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Expression Patterns of *Engrailed*-Like Proteins in the Chick Embryo

CHARLES A. GARDNER AND KATE F. BARALD
Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, Michigan 48109

ABSTRACT The protein products of both of the identified chick *engrailed*-like (*En*) genes, *chick En-1* and *chick En-2*, are localized in cells of the developing brain, mandibular arch, spinal cord, dermatome, and ventral limb bud ectoderm, as demonstrated by labeling with the polyclonal antiserum α*Enhb-1* developed by Davis et al. (Development 111:281–298, 1991). A subpopulation of cephalic neural crest cells is also *En*-protein-positive. The monoclonal antibody *4D9* recognizes the *chick En-2* gene product exclusively (Patel et al.: Cell 58:955–968, 1989; Davis et al., 1991) and colocalizes with *chick En-2* mRNA in the developing head region of the chick embryo as shown by in situ hybridization (Gardner et al.: J. Neurosci. Res. 21:426–437, 1988). In the present study we examine the pattern of α*Enhb-1* and *4D9* localization throughout the chick embryo from the first appearance of antibody (Ab)-positive cells at stage 8 (Hamburger and Hamilton: J. Morphol. 88:49–92, 1951) through stage 28 (1–5.5 days). We compare the localization patterns of the two Abs to each other, as well as to the localization of the monoclonal Ab, HNK-1, which recognizes many neural crest cells, using double- and triple-label fluorescence immunohistochemistry. Most *En* protein-positive cells in the path of neural crest cell migration are not HNK-1 positive. In detailed examination of α*Enhb-1* and *4D9* localization, we find previously undetected patterns of *En* protein localization in the prechordal plate, hindbrain, myotome, ventral body-wall mesoderm, and extraembryonic membranes. Based upon these observations we propose: 1) that *En* expression in the mesoderm may be induced through interaction with *En* expressing cells in the neuroectoderm; 2) that *En* expression in the head mesenchyme is associated with somitomere 4; and 3) that *En* expression may be involved in epithelial-mesenchymal cell transformations.
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Key words: Neural crest, Development, Homeobox

INTRODUCTION

All vertebrate *En* genes share sequence similarity with the *Drosophila* developmental gene, *engrailed* (Joyner et al., 1985; Darnell et al., 1986; Joyner and Martin, 1987; Fjose et al., 1988; Holland and Williams, 1990; Davis et al., 1991; Hemmati-Brivanlou et al., 1991). *En* proteins contain a homeodomain, a helix-loop-helix containing region thought to bind DNA. Because *En* proteins also localize to cell nuclei, they may act as transcription factors (for reviews of homeodomain function see Levine and Hoey, 1988; Scott et al., 1989). For these reasons and because of their highly conserved patterns of expression during fish, amphibian, avian, and mammalian development it is thought that *En* genes may play important roles during vertebrate embryogenesis (Davis et al., 1988, 1991; Gardner et al., 1988; Davidson et al., 1988; Davis and Joyner, 1988; Njolstad and Fjose, 1988; Patel et al., 1989; Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1991; Hatta et al., 1991; Gardner and Barald, 1991).

There are two *En* genes in the chick, *chick En-1* and *chick En-2* (Darnell et al., 1986; Davis et al., 1991). We have previously reported the localization of *chick En-2* mRNA by in situ hybridization (Gardner et al., 1988) and protein localization by antibody staining with the monoclonal antibody (MAb) *4D9* (Gardner et al., 1988, 1989). This antibody was developed by Coleman and Kornberg and its extensive species cross-reactivity was described by Patel et al. (1989). Those studies demonstrated that *chick En-2* expression is restricted to the head region; specifically, to neuroepithelial cells of the mesencephalic-metencephalic region of the developing brain, to cells in the path of neural crest migration, and to cells of the mandibular arch. No *chick En-2* (*4D9*) immunoreactivity was detected caudal to the metencephalon.

Because 4MAb *4D9* does not label the *En* protein product of the two mouse genes, Davis et al. (1991) have developed a polyclonal antiserum, α*Enhb-1*. They have also demonstrated that the antiserum recognizes the conserved homeodomain region of **both** *chick En* genes (Davis et al., 1991). The pattern of α*Enhb-1* localization, therefore, has to be inferred from the combined patterns of both *chick En-1* and *chick En-2* protein expression. Since *chick En-2* protein is restricted

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Address reprint requests/correspondence to Kate F. Barald, Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109.

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to the head region rostral to the myelencephalon, any α*Enhb-1* immunoreactivity caudal to this region reflects expression of only the *chick En-1* protein.

Chick En-1 protein localization has previously been observed in rhombomere 4 of the mouse hindbrain, in the spinal cord, in the dermatome of developing somites, and in the ventral ectoderm of the limb buds by Davis et al. (1991). This report represents a detailed comparative study of the two *chick-En* products' localization during the early development of the avian embryo. By performing double- and triple-label immunofluorescent antibody localization studies using *4D9*, α*Enhb-1,* and the MAb HNK-1, which recognizes many migratory neural crest cells in the chick embryo (Vincent et al., 1983; Vincent and Thiery, 1984; Tucker et al., 1984; Bronner-Fraser, 1986; Loring and Erickson, 1987; Noden, 1988), we show that a subpopulation of neural crest cells also expresses these proteins. Implications for the use of *En* probes as markers for cell segregation and migration are discussed.

Previous work has led to proposals that *chick En* genes play a role in neurogenesis and the regionalization of the chick cranial neuroepithelium (Gardner et al., 1988; Patel et al., 1989; Martinez and Alvarado-Mallart, 1990; Alvarado-Mallart et al., 1990; Martinez et al., 1991; Davis et al., 1991; Gardner and Barald, 1991). Based on our new observations we suggest that *chick En* protein may also be involved in inductive interactions between the germ layers, in the segmentation of the head mesenchyme, and in the behavior of cells within the somitic mesoderm.

RESULTS

Changing Patterns of α*Enhb-1* and *4D9* Localization in the Head Region (Figs. 1–8)

Both the MAb *4D9* and the polyclonal antiserum α*Enhb-1* localized to cells in the head region. In general, *4D9* and α*Enhb-1* immunolocalization in the developing head was roughly coextensive, though early stages differed slightly in the timing of appearance and extent of localization of the two Abs. As previously noted for *4D9* (Gardner et al., 1988) and for α*Enhb-1* (Davis et al., 1991), no immunoreactivity was detected by either Ab in embryos before stage 8 (4 somites).

Both Abs recognized cells of the dorsal ectoderm, neural crests, and neuroepithelium just prior to and during neural tube closure (stages 8–9, Fig. 1a–d) in the presumptive mes-metencephalic region. Development of *4D9* (*chick En-2*) immunoreactivity lagged behidn that of α*Enhb-1* (*chick En-1+2*) and was first seen only in the dorsal ectoderm and neural crests (Fig. 1c). Immunoreactive cells were detected by both Abs in the ectoderm overlying the mes-metencephalic region at all subsequent stages examined. From stage 10 both Ab types clearly recognized cells in the neuroepithelium of the mes-metencephalic region (Figs. 1e–i, 2–4). A gradient of immunoreactivity was seen in the mesencephalic vesicle, increasing caudally. This gradient was more obvious in *chick En-2* localization. Localization of *chick En-1+2* in the mesencephalon extended somewhat further rostral than that of *chick En-2* (Figs. 1i, 4c,d, 5e).

Localization of the two Abs along the ventral midline changed considerably with time (Figs. 1–3). During stages 8–9 neither Ab extended to the ventral midline. The border between *chick En-1+2*-reactive cells and cells of the ventral midline by stage 9 was quite sharp (Fig. 1a,c). By stage 10 *chick En-1+2* immunoreactivity did extend into the ventral midline of the metencephalon while the ventral midline of the mesencephalic vesicle did not contain *chick En-1+2*-positive cells. *Chick En-2*-positive cells were not seen in the ventral midline of the metencephalon until stage 11. Localization of *chick En-1+2* in the ventral midline of the mesencephalic vesicle developed in a caudorostral direction from stage 11 through stage 12 until by stage 13 intensely labeled cells extended rostral to the mesencephalic vesicle (Fig. 2). This ventral tongue of cells was seen at all subsequent developmental stages examined. Localization of *chick En-2* in the ventral midline of the mesencephalon became apparent only after stage 13.

Cells in the head mesenchyme were detected by both *En* protein-specific Abs. At stage 10 α*Enhb-1* (which recognizes *chick En-1+2*) localized to from 92 to 170 mesenchymal cells in each embryo, whereas in embryos incubated with *4D9* (*chick En-1*), from 22 to 40 cells were detected in this region. Beginning at stage 9, immunoreactive cells appeared dorsal to the mes-metencephalic region of the neural tube (Figs. 1c–i, 7). Roughly one fourth of all immunoreactive cells were observed lateral or ventrolateral to the neural tube by stage 10 (Fig. 1e,g). Thus, there appeared to be a mediolateral progression in the appearance of *En*-immunoreactive cells in the head mesenchyme at a time when cephalic neural crest cells are known to be migrating from the dorsal midline (di Virgilio et al., 1967; Tosney, 1982). The surface ectoderm was also quite heavily labeled by *4D9* (Fig. 1i). By stage 13 a strongly immunoreactive group of cells had collected in the mesenchyme dorsolateral to the mes-metencephalic border. Ab-positive cells were seen in this region in all subsequent stages examined (not shown). Also at stage 13, a more ventral collection of immunoreactive mesenchymal cells could be seen staining faintly near the developing mandibular arch. This group of cells was seen more easily in stage 14 embryos (Fig. 5a,b). In older embryos immunoreactive cells were distributed into a loosely associated mesenchymal cell mass just ventral to the trigeminal ganglion, continuous with a more densely associated cell mass extending into the center of the mandibular arch and surrounding the mandibular branch of the trigeminal nerve (Fig. 5c–h). The bilateral populations of immunoreactive cells sometimes appeared to be continuous across the midline in a faintly labeled bridge of cells. Cell nuclei were also detected by both Abs in the trigeminal ganglia of all embryos after stage 14 (Fig. 5c,e,f).

