Examining pattern formation in mouse, chicken and frog embryos with an
En-specific antiserum

CLAYTUS A. DAVIS1,2, DOUGLAS P. HOLMYARD3, KATHLEEN J. MILLEN1,2 and ALEXANDRA L. JOYNER1,2

1Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave.,
Toronto, Canada MSG 1X5
2Department of Medical Genetics, University of Toronto, Toronto, Canada
3Connective Tissue Research Group, Samuel Lunenfeld Research Institute, and Department of Pathology, Mount Sinai Hospital, 600
University Ave., Toronto, Canada MSG 1X5

Summary

We have raised an antiserum, designated aEnhb-1, to a portion of the mouse En-2 protein containing the
homeodomain. The antiserum detects both the En-1 and En-2 proteins in mouse, chick and Xenopus embryos by
Western blot analysis. Using whole-mount immunohistochemistry, combined in some cases with scanning
electron microscopy, we have examined the distribution of the proteins in the early embryos of these species. The
major features of expression were similar. The initial production of En protein occurred, just before or during
the formation of the first somites, in a band of the anterior neural plate in the prospective mid/hindbrain
region. Later in development En-1 protein accumulated in the ventral ectoderm of the developing mouse and
chick limb buds, indicating that a dorsal-ventral polarity is present as soon as any limb bud swelling is
apparent and that, at least in the mouse, this polarity is established independently of the apical ectodermal
ridge. In all three species, aEnhb-1 bound to a subset of ventro-lateral differentiating neurons in the spinal cord
and hindbrain and their pattern of birth in the mouse reflected the division of the hindbrain into rhombo-
meres. En-1 protein also accumulated in a lateral stripe of dermatome in the mouse and chick, indicating a
dorsal-ventral subdivision of this tissue. The results show that En expression is a good marker for pattern
formation in a variety of tissues and will be useful in experimental studies designed to characterize further
these processes.

Key words: immunohistochemistry, homeobox, limb, CNS, somites, development, scanning electron microscopy.

Introduction

The expression patterns of the vertebrate homeobox-containing genes have been used largely to determine in
which embryonic processes the genes may function. A second important, but under-exploited, application is to
use these genes to explore the nature of the developmental processes in which their expression is a feature.
For instance, analyses of the distribution of transcripts of several mouse homeobox- and zinc-finger-containing
genes have shown that they display rhombomere specific patterns (Wilkinson et al. 1989a; Wilkinson et
al. 1989b; Murphy et al. 1989). Together with studies of neurofilament expression (Lumsden and Keynes, 1989)
and lineage analysis (Fraser et al. 1990), they provide convincing evidence that the vertebrate hindbrain is
segmented. Gene expression may also provide a useful marker of tissue differentiation status following exper-
imental manipulation. By following the expression of XlHbox6 in isolated Xenopus ectoderm in response to
neural induction, it has been shown that the presumptive neural plate may be predisposed to induction
(Sharpe et al. 1987). Finally, gene expression may be used to study cell movement during development, as
alkaline phosphatase activity has been used to follow migrating germ cells (Chiquoine, 1954). Since the
vertebrate homeobox-containing genes show a rich variety of expression patterns in all three germ layers
they should provide novel developmental probes, particularly as good antisera become available.

Comparing the expression patterns of a gene in different species may provide insight into both the
evolution of the gene's function as well as the evolution of the developmental processes which involve its
expression. Patel et al. (1989) considered the evolution of En gene function by comparing En gene expression
in a wide range of metazoans and concluded that the ancestral gene was most likely involved in the develop-
ment of the nervous system. Oliver et al. (1988a) followed the distribution of the XlHbox1 protein in
**Xenopus** and its murine homolog in mouse embryos and suggested that there may be a rostral-caudal shift of mesoderm with respect to the CNS in the mouse that does not occur in **Xenopus**.

We and others have described the cloning and RNA distribution during development of two murine genes, **En-1** and **En-2**, which contain a homeobox and three other protein domains conserved in the *Drosophila* *enlarged* and *inverted* genes (Davidson et al. 1988; Joyner and Martin, 1987; Joyner et al. 1985; Davis et al. 1988; Davis and Joyner, 1988). Briefly, both **En-1** and **En-2** were found to be expressed in a band of the early neural plate around the mid/hindbrain junction and continued to be expressed in this region throughout development. **En-1** was also expressed in tissues derived from the dermatome and sclerotome portions of the somites, in two ventrolateral stripes along the spinal cord and hindbrain, and in the limb buds. While the patterns suggested roughly what processes the genes may function in, their use as developmental probes suffered from the laborious nature of the *in situ* procedure, as well as its low resolution.

In this paper, we report the production of a polyclonal antiserum, α**Enhb-1**, specific for the homeobox region of the **En-1** and **En-2** proteins in several vertebrates and describe its binding patterns during mouse, chick and **Xenopus** embryogenesis. The main objective of the study was to examine the embryonic processes featuring **En** expression and to compare these processes in the different vertebrate species. **En** protein localization was followed during four developmental events; the spatial subdivision of the central nervous system, neurogenesis in the hindbrain and spinal cord, the development of the somites, and the patterning of the limb buds. The patterns of **En** protein distribution indicated some common features of these processes in the different species, although there were a number of species-specific variations.

### Materials and methods

**Isolation of an En-2 homeodomain–bacterial TrpE fusion protein**

A *TrpE–En-2* fusion protein was made by established protocols (Sadowski and Pawson, 1987). A 590 bp Accl–BglII **En-2** cDNA restriction fragment encoding 117 amino acids from the carboxyl terminus of the **En-2** protein was cloned in frame with the *TrpE* sequences in a *path* bacterial expression vector. This produced a $5 \times 10^3 M_\text{r}$ fusion protein including the homeodomain and most of the sequences conserved among the *enlarged*–like genes. Following induction, bacterial cultures were lysed and heated in an SDS/Urea buffer. The fusion proteins were purified by preparative SDS–PAGE (Laemmli, 1970) and electroelution. The protein was then dialyzed extensively against an ammonium bicarbonate buffer and lyophilized.

