher level of induction within the neuroectoderm. Recently, Ruiz i Altaba and Jessell (1991b) have shown that when *Xenopus* neurulae are treated with retinoic acid, neurons containing the homeodomain protein Xhox3 or the neurotransmitter serotonin are found at more anterior locations within the central nervous system. This anterior shift, however, is not due to the loss or diminution of anterior regions, but represents a change in the differentiation of these cell types. For example, retinoic acid treatment was shown to induce neurons bearing these markers to appear in the forebrain, where neither cell type is normally found. In our own studies, however, ectopic expression of Ghox 2.9 is not seen

within structures anterior to rhombomere 4 such as the forebrain and midbrain. Results similar to these have also been obtained in mouse, using Hox 2.9 and Krox 20 mRNAs as positional markers for the hindbrain (Morriss-Kay et al., 1991).

This report and a series of studies in *Xenopus* (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Sive and Cheng, 1991) and mice (Morriss-Kay et al., 1991) demonstrate a profound effect of exogenously provided retinoic acid on the pattern of the central nervous system. This raises the question of whether endogenous retinoic acid or a related retinoid has a physiological role in regulating central nervous system patterning or whether applied retinoic acid mimics other completely unrelated endogenous signals. It is now well established that retinoic acid acts through specific retinoic acid receptors (RARs) that are ligand-regulated transcription factors (Petkovich et al., 1987; Giguère et al., 1987). Vertebrates contain three RAR genes encoding three distinct receptor types known as α-RAR, β-RAR and γ-RAR. Recent in situ hybridization studies in mouse and chick show that β-RAR is expressed in the region of the primitive streak (Ruberte et al., 1991; Smith and Eichele, 1991). Moreover, in both species, the anterior border of β-RAR expression is located in front of Hensen's node. Thus, there is overlap between the expression domain of β-RAR and that of Ghox 2.9. One apparent problem is that β-RAR is not present in the region where Ghox 2.9 will be ectopically induced. However, there is good evidence that exogenously applied retinoic acid can also induce β-RAR (Noji et al., 1991). This induction is a direct response since the β-RAR gene contains a retinoic acid response element in its promoter region (de Thé et al., 1990; Sucov et al., 1990).

It is also very likely that gastrula-stage embryos contain retinoic acid. One piece of evidence comes from the demonstration that *Xenopus* embryos apparently contain several endogenous retinoids (Durston et al., 1989). In addition, Rossant et al. (1991) using a gene reporter system (see below) have provided good evidence for the presence of retinoic acid in primitive-streak-stage and older mouse embryos. Lastly, work in our laboratory indicates the presence of enzymes able to synthesise retinoic acid from precursor compounds (R. Janocha, O.S. and G.E., unpublished data). In sum, ligand and receptor, two essential components of the retinoic acid signal transduction system, are present in primitive-streak-stage embryos.

Assuming that retinoic acid operates as an endogenous signalling molecule, what is its mechanism of action? One possible scenario is that retinoic acid or a related retinoid is differentially distributed within these embryos, perhaps secreted by Hensen's node or by the distinctive region of refractile mesoderm. Rapid degradation of the retinoid could insure that it functions as a short-range signal acting upon *Ghox 2.9* and perhaps other *labial* genes (Sive and Chen, 1991) in the nearby tissue. A second possible scenario is that endogenous retinoids function by synchronizing gene expression during development, along the lines of a classical hormone. In this scheme, retinoic acid is essentially absent until the beginning of streak regression, at which time its levels increase throughout the embryo. Obviously, in this case, other factors would control the shape of the Ghox 2.9 expression domain, and retinoic acid would act as a permissive agent.

A recent study (Rossant et al., 1991) suggests that retinoic acid may provide both spatial and temporal cues in the early embryo. These experiments sought to detect endogenous retinoic acid by making transgenic mice carrying a *lacZ* construct driven by retinoic acid response elements. In the primitive-streak mouse embryo, corresponding roughly to stage 4 of chick, the *lacZ* reporter was not active. By the mouse head-fold stage, corresponding to chick stage 5, the reporter was turned on within all tissue layers in a posterior zone of the embryo region which probably corresponds to the domain of Ghox 2.9 expression. These results raise the possibility that endogenous retinoic acid or a related molecule regulates both the onset of Ghox 2.9 expression and its spatial distribution. Retinoic acid is therefore a candidate for a factor that normally regulates the expression of *Ghox 2.9* and possibly other homeobox genes in the early vertebrate embryo.

We wish to thank Beat Lutz and Shigeru Kuratani for reading the manuscript and for many helpful suggestions. This work was funded by the American Cancer Society, with additional support from Hoffmann-LaRoche and the McKnight Foundation.

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(*Accepted 20 December 1991*)