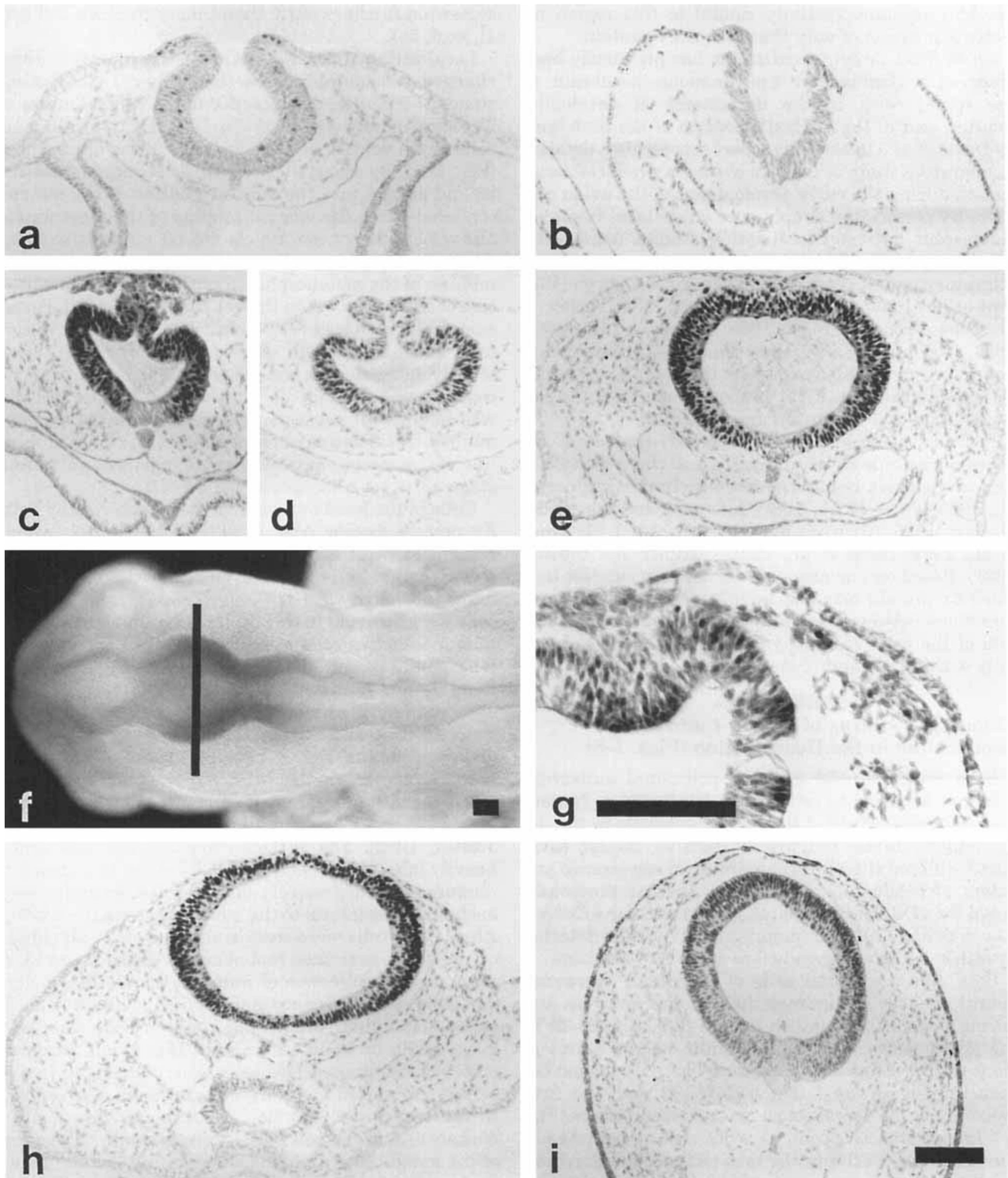

Fig. 1. Development of *chick En* immunoreactivity in mid-mesencephalic vesicle transverse sections from stage 8 through stage 13. Immunoreactive cells can be seen in the neuroepithelium, in the overlying ectoderm, and in the path of neural crest migration. **a,b:** Stage 8, 5 somites, *αEnhb-1* (*chick En-1+2*) localization and *4D9* (*chick En-2*) localization, respectively. **c,d:** Stage 9, 7 somites, *αEnhb-1* localization. **e:** Stage 10, 10 somites, *αEnhb-1* localization; immunoreactive cells can be detected lateral to the neural tube in the head mesenchyme. **f:** The approximate level of section is shown in a chick embryo stained in whole mount with *4D9*. **g:** Stage 10, 10 somites, *4D9* localization in frozen section; several immunoreactive cells can be seen lateral to the neural tube in the path of neural crest cell migration. **h,i:** Stage 13, 18 and 19 somites, *αEnhb-1* localization and *4D9* localization, respectively. Scale bar for a–e,h,i shown in i = 100 μm. Scale bar for f = 100 μm. Scale bar for g = 100 μm.

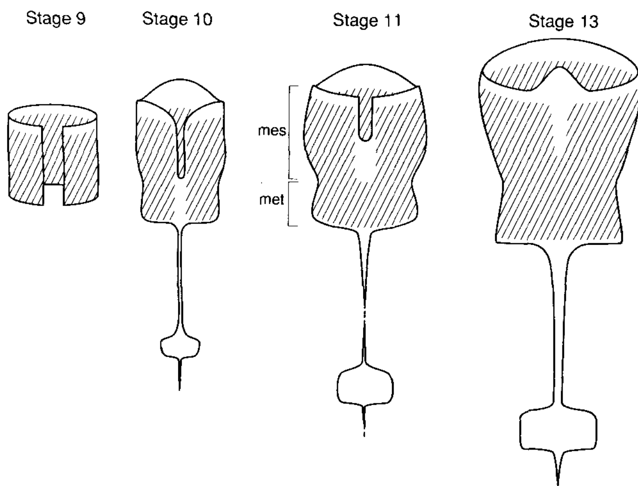

Fig. 2. Development of *chick En* immunoreactivity in the brain, seen from the ventral surface. Rostral is toward the top, caudal toward the bottom. Only the region of the neuroepithelium in which *chick En* protein was detected is shown. The entire outline represents localization of *αEnhb-1* (*chick En-1+2*). Cross-hatching represents localization of *4D9* (*chick En-2*). Immunoreactivity in the ventral midline of the mes-metencephalon developed from caudal to rostral. Localization of *4D9* lagged behind that of *αEnhb-1*. At stage 10, immunoreactivity appeared in the floor plate of the myelencephalon (the "tail" extending below the main region of immunoreactivity at stages 10, 11, and 13). An expansion of floor plate immunoreactivity was seen in rhombomere 4. mes, mesencephalon; met, metencephalon.

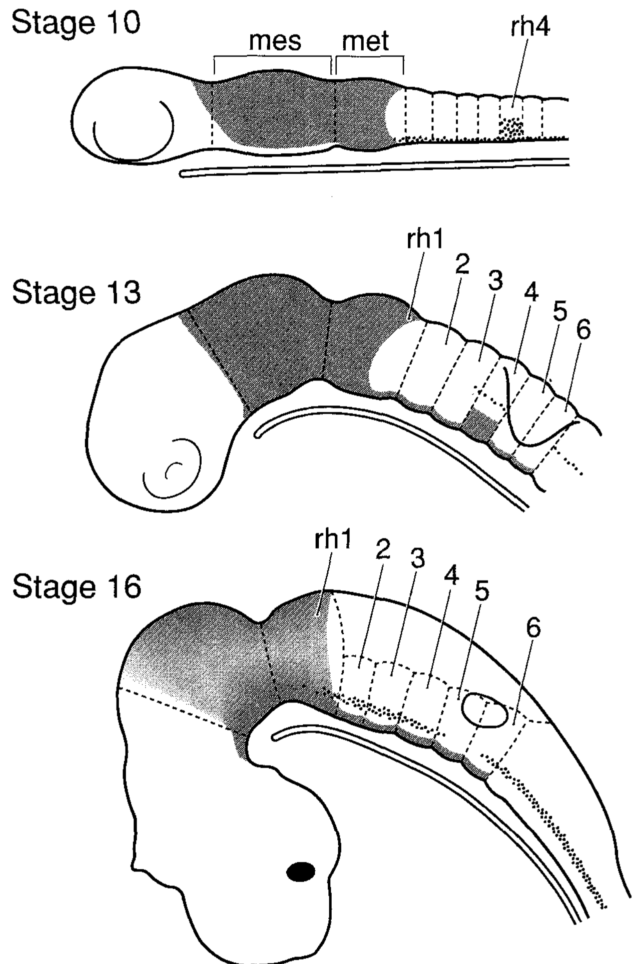

Fig. 3. Development of *chick En-1+2* immunoreactivity in the brain (stipple), side view of the neural tube with ectoderm and mesoderm removed. Rostral is to the left, caudal to the right. The otic pit and vesicle are shown at stages 13 and 16, respectively, at the level of rhombomeres 5 and 6. The stage 16 embryo is adapted from Vaage (1969). mes, mesencephalon; met, metencephalon, rh, rhombomere.

Beginning at stage 12, *En* protein-positive cells could be detected in the region of the prechordal plate and/or rostral notochord of some embryos. In embryos stained with *αEnhb-1* where the development of these cells was followed most closely, the number of immunoreactive cells in this region varied. In 9 of 34 embryos between stages 12 and 17 no stained cells were seen in this region. In each of 2 embryos, stages 13 and 14, respectively, more than 20 strongly immunoreactive cell nuclei were observed in the prechordal plate and anterior notochord. In most embryos 5–10 *En* protein-positive cell nuclei were seen (Fig. 6a,b). All embryos older than stage 17 contained a substantial number of immunoreactive cells in the prechordal plate (Fig. 6d). Although the prechordal plate cells are all presumably of the same embryonic origin, there may be a subpopulation of these cells that is labeled.

Colocalization of *αEnhb-1, 4D9,* and HNK-1 in the Head Mesenchyme (Fig. 7)

To determine whether *En* protein-positive cells in the path of crest migration were a part of the HNK-1-positive neural crest population, triple-label immunolocalization experiments were performed using *αEnhb-1, 4D9,* and HNK-1 Abs. Almost all *En* protein-positive cells seen in the head mesenchyme of embryos ranging from stage 9 (7 somites) to stage 12 (17 somites) were not HNK-1-positive (n = 10 embryos). A total of 83 sections were examined. More than 250 *αEnhb-1*-positive mesenchymal cells were identified. Approximately half of *αEnhb-1*-reactive cells were also *4D9*-reactive. However, after careful examination of these cells under both fluorescent illumination and Nomarski optics we determined that only 1 of these *En* protein-positive cells was labeled by both *αEnhb-1* and HNK-1 Abs. Thus, the majority of *En* protein-positive cells seen in the path of neural crest migration did not also present the epitope recognized by HNK-1, purportedly a marker for migrating neural crest cells (Vincent et al., 1983; Vincent and Thiery, 1984; Tucker et al., 1984; Bronner-Fraser, 1986; Loring and Erickson, 1987; Noden, 1988).

Localization of *αEnhb-1* in the Floor Plate of the Hindbrain (Figs. 2, 3, 8)

Neither *chick En-2* mRNA (Gardner et al., 1988) nor the *chick En-2*-specific Ab *4D9* (Gardner et al., 1988; Patel et al., 1989) have been detected caudal to the

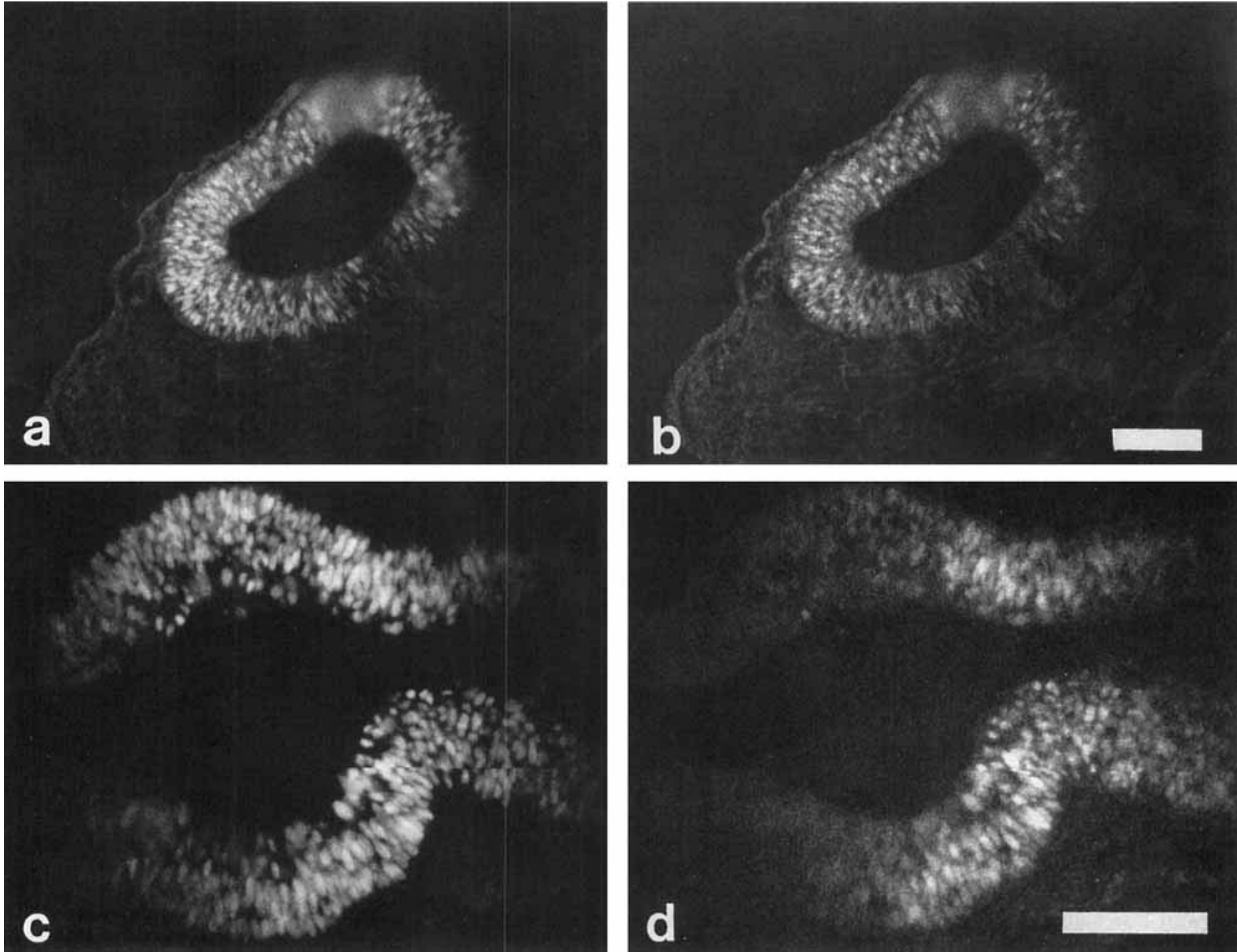

Fig. 4. Immunofluorescence localization of (**a,c**) *αEnhb-1* (*chick En-1+2*) and (**b,d**) *4D9* (*chick En-2*). Panels a and b are the same transverse section through the mid-mesencephalic vesicle, observed through different filters. Panels c and d are the same frontal section through the mes-metencephalic region; rostral is to the left, caudal to the right. The section shown in c and d cuts through a more ventral part of the neuroepithelial wall in the top of the image than in the bottom. Scale bars for a,b shown in b and for c,d shown in d = 100 μm.

midbrain-hindbrain region of the embryo. This allows the assignment of all more caudal *αEnhb-1* immunoreactivity to expression of the *chick En-1* protein. Localization of *chick En-1* immunoreactivity was first seen in the ventral midline or floor plate of the hindbrain in a stage 9^+ embryo (8–9 somites). As discussed above, at that time the more prominent and more rostral mes-metencephalic immunoreactivity did not yet reach the ventral midline. Scattered lightly stained nuclei were seen in the floor plate of the hindbrain of all older embryos examined (Figs. 2, 3, 8d). Beginning at stage 9^+ and in all older embryos until about stage 15, this ventral midline expression pattern was continuous with an expansion of *chick En-1* immunoreactivity into the lateral walls of rhombomere 4 (Fig. 8a–c). The dorsal-most of the immunoreactive nuclei in rhombomere 4 were located in the mantle zone, indicative of postmitotic cells. The caudal extent of *chick En-1* protein localization in the floor plate was variable beyond rhombomere 4, in no case reaching further than rhombomere 6. A cross-section from the hindbrain of an older embryo (stage 21) is shown in Figure 8d. Immunoreactive cells can be seen: in the floor plate, lateral to the floor plate on either side of the midline (see below), and, in rhombomere 4 only, in a still more lateral group of immunoreactive cells at the base of cranial nerves VII–VIII (see also Fig. 5f). Similar immunoreactive cells were not seen at the base of cranial nerves V or IX.

Ventrolateral Localization of *αEnhb-1* in the Hindbrain and Early Spinal Cord (Figs. 2, 8c,d, 9)

Figure 9 demonstrates the development of *chick En-1* protein localization in the ventrolateral walls (basal plate) of the spinal cord at the level of the 8th somite at

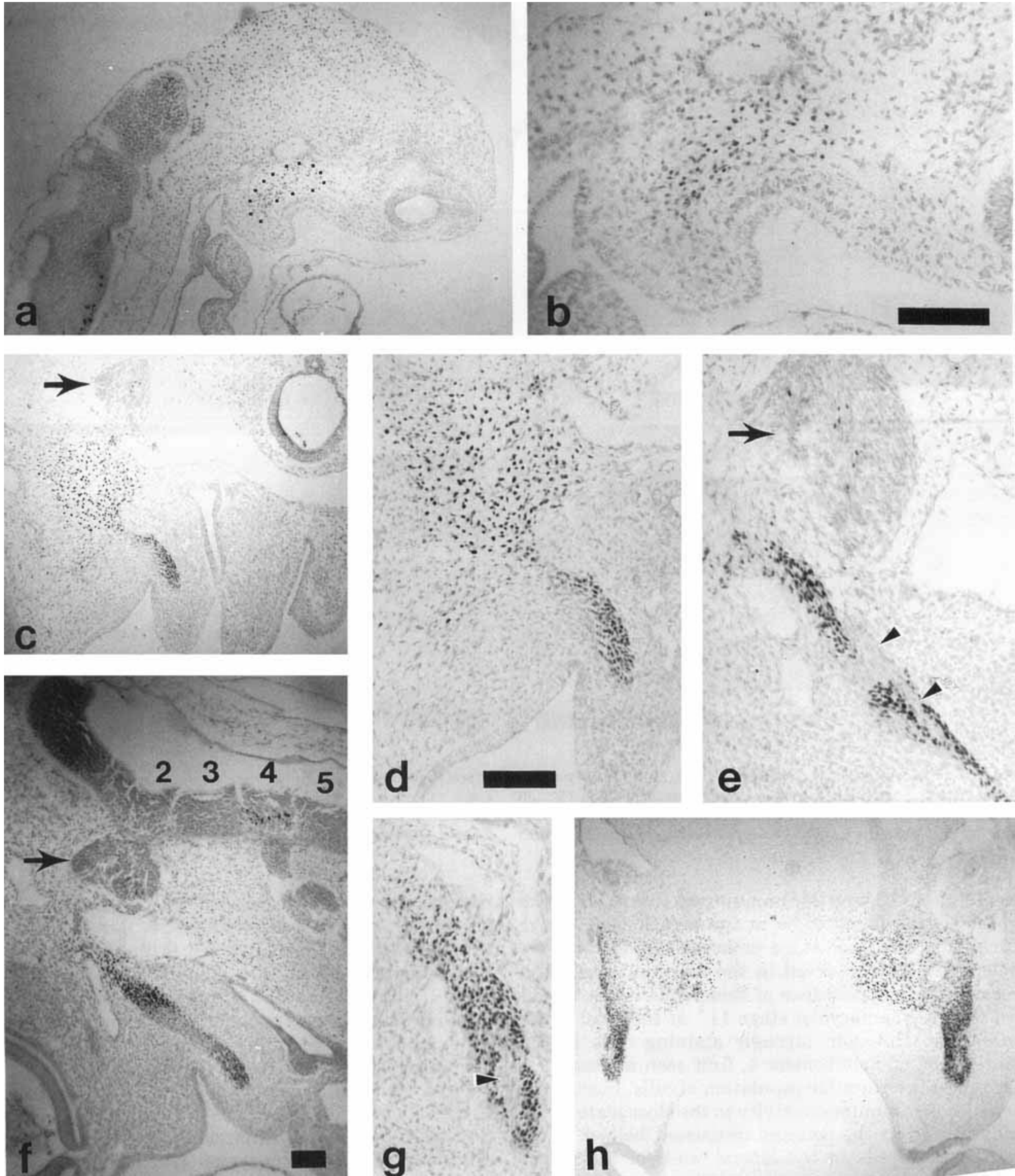

Fig. 5. Immunolocalization of *αEnhb-1* (*chick En-1+2*) in the head mesenchyme and trigeminal ganglion. **a,b:** Stage 14, 22 somites, parasagittal section (rostral to the right). **c,d:** Stage 18, parasagittal section (rostral to the left). **e–g:** Stage 20, parasagittal section (rostral to the left). **h:** Stage 20, coronal section cut perpendicular to the plane of immunoreactive cells which crosses diagonally in c–f. In a, faintly immunoreactive cells are outlined in the developing mandibular arch, shown at higher power in b. First, second, and third arches are visible in c; first arch shown at higher power in d. Arrows in c, e, and f indicate trigeminal ganglion in which a few immunoreactive cells can be seen. Arrowheads in e and g indicate manidbular branch of the trigeminal nerve, surrounded by immunoreactive cells of the mandibular muscle condensation. Numbers in f refer to rhombomeres. Scale bar for a,c,f,h shown in f = 100 μm. Scale bar for b = 100 μm. Scale bar for d,e,g shown in d = 100 μm.

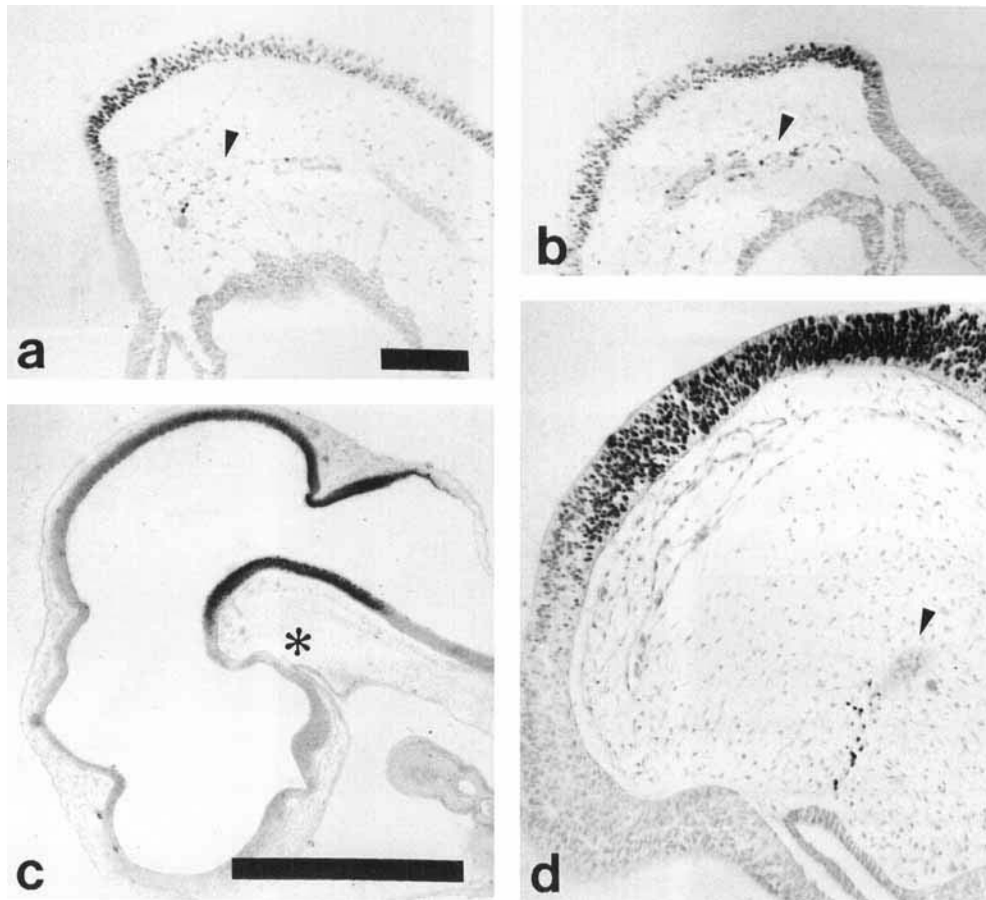

Fig. 6. Immunolocalization of *αEnhb-1* (*chick En-1+2*) in the prechordal plate and anterior notochord. **a:** Sagittal section, stage 15^+, 26 somites (rostral to the left). **b:** Sagittal section, stage 14, 22 somites (rostral to the right). The location of the prechordal plate near Rathke's pouch is shown in **c** by an asterisk (stage 18, mid-sagittal section). **d:** Stage 18, same magnification as in a and b. Arrowheads in a and b indicate prechordal plate. Arrowhead in d indicates rostral tip of the notochord. Scale bar in a,b,d shown in a = 100 μm. Scale bar in c = 1 mm.

stages 14, 15, 19, and 24. Immunoreactive nuclei were almost always found to be in the mantle zone of the neural tube. In later stage embryos immunoreactive nuclei were also observed in the marginal zone. The first definitive appearance of these cells in the spinal cord was in an embryo at stage 11^+ at the level of the 4th somite. However, strongly staining cells in the mantle zone of rhombomere 4, first seen at stage 9^+, may represent a similar population of cells. In contrast to *chick En-1* immunoreactivity in the floor plate of the hindbrain or in the somites (discussed below), *chick En-1*-positive cells in the lateral walls of the spinal cord were intensely immunoreactive from the time they were first observed.

By stage 13 (19 somites) a stripe of *chick En-1*-reactive cells extended from rhombomere 4 rostral through rhombomeres 3 and 2. Except for an occasional faintly stained nucleus in the floor plate of rhombomeres 5 and 6, the hindbrain adjacent to the otic vesicle contained no immunoreactive cells. In the caudal direction *chick En-1*-positive cells extended from rhombomere 7 to the level of the 10th somite. Two embryos, each with 23 somites (stage 14^+), each contained a single *chick En-1*-reactive cell in the lateral wall of rhombomere 5. From 23 to 28 somites (stage 14^+ to stage 16) the ventrolateral immunoreactivity in rhombomeres 5 and 6 was variable. Figure 2 shows the early appearance of Ab-positive cells in rhombomeres 5 and 6, at stage 16.

From 29 somites on (stage 17), the ventrolateral stripes of *chick En-1* protein localization in the spinal cord were continuous through this area. The stripes continued into the metencephalon as well, reaching as far as the mes-metencephalic border, clearly distinguishable from surrounding less intensely immunoreactive cells. By stage 17 a second, more ventral, population of cells made its first appearance (Fig. 9c,d). A lateral view at this stage presented the appearance of two stripes down the length of the neural tube: one thicker and more dorsal, the second ventral and composed of fewer cells. By stage 21 a more dorsal population of cells in the marginal zone was also observed. In

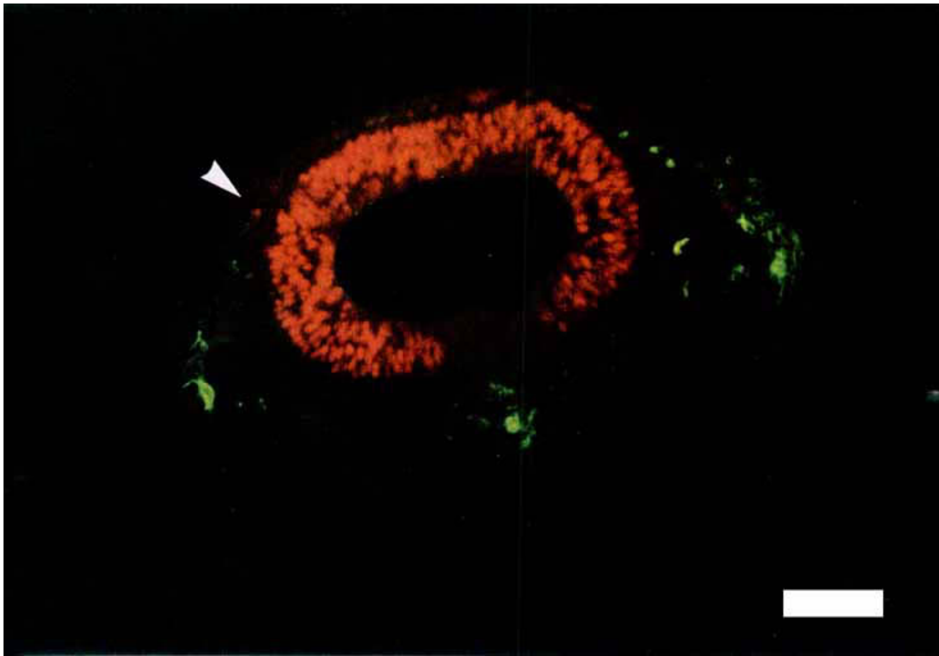

Fig. 7. Double-label immunofluorescence localization of *αEnhb-1* (red) and a marker for migrating neural crest cells, HNK-1 (green). Section is transverse through the mesencephalon of a stage 10 embryo, 9–10 somites. Neural crest cells (green) can be seen lateral to the neural tube. Arrowhead indicates the nucleus of an *αEnhb-1*-reactive cell in the path of neural crest migration. Neither this cell nor the two *αEnhb-1*-reactive cells on the other side of the neural tube are labeled by the cell surface marker HNK-1. Scale bar = 100 μm.

the oldest embryos examined (stage 28), *chick En-1* immunoreactivity did not extend into the secondary neural tube.

Camera lucida reconstruction of a stage 14 embryo (21 somites) showed no evidence of a periodic pattern among *chick En-1*-reactive cells along the rostrocaudal axis of the neural tube (not shown). In older embryos, from approximately stage 20, some if not all of the strongly *αEnhb-1*-reactive cells in the lateral walls of rhombomere 2 were also *4D9*-reactive. This represented the caudal-most extent of *chick En-2* localization.

Chick En-1 Protein in the Somites (Figs. 9, 10)

Localization of *chick En-1* protein in cells of the already formed somites was first seen at stage 13 (19 somites). Immunoreactivity quickly appeared in the first 4 or 5 somites and then developed more slowly in a rostrocaudal direction. As somites developed from the segmental plate the earliest *chick En-1* immunoreactivity observed (the most caudal immunoreactive somite in the embryo) was always seen at the rostral border of the somite (Fig. 9a). As the immunoreactivity increased it spread across the dermatome (Figs. 9b, 10a). The dorsal and ventral borders of the dermatome did not stain (Fig. 9b,d). However, cells of the rostral border of each somite continued to be Ab-positive (Fig. 10b). As somites matured, *chick En-1*-reactive cells appeared in the myotome (Fig. 10c–h). Older somites contained many deeply stained *chick En-1*-positive cells in the myotome, though spindle-shaped cells, probably early myocytes, stained faintly or not at all.

Rostrocaudal Development of *αEnhb-1* Localization in the Neural Tube and Somites

The appearance of *chick En-1* protein localization in both the neural tube and the somites was followed closely in serial sections from 36 embryos ranging in development from 14 to 39 somites. For each tissue the extent of *chick En-1* protein localization was noted as the caudal-most somite level reached by immunoreactivity in that tissue. The total number of somites in the embryo was also counted. Regression analysis revealed a linear relationship of the development of both neural tube and somite immunoreactivity to somite formation [$t(34) = 54.370$, $P < 0.0000$; $t(34) = 26.778$, $P < 0.0000$, for neural tube and somites, respectively]. When plotted against the number of somites in the embryo the slopes of the two lines were greater than 1.0 (1.2606 and 1.3446, respectively; not shown). The difference between the two slopes (0.08403 = 0.04783) was not statistically significant [$t(34) = 1.7570$, $P = 0.0879$]. Therefore, the two expression patterns progressed in a rostrocaudal direction in synchrony with one another, though both developed at a slightly faster pace than did the somites themselves.

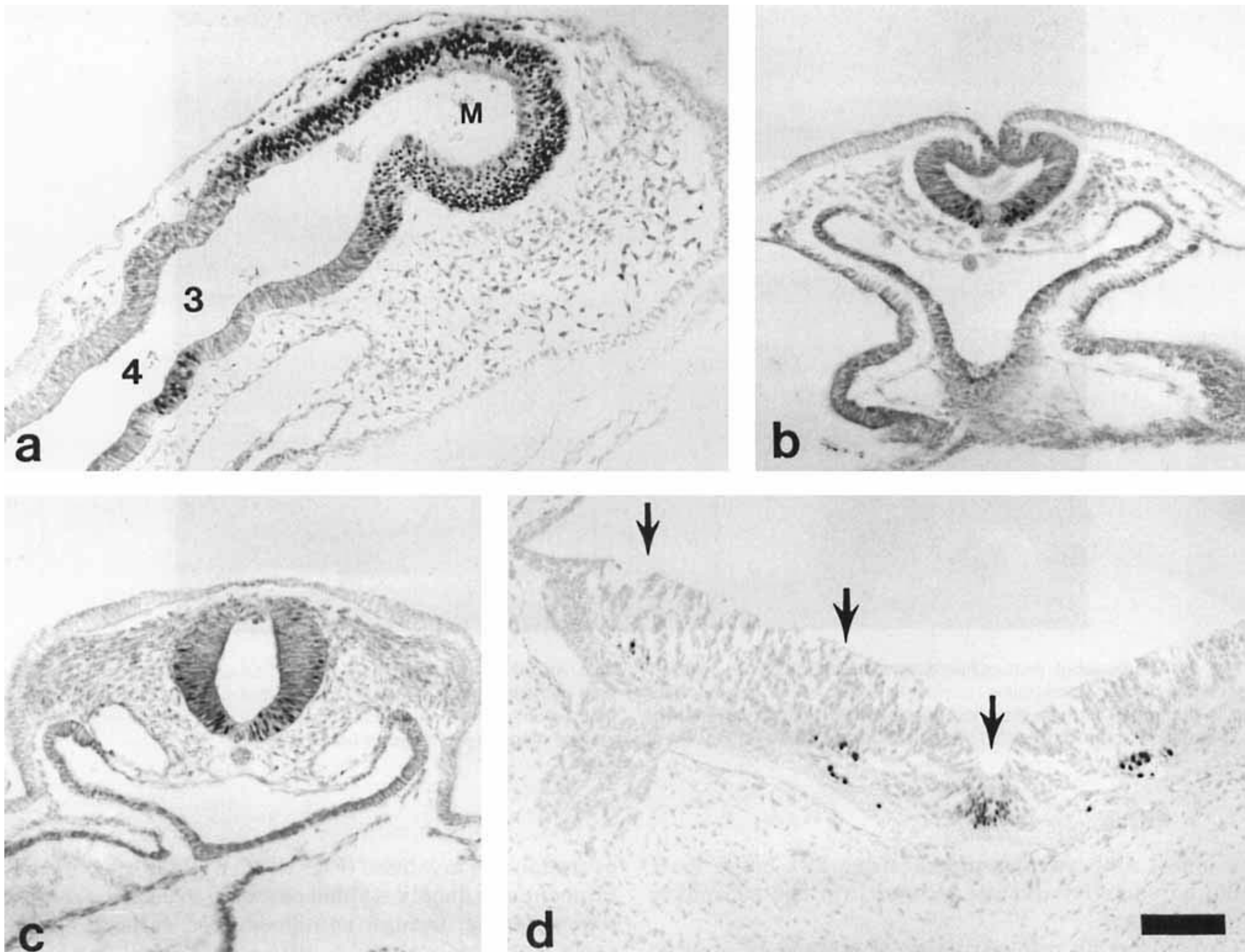

Fig. 8. Localization of *chick En-1* protein in rhombomere 4. **a:** Section is transverse/frontal through the neural tube of a stage 12 embryo (15 somites). **b,c:** Sections are transverse through rhombomere 4 at stage 9^+ (8–9 somites) and stage 12 (16 somites), respectively. **d:** Section is transverse through the hindbrain at the level of rhombomere 4 (stage 21, 43 somites). Arrows in d indicate, from left to right, *chick En-1* expression: 1) at the root of cranial nerve VII–VIII, 2) in a cell population that is continuous with a ventrolateral stripe of neural tube immunoreactivity that extends throughout the length of the neural tube, and 3) in the floor plate. M, mesencephalon. Numbers in a refer to rhombomeres. Scale bar = 100 μm.

Localization of α*Enhb-1* in the Surface Ectoderm (Figs. 1i, 11)

The earliest immunoreactivity was observed in the dorsal ectoderm of stage 8 embryos (Fig. 1i). As mentioned above, some cells in this region, overlying the mesencephalic and metencephalic vesicles, remained Ab-positive at all stages examined. Localization of *chick En-1* protein in the ventral ectoderm of the presumptive wing bud began faintly by stage 15. As the first swellings of the limb buds appeared each was strongly labeled on its ventral surface. The dorsoventral border of *chick En-1* protein localization was within the apical ectodermal ridge (Fig. 11a,b). By stage 16 ectodermal localization of *chick En-1* protein extended throughout the length of the ventral body wall (ventral half of the lateral body fold) from the wing buds to the leg and tail buds, as well as onto the amnion layer of the extraembryonic membranes (Fig. 11a,c). In addition to ectodermal immunolocalization in the wing bud, as Figure 11a,c demonstrate, cells of the ventral mesoderm (somatopleure of the lateral plate) at the level of the wing buds became *chick En-1*-positive at approximately stage 17. Ab-positive cells in the corresponding area of leg bud mesoderm were never detected.

DISCUSSION

We have used two Abs to compare the expression of chicken *engrailed*-like (*En*) proteins in the avian embryo: an *En*-specific MAb that recognizes the product of the *chick En-2* gene (Patel et al., 1989) and an *En*-specific polyclonal antiserum that recognizes the prod-

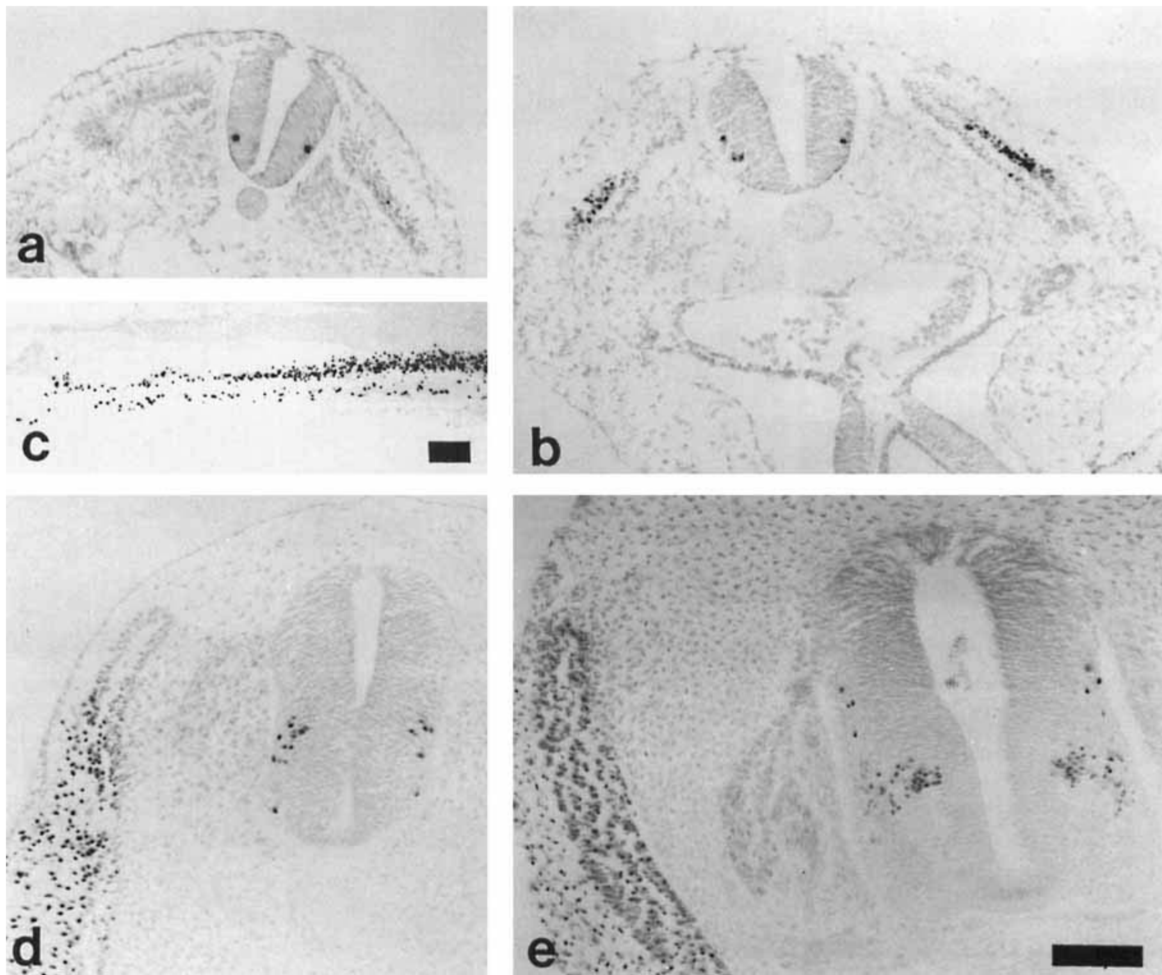

Fig. 9. Ventrolateral immunolocalization of *chick En-1* protein in the spinal cord. Transverse sections (**a,b,d,e**) are at the level of the 8th somite. Embryos are stage 14 (22 somites), stage 15 (26 somites), stage 19 (39 somites), and stage 24 (4 days incubation), respectively. Section in (**c**) is parasagittal through the wall of the neural tube in the cervical region of a stage 20 embryo; the section is tangential toward the left (rostral) but more through the center of the immunoreactive cells toward the right (caudal). Also visible in a is a single immunoreactive cell in the somite on the right. This section is through the rostral edge of the somite and no immunoreactivity could be seen in more caudal somites. Immunoreactivity can be seen in the dermatome of somites in b–e, as well as in the myotome (more ventromedial). Scale bars for a,b,d,e shown in e and for c = 100 μm.

ucts of both the *chick En-1* and *chick En-2* genes (Davis et al., 1991). Complex and dynamic patterns of *En* protein expression during embryogenesis are detailed here that have not been reported previously. These investigations highlight new features of *engrailed*-like (*En*) protein expression in the ventral midline of the midbrain, head mesenchyme, and mandibular arches, and also demonstrate *chick En-1* protein expression for the first time in the trigeminal ganglion, prechordal plate, floor plate of the hindbrain, myotome of developing somites, ventral body-wall mesoderm, and amnion. We have also detailed the timing of expression of *chick En-1* protein as it progresses in a rostrocaudal direction through the neural tube and somitic mesoderm.

These findings will aid in the comparison of *En* expression patterns among vertebrate species. Both *4D9* and *αEnhb-1* are increasingly used a markers for *En* protein in a wide range of vertebrate species and under a wide range of experimental conditions (Gardner et al., 1988, 1989; Patel et al., 1989; Kavka et al., 1989; Hemmati-Brivanlou and Harland, 1989; Martinez and Alvarado-Mallart, 1990; Hatta et al., 1990, 1991; Hemmati-Brivanlou et al., 1990; Davis et al., 1991; Joyner et al., 1991; Hemmati-Brivanlou et al., 1991; Martinez et al., 1991; Gardner and Barald, 1991).

The comparisons between localization of *chick En-1* and *chick En-2* protein products also suggest new aspects of *En* gene function, which can be tested experimentally in the quail/chick transplant system that has already been used successfully for such experiments (Kavka et al., 1989; Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991; Gardner and Barald, 1991).

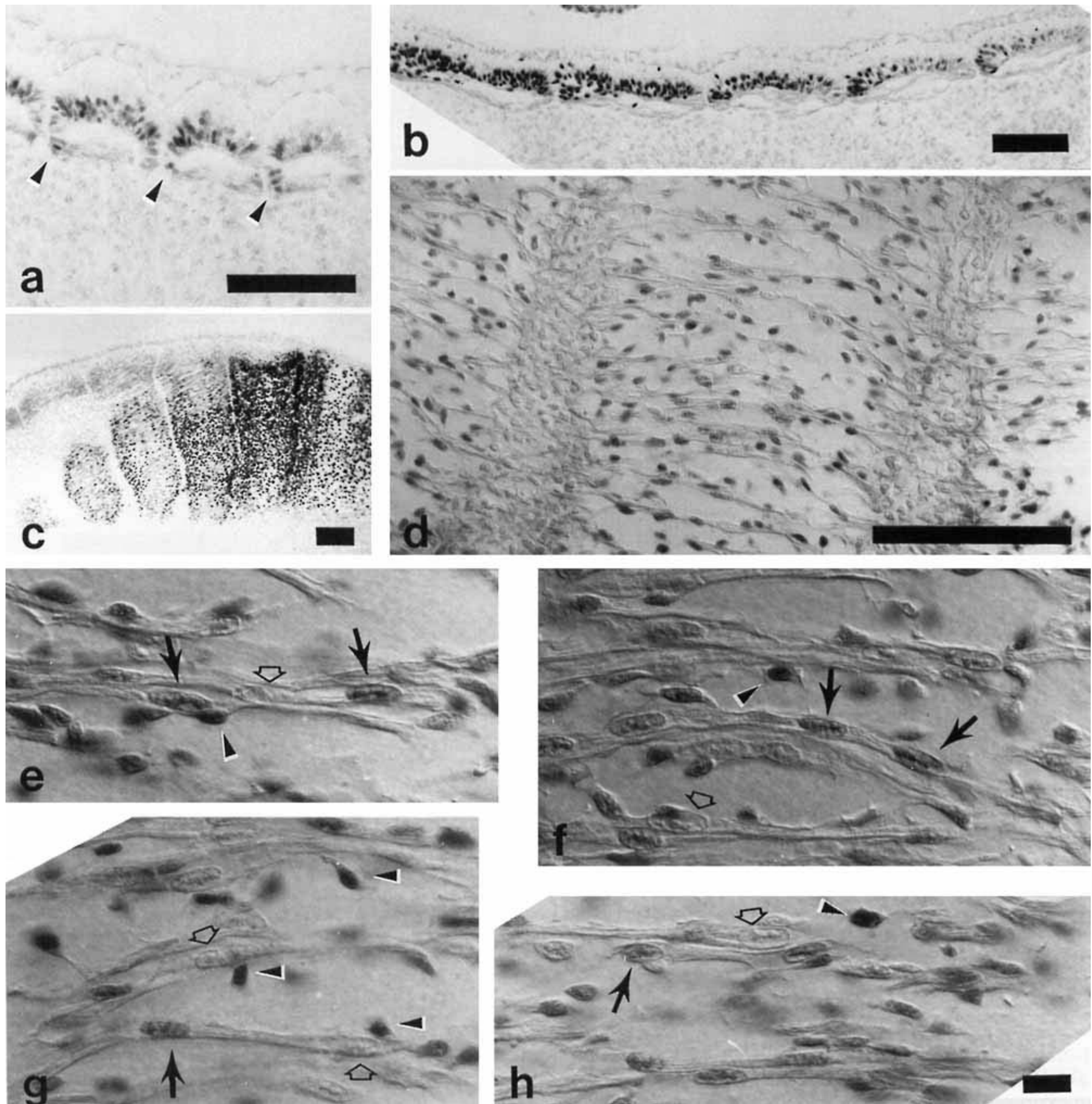

Fig. 10. Immunolocalization of *chick En-1* protein in the dermatome and myotome of the somites. In all sections, rostral is to the left, caudal is to the right. **a:** Frontal section demonstrates *chick En-1* expression in the dermomyotomes (top layer) of the 7th–9th somites of a stage 16 embryo (27 somites). Immunoreactivity is restricted to the rostral borders of the somites. Arrowheads in a indicate immunoreactive cells at the rostromedial border of the somites, the region from which myotome originates. **b:** A frontal section through the somite region (somites 13–17) of an older embryo (stage 20). Immunoreactive cells can clearly be seen in the myotome, as well as in the more prominent dermatome. Most of the *dermatome remains epithelial* in character. The appearance of greater immunoreactivity in the rostral part of each dermatome in b is due to the plane of section. **c,d:** These sections Stage 19, 2nd–7th somite and stage 28, cervical level, respectively, are cut parallel to the dermatome/myotome plate. Due to the plane of section in c, lateral immunoreactive dermatomal cells (now a loose mesenchyme) can be seen on the right while the more medial immunoreactive cells of the myotome are observable on the left. **e–h:** These sections are high power views of the myotome at stage 28. At least three cell types can be distinguished by the shape of the nucleus, cell body, and the level of *chick En-1* protein in the nucleus. Non-immunoreactive nuclei within spindle-shaped cell bodies are indicated by open arrows. These are probably myocytes. Faintly immunoreactive nuclei within spindle-shaped cell bodies are indicated by solid arrows, also probably myocytes. Intensely immunoreactive round nuclei within rounded small cell bodies (arrowheads) may be muscle precursor cells. Scale bars in a–d = 100 μm. Scale bar for e–h shown in h = 10 μm.

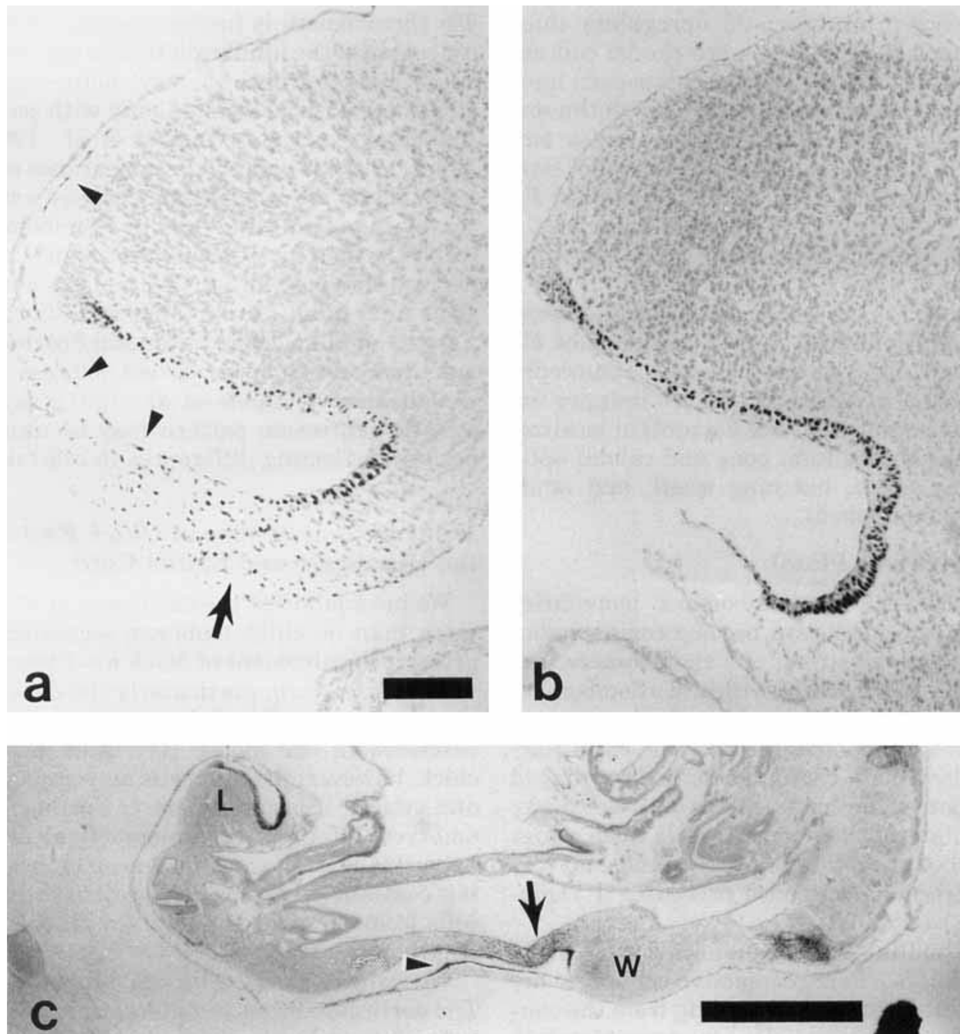

Fig. 11. Immunolocalization of *chick En-1* protein at stage 20 in the wing bud ventral ectoderm (**a**) and the leg bud ventral ectoderm (**b**). Immunoreactivity in the ventral body-wall mesoderm of the wing bud region is indicated by arrows in a and **c**. Cells of the ventral ectoderm of the body wall are continuous with the amnion, shown in a (arrowheads) and in c (arrowhead). L, leg bud; W, wing bud. Scale bar for a,b shown in a = 100 μm. Scale bar for c = 1 mm.

In using the experimental results described here to propose putative relationships and interactions among cell populations, our speculations are limited to what we believe to be, based on this evidence, the most likely, but certainly not the only, explanation of our results.

Chick En Protein in the Brain

In the head region, rostral to rhombomere 2, both Abs localized to the same cell populations. The only observable difference between the two Abs in the neuroepithelium was in the initial development of immunolocalization—*4D9* (*chick En-2*) lagged behind α*Enhb-1* (chick *En-1* and *-2*)—and in the rostral extent of early immunolocalization in the dorsal midbrain. In the mouse a similar pattern is observed: *En-1* mRNA is expressed before *En-2* mRNA (Joyner, personal communication). Comparison of the localization of the 2 Abs in the chick indicates that *chick En-1* protein is expressed in the dorsal mesencephalon over a slightly broader rostral extent than *chick En-2* protein. In the mouse, however, the opposite appears to be true. *En-2* mRNA is expressed over a larger proportion of the mesencephalon than *En-1* mRNA (Davidson et al., 1988; Davis and Joyner, 1988). This raises the possibility that the differences observed between α*Enhb-1* and *4D9* localization may be produced by a lower sensitivity in the detection of *4D9*. This possibility should be of concern to investigators using *4D9* alone in experimental manipulations of the chick embryo.

For example, Martinez and Alvarado-Mallart (1990) reversed the alar plate of the mesencephalic vesicle in quail/chick chimeric embryos (stages 12–14). They con-

cluded that the rostral mesencephalon, which was not *4D9*-reactive before grafting, could upregulate *chick En-2* protein when placed into a more caudal cell environment. Davis et al. (1991) and we (this report) have shown that α*Enhb-1* reacts strongly to cells in the rostral mesencephalic vesicle of stages 12–14 chick embryos (as well as in quail embryos; Gardner and Barald, 1991), indicating that at least one of the *chick En* genes was already expressed in the rostral mesencephalon **before** that region was grafted in the above experiments.

As with *En-1* and *-2* mRNA localization in the mouse (Davis et al., 1988; Davis and Joyner, 1988), *chick En* proteins continue to be expressed in mes-metencephalic derivatives even to adulthood. In preliminary experiments we have observed *chick En* protein localization in cells of the cerebellum, pons and caudal optic tectum of 15 day chick, hatching quail, and adult chicken brains (unpublished).

Ventral Midline (Floor Plate)

With the appearance of rhombomeric boundaries (stage 9), cells of the hindbrain become compartmentalized. They cannot cross from one rhombomere into another. Cells can move freely within a rhombomere and, interestingly, can also cross from the basal plate into the floor plate. But once in the floor plate they remain there. Floor plate cells that have been tagged give rise to elongate clones restricted to the floor plate. Unlike the more lateral rhombomeric cells, such clones do not respect rhombomeric boundaries and may extend over a rostrocaudal distance exceeding 1 rhombomere (Fraser et al., 1990).

In the ventral midline or floor plate of the brain the first scattered α*Enhb-1* immunoreactive cells were detected simultaneously in a line spanning from the caudal border of the metencephalon to approximately rhombomere 6 (stage 9^+). After initiation of *chick En-1* protein expression in floor plate cells of the hindbrain at stage 9^+, therefore, continued immunoreactivity in the floor plate may be due to the movement of *chick En* expressing cells into the floor plate from the lateral walls of the metencephalon and of rhombomere 4 (Fraser et al., 1990). Indeed, we observed the lowest number of immunoreactive floor plate cells midway between the metencephalon and rhombomere 4 in younger embryos.

Expression of *chick En-1* protein in the ventral and lateral walls of rhombomere 4 beginning at stage 9^+ may well be related to hindbrain segmentation. In addition to compartment boundary restrictions in the hindbrain (Fraser et al., 1990; Guthrie and Lumsden, 1991), each compartment, or rhombomere, exhibits a characteristic pattern of gene expression and early cell differentiation (reviewed by Wilkinson, 1989; Wilkinson et al., 1989; Lumsden, 1990; Wilkinson and Krumlauf, 1990; Keynes and Lumsden, 1990). In both mouse and chick embryos, for instance, *Hox-2.9* mRNA is expressed specifically in rhombomere 4 (Murphy et al., 1989; Wilkinson et al., 1989; Maden et al., 1991). The 4th rhombomere is further distinguished as the earliest region of the hindbrain to develop post-mitotic cells, since the appearance of acetylcholinesterase (AChE) at stages 10–11 is associated only with post-mitotic cells (Layer et al., 1988; Weikert et al., 1990; Layer and Alber, 1990). However, the appearance of AChE is seen a few hours after *chick En-1* protein expression is observed in post-mitotic cells in rhombomere 4, as indicated by their position in the mantle zone. AChE is expressed in post-mitotic neuroblasts about 15 hr **after** their final mitotic cycle (Miki and Mizoguti, 1982; Mizoguti and Miki, 1985; Layer and Sporns, 1987). Mouse and *Xenopus* embryos do not express *En* protein in rhombomere 4 (Davis et al., 1991), so this segment-specific expression pattern may be unique to avians, perhaps reflecting differences in cell fate.

Ventrolateral Stripes of *Chick En-1* Protein in the Hindbrain and Spinal Cord

We have followed the development of this pattern in more than 60 chick embryos, sectioned serially. Progressive development of *chick En-1* protein expression in the spinal cord, particularly the delayed expression in rhombomeres 5 and 6, was nearly identical to that described in the mouse (Davis et al., 1991). In the chick, however, the first cells may appear rostral to the otic vesicle, in rhombomere 4. Further, in older chick embryos, intensely immunoreactive cells extended as far rostrally as the mes-metencephalic boundary, forming continuous stripes of *chick En* protein expressing cells from the rostral hindbrain all the way down the length of the embryo.

We believe these cells are probably interneurons. The earliest neurons to differentiate within the neural tube are reticulospinal (intersegmental) neurons (Windle and Austin, 1936; Lumsden and Keynes, 1989; Weikert et al., 1990). These cells project axons from the ventrolatral wall of the hindbrain at stages 11–12, approximately 10 hr after AChE activity is observed in their cell bodies (Weikert et al., 1990). In the spinal cord the first axonal projections arise later, also from intersegmental neurons (for recent reports see Holley, 1982; Holley and Silver, 1987; Oppenheim et al., 1988; Schlosser and Tosney, 1988; Yagunima et al., 1990). In the spinal cord of zebrafish embryos *En* protein is expressed in interneurons (Hatta et al., 1991).

Until approximately stage 16 the ventrolateral stripes of *chick En-1* protein expression are broken by a gap in the hindbrain between rhombomeres 4 and 7. This pattern appears to reflect the known timing of cell differentiation in the rhombomeres (Layer et al., 1988; Lumsden and Keynes, 1989; Weikert et al., 1990; Layer and Alber, 1990). Layer and Alber (1990) have postulated that rhombomeric differentiation emanates both rostrally and caudally from the otic vesicle, while in the vicinity of the otic vesicle (rhombomeres 5 and 6), development is delayed.

Expression of *chick En-1* protein in the spinal cord reflects the overall rostrocaudal gradient of growth and differentiation of the embryo. The appearance of immunoreactivity in the spinal cord proceeds at a slightly more rapid pace than the rate at which new somites are formed, possibly reflecting a slowing of the rate of new somite formation in the caudal part of the embryo.

Expression of *Chick En* Protein in the Neural Crest

Labels for *chick En* protein may be useful markers for some neural crest cell migrations, since *chick En* protein-positive cells were clearly seen in the neural crest before neural tube closure and later, in the path of neural crest migration. However, we found that most *αEnhb-1*-reactive cells were not also labeled by HNK-1, a purported marker for migrating neural crest cells (Vincent et al., 1983; Vincent and Thiery, 1984; Tucker et al., 1984; Bronner-Fraser, 1986; Loring and Erickson, 1987; Noden, 1988). While other explanations are possible, we suggest that *chick En* protein is expressed in an HNK-1-negative neural crest subpopulation of substantial size (as many as 170 cells per embryo at stage 10). After these cells have reached their destinations (stages 13–14), paraxial mesoderm cells associated with the mandibular arch (which are not neural crest derivatives) could be influenced to express *chick En* protein by interaction with the neural crest subpopulation. Those neural crest cells might then contribute to the connective tissue of the first arch muscles.

In support of this explanation it is highly probable that not all cephalic neural crest cells are HNK-1-positive (D.M. Noden, D. Newgreen, P. Layer, personal communication). It has recently been shown that a subpopulation of metencephalic neural crest cells is not HNK-1-reactive at stages 10–11 (Layer, personal communication). In addition, by performing in situ hybridization in primary cell cultures we showed (Gardner et al., 1988) that a subpopulation of mesencephalic neural crest cells expresses *chick En-2* mRNA. Hemmati-Brivanlou et al. (1991) and Davis et al. (1991) have suggested that some *En* protein-positive cells in the head mesenchyme are neural crest-derived.

Furthermore, neural crest cells from the mesencephalic vesicle colonize the first arch where they give rise, among other things, to the connective tissue of the first arch muscles (Johnston, 1966; Noden, 1975, 1983a,b; reviewed by Le Douarin, 1983; Noden, 1988). Noden (1983a) transplanted mesencephalic neural crest cells caudally so that they migrated into the second rather than the first arch. There they seemed to instruct the second arch mesoderm to make first arch-like muscles. It would be interesting to determine whether *chick En* expression was induced in the second arch mesoderm of these embryos.

Alternatively, the HNK-1-negative, *chick En*-positive cells observed in the neural crest prior to neural tube closure are cells that contribute only to the neural tube and the dorsal ectoderm. After neural tube closure the ectoderm overlying the mes-metencephalic region was in fact *chick En* protein-positive at all stages examined. Most immunoreactive cells observed in the path of neural crest migration would then be mesodermal and contribute directly to the immunoreactivity associated with the mandibular arches. Development of the head mesenchyme involves a ventrolateral movement not only of neural crest cells but of paraxial mesoderm as well (Noden, 1984). Thus, lateral *chick En* protein expressing cells at stages 10–12 may be in a position to contribute to the mandibular muscle condensation by stages 13–14.

In support of this possibility, in zebrafish, Hatta et al. (1990) did not find *En* expression in neural crest cells. *En* expression was observed in the paraxial mesenchyme as mesoderm-derived muscle-precursor cells migrated away from the mes-metencephalic region of the neural tube toward and into the mandibular arch, where they condensed and differentiated into 2 functionally related jaw muscles which continued to express *En* protein even after differentiation.

Expression of *Chick En* Protein in the Head Mesenchyme

In the chick embryo the mandibular muscle condensation fills the center of the mandibular arch and gives rise to the jaw closing muscles (McClearn and Noden, 1988; reviewed by Noden, 1988). Meier (1979, 1981), Meier and Tam (1982), and Tam and Meier (1982) have argued that the early cephalic mesoderm is composed of 7 incompletely segmented cell populations which they termed "somitomeres." Noden (1983b, 1988) has shown that the jaw closing muscles of the chick derive from paraxial mesoderm at the rostrocaudal level of somitomere 4, adjacent to the mes-metencephalic region of the brain (Anderson and Meier, 1981). Thus, as in the zebrafish, mesodermal cells that later express *chick En* protein and contribute to the jaw muscles are originally situated adjacent to the region of *chick En* protein expression in the brain. Hatta et al. (1990) have suggested that neuroepithelial-mesenchymal interactions during the close approximation of these cell populations in the zebrafish may be involved in later neuromuscular target recognition between trigeminal axons and the jaw muscles. In this light, it is intriguing that we have observed *chick En* protein-positive cells in the trigeminal ganglion itself. Immunoreactive cells were also observed in rhombomere 2, from which motor axons of the trigeminal nerve take their origin.

The importance of somitomeres has been controversial (Wachtler and Jacob, 1986; Keynes and Stern, 1988). Molecular evidence will be required to determine a definitive role in development. Our observation that *chick En* protein expression may be associated with somitomere 4 raises the possibility that the Abs *4D9* and *αEnhb-1* could be used as markers to investigate the somitomeric organization of the head.

Expression of *Chick En* Protein in the Prechordal Plate

During gastrulation the prechordal plate precedes the notochord, ingressing from the anterior part of Hensen's node. At later stages it remains an irregular mass of cells extending from the anterior notochord to the foregut (Adelmann, 1922, 1926, 1927; Meier, 1981). In the mouse, *En-2* mRNA but not *En-1* mRNA is expressed near Rathke's pouch (Davis et al., 1988; Davis and Joyner, 1988). In the chick we have previously noted immunolocalization of *4D9* near the ventral midline, beneath the mes-metencephalic area (Gardner et al., 1988). We can now identify these cells as prechordal plate. Immunoreactive cells were never seen in the anlagen of the extrinsic eye muscles which are thought by some (Wachtler et al., 1984; Jacob et al., 1984; Wachtler and Jacob, 1986) but not by others (Johnston et al., 1979; Noden, 1982, 1983a,b) to arise from cells of the prechordal plate.

In *Xenopus,* the anterior notochord can induce *En* expression in overlying neuroectoderm (Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1990). However, notochordless chick embryos have a normal overall pattern of *chick En-2* protein expression (Darnell and Ordahl, personal communication). We have also found this to be the case (unpublished observations). It should be noted that in normal chick development *chick En* protein appears in the prechordal plate and anterior notochord some time **after** expression is seen in the mes-metencephalic neuroepithelium above.

Homeogenetic Induction of *En*

Hunt et al. (1991) have found that neural crest mesenchymal cells in branchial arches 2–4 express specific combinations of *Hox 2* genes that reflect their rhombomeric segmental origins. Following the migration of neural crest cells into the arches the overlying ectoderm of each arch (2–4) begins to express the same segment-specific combination of *Hox 2* genes found in the mesenchyme below it (*Hox 2* genes are not expressed in the first arch). These authors proposed that positional information is transferred from the mesenchyme to the overlying ectoderm. This cross-talk is a specific example of the classical principle of "homeogenetic induction" (see review by De Robertis et al., 1989), meaning in this case "like-begets-like" in the specification of similar positional values across germ layers.

We propose that homeogenetic induction of *chick En* expression occurs in the opposite direction, from the ectoderm (or neuroectoderm) to the mesoderm, both in the head and in the trunk. In the trunk, e.g., *chick En* expression in the neural tube may induce *chick En* expression in the nearby somites. The neural tube is known to influence the somitic mesoderm (Lipton and Jacobson, 1974; Bellairs et al., 1980; Teillet and Le Douarin, 1983; Stern and Bellairs, 1984). This hypothesis will be tested through microsurgical approaches which offer a means to explore subtle germ layer interactions that lead to the induction of *En* proteins (Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1990; Gardner and Barald, 1991).

Chick En Expression May Be Associated With Mesodermal Cell Migration or Epitheliomesenchymal Transformation

Later stage somites are composed of the ventral sclerotome and a dorsal wall made up of 2 layers (reviewed by Lash and Ostrovsky, 1986; Keynes and Stern, 1988). The outer dermatome layer remains epithelial. The inner myotome layer is composed of spindle-shaped cells (myocytes or early myotubes) arranged longitudinally. Cells in the midregion of the dermatome eventually loose their epithelial character. These cells "move away from" the epithelial dermatome and spread out as a loose mesenchyme just under the ectoderm, while rostral, caudal, medial, and lateral edges of the original dermatome plate remain epithelial. Early *chick En-1* protein expression in the dermatome precisely defines the mediolateral region of the epithelium that will later become mesenchymal.

Kaehn et al. (1988) have shown that myotome cells arise from the rostromedial border of the dermatome. Cells elongate and migrate caudally under the dermatome layer. With time, more lateral regions of the myotome are filled in. We have observed expression of *chick En-1* protein at the rostral border of the dermatome of early somites. Later, immunoreactive cells were seen in the myotome. Intensely immunoreactive cells were not spindle shaped, though some spindle-shaped cells did contain faintly stained nuclei. One explanation for these observations is that at least some myotome-forming cells express *chick En-1* protein. As these cells enter the myotome, some downregulate *chick En-1* expression and differentiate into myocytes while others continue to express *chick En-1* protein and do not immediately enter the path toward myocyte formation. This last cell population might contribute to connective tissue and/or muscle precursor cells. Evidence exists that the majority of non-spindle-shaped cells in the early myotome are in fact muscle precursor cells (Sassoon et al., 1988, 1989; Bober et al., 1991). In the differentiation of both dermatomal and myotomal cell populations *chick En-1* protein is associated with a loss of epithelial cell character and a movement of mesodermal cells.

Until now, *En* expression in the myotome has been described only in *Xenopus* (Davis et al., 1991) and zebrafish (Hatta et al., 1991) embryos. Our observation that *chick En-1* protein is also expressed in the myotome of chick embryos reinforces the concept that *En* expression patterns have been highly conserved during vertebrate evolution (see, e.g., Patel et al., 1989; Davis et al., 1991). Further support for our observation comes from Sassoon (personal communication), who has recently observed localization of *En-1* mRNA in the myotome of mouse embryos.

Chick En-1 Protein Expression in Limb Development

In the mouse, *En-1* mRNA accumulates in the limb buds (Joyner and Martin, 1987; Davidson et al., 1988; Davis and Joyner, 1988). Davis et al. (1991) have reported *En-1* protein localization specifically in the ventral ectoderm of mouse limb buds and the body wall beginning with the earliest visible limb bud swellings. Expression of *chick En-1* protein in the wing and leg buds was found to be similar. We have now further noted immunoreactivity in the ventral body-wall mesoderm of the wing bud region and in the amnion. Thus, *chick En-1* proteins provide useful markers for dorsal/ventral polarity in the limb.

EXPERIMENTAL PROCEDURES

Chickens

Fertile White Leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Dave's Eggs and Poultry (Ann Arbor, MI). All eggs were maintained in a force-draft incubator at 38–39°C. Embryos were staged according to Hamburger and Hamilton (1951) and either prepared for whole mount immunolocalization or frozen for cryosectioning.

Immunolocalization in Whole Chick Embryos

For α*Enhb-1* localization in whole mount, embryos were fixed and stained according to a modification of the method used by Davis et al. (1991). Embryos were fixed overnight in methanol:dimethylsulfoxide (DMSO) (4:1) at 4°C, followed by bleaching in methanol:DMSO:30% H_2O_2 (4:1:1) for 4–5 hr at room temperature. This destroyed endogenous peroxidase activity in the tissue. Embryos were then stored at −15°C for up to 6 weeks. For immunolocalization, embryos were hydrated and washed in phosphate buffered saline (PBS) containing 0.1% Triton X-100 and 2 mg/ml bovine serum albumin (BSA) (PBT). A part of the telencephalon, where immunoreactivity was never seen, was sometimes cut open for better penetration of Ab and reaction solutions. Embryos were incubated in α*Enhb-1* diluted either 1:50 or 1:75 in PBT on a rotator for 2–3 days at 4°C. The embryos were then washed at least 5 times for 1 hr each in PBT and incubated in peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), diluted 1:200 in PBT, for up to 2 days on a rotator at 4°C. Final washes were performed as above. After washing, embryos were incubated for 40–45 min in an incubation mixture containing diaminobenzidine (0.3 mg/ml), $CoCl_2$ (0.025% aq.), and $Ni(NH_4)_2(SO_4)_2$ (0.02% aq.). This incubation solution was then replaced with a thoroughly mixed fresh solution of the same composition but containing 0.003% H_2O_2 (reaction solution). The reaction was quenched after 2 min by passing the embryos through several washes of PBT.

For *4D9* localization in whole mount, embryos were fixed in 0.1m PIPES/2mm EDTA/1mm Magnesium sulfate/3.7% Formaldehyde (PEM-FA) (Patel et al., 1989) and stained according to a protocol developed by Patel (Patel et al., 1989). Partial embryo dissection, incubation times, washes, incubation, and reaction solutions were modified from Patel et al. (1989) as above. After whole mount immunolocalization with either α*Enhb-1* or *4D9*, embryos were embedded in TissuePrep 2 (Fisher Scientific #T555, Fair Lawn, NJ) and sectioned (7 μm). Serial sections were mounted directly on gelatin-subbed slides for observation of the diaminobenzidine-peroxidase reaction product. Some embryo sections were lightly counterstained with hematoxylin and eosin.

Immunolocalization in Tissue Sections

Embryos were examined in transverse, frontal, and sagittal sections (n = 204 embryos, stages 2–28). Half (102 embryos) were examined for α*Enhb-1* localization and half for *4D9* localization. To verify that patterns of Ab localization observed in whole mount were not due to uneven penetration of Abs, incubation, or reaction solutions, some embryos were sectioned, mounted, and labeled with Ab solutions directly on slides (for α*Enhb-1*, n = 10 embryos, stages 9–12, and n = 5 embryos, stages 22–23; for *4D9* n = 36 embryos, stages 9–12, and n = 7 embryos, stages 22–23). Embryos to be sectioned and incubated with α*Enhb-1* alone were first fixed as for α*Enhb-1* whole mounts. Embryos to be sectioned and incubated with *4D9* alone, or with any combination of *4D9*, α*Enhb-1*, and HNK-1 on the same section, were fixed as for *4D9* whole mounts. After fixation, embryos were rehydrated if necessary, washed with PBT, cryoprotected in PBT with 25% sucrose, and infiltrated with a 1:1 mixture of OCT (Tissue-Tek, Miles, Elkhardt, IN.) and PBT containing 25% sucrose. Frozen sections were cut in a cryostat (12 μm) and arranged, 2 per slide, on gelatin-subbed slides. Sections were stored at −90°C until needed.

For immunohistochemistry, sections were incubated in primary Abs at the same concentrations used for whole mount immunolocalization. In double- or triple-label experiments (n = 10 embryos), *4D9*, α*Enhb-1*, and/or HNK-1 (diluted 1:20, kindly supplied by Claudio D. Stern, Oxford University) were mixed into a single PBT solution. One section on each slide was incubated with the primary Ab solution (75 μl) while the other was incubated in PBT alone. During incubation and between washes, slides were kept in a sealed humidified box. Primary incubation was overnight. After gentle washing (3 × 1 hr each), both control and experimental sections were incubated in secondary Abs (75 μl, overnight).

In peroxidase labeling experiments, secondary Ab solutions were made as for whole mount immunolocalization. For fluorescent labeling the following secondary Abs were used (Jackson ImmunoResearch): for α*Enhb-1* localization, Texas Red conjugated goat anti-rabbit IgG with minimum cross-reactivity to human serum protein (#111-075-045); for *4D9* localization,

fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG with minimum cross-reactivity to human, bovine, and horse serum proteins (#115-095-062); and for HNK-1 localization, either 7-amino-4 methylcoumarin-3 acetic acid (AMCA-) or FITC-conjugated goat anti-mouse IgM with minimum cross-reactivity to human, bovine, and horse serum proteins (#115-155-020 or 115-095-020, respectively). In triple-label procedures, the two *chick En* protein products are nuclear, and the HNK-1 labeling is on the cell surface.

Control experiments showed that there was no detectable cross-reactivity between the anti-rabbit IgG and *4D9* (a mouse mAb) or between the anti-mouse IgG and α*Enhb-1* (a rabbit antiserum). Minor cross-reactivity was occasionally observed between the *4D9*/anti-mouse IgG and HNK-1/anti-mouse IgM combinations. Because *4D9* localizes to cell nuclei, whereas HNK-1 recognizes a cell surface epitope (Tucker et al., 1984; Kruse et al., 1984), this minor and variable cross-reactivity between the 2 anti-mouse secondary Abs did not affect our interpretation of data.

Control experiments were also performed to examine possible competition between α*Enhb-1* and *4D9* for *En* epitopes within the tissue. Cross-sections from a stage 11^+ embryo (14 somites) were labeled normally with 1 *En*-specific Ab and its secondary Ab, followed by labeling with the other *En*-specific Ab and its secondary Ab. Adjacent cross-sections were labeled in reverse order. A Leitz ORTHOMAT E photometer in integral metering mode was used to compare the relative fluorescences of the two secondary Abs under each of these experimental conditions. No difference in fluorescence could be detected whether sections were incubated first in α*Enhb-1* or *4D9*. Nor was any difference in brightness detected between these sections and those labeled by primary and secondary Ab mixtures.

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