**Generating an affinity-purified antiserum**

500 μg of the purified fusion protein was dissolved in water and injected as an emulsion with complete Freund’s adjuvant into the lymph nodes of rabbits. The rabbits were boosted with protein at monthly intervals until a good immune response resulted. The serum was affinity purified through three columns. First, the immunoglobulins were bound to protein A–sepharose. Eluted immunoglobulins were then passed through a column containing bound protein isolated from bacteria expressing *TrpE* to remove antibodies against *TrpE* and other bacterial proteins. Finally, the flowthrough was passed through a column containing bound fusion protein. Antibodies bound to this column were then eluted and dia lyzed against PBS. The antiserum was designated α**Enhb-1**.

**Western analysis**

Tissue samples from mouse embryonic forebrain, mid/hindbrain, spinal cord and limb buds; adult mouse forebrain and cerebellum; chick embryonic brain, hindbrain and spinal cord, and limb buds; and **Xenopus** mid/hindbrain were lysed by sonication in 10 volumes of a solution containing 10% glycerol, 5% beta-mercaptoethanol, 2.3% SDS and 62 nm Tris pH 6.8 and heated for 5 min at 95°C. The proteins were separated by SDS–PAGE and transferred to a nitrocellulose filter. Bound **En** proteins were detected by first incubating the filter with α**Enhb-1**, and then with an alkaline-phosphatase-conjugated anti-rabbit IgG secondary antibody, and finally with the enzyme substrates, 5-bis-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Whole-mount immunohistochemistry**

The immunohistochemistry protocol was a combination of two procedures (Dent et al. 1989; LeMotte et al. 1989). Embryos were fixed in methanol:DMSO (4:1) overnight at 4°C, bleached in fix including 5% H2O2 for 4–5 h at room temperature and stored in methanol at −20°C. They were then hydrated through a methanol series to PBS, washed twice in PBS containing 0.5% Triton X-100 and 2% skim milk powder (PBSMT), and incubated overnight at 4°C with α**Enhb-1** diluted 1:50 in PBSMT. The embryos were washed five times for one hour each in PBSMT and incubated overnight at 4°C with goat anti-rabbit IgG antiserum conjugated with horseshadish peroxidase (Jackson Immunoresearch), diluted 1:200 in PBSMT. They were then washed as before, rinsed in PBS with 0.5% Triton X-100 and 2% BSA (PBST), incubated for 30 min with 0.3 mg ml$^{-1}$ dianibomindine (and optionally 0.5% NiCl2 for enhanced signal) in PBST. H2O2 was then added to 0.03%, the reaction monitored and then stopped when a good staining intensity developed by rinsing in PBST and dehydrating through an ethanol series. If necessary, the embryos were cleared in benzyl alcohol:benzyl benzoate (1:2). If a higher resolution was needed then the stained embryos were embedded in paraaffin wax, sectioned at 8 μm, mounted on slides, dewaxed and counterstained with methyl green.

**Localization of the En proteins during scanning electron microscopy**

The **En** proteins were first localized in whole mouse embryos with open neural folds by the whole-mount immunohistochemistry procedure with the NiCl2 enhancement step included. The embryos were then dehydrated through an ethanol series and dried in a Ladd no. 2800 critical point dryer according to the manufacturer’s instructions. The embryos were then mounted on carbon stubs with epoxy cement and coated lightly with carbon in a Denton Vacuum Benochtop Turbo according to the manufacturer’s instructions. To visualize both the embryo and the immunohistochernical reaction product, an Hitachi S320 scanning electron microscope with a Link Systems AN10000 X-ray emission detector and element analysis system was used. The specimens were
oriented such that the surface of the neural plate was visible to both the viewer and the X-ray detector. Nickel deposited near the surface by the immunohistochemical procedure was detected by its characteristic Kα X-ray emission at 7.472 KeV caused by the scanning beam electrons. For each sample 512×512 pixel SEM and nickel maps were collected using a 20KeV electron beam with a point dwell time of 40μs and 25–30% detector dead time. The nickel emission map was then superimposed onto the SEM map of the surface.

Results

Specificity of the αEnh-1 antisem

A high-affinity antiserum to a TrpE fusion protein encoding the En-2 homeodomain was generated after inoculating and boosting rabbits 4 to 5 times (see Materials and methods). The serum was purified to yield antibodies recognizing only the En-2 peptide in the fusion protein and named αEnhb-1. By Western blot analysis the affinity-purified antiserum bound strongly to protein of the correct size in the bacterial lysates containing the fusion protein and only very weakly to bacterial lysates containing TrpE (data not shown).

The region of the En-2 protein against which αEnhb-1 was made is extremely well conserved in the En genes of all vertebrate species analyzed (Fjose et al. 1988; A. Brivanlou and R. M. Harland, personal communication; C. Logan, D. Nallainathan and A. Joyner, unpublished data). We examined the binding of the antiserum to proteins of different species by Western blot analysis and wholemount immunohistochemistry. To establish the identity of the proteins that bound the antibody, these results were compared with those of previous analyses of En expression by Northern blot, RNA in situ and whole-mount immunohistochemistry techniques. In the Western blot analysis, we observed consistent binding of αEnhb-1 to a set of bands in chick and mouse protein samples and weak but consistent binding to two bands in Xenopus. Weak bands of different sizes were occasionally apparent. These are probably due to either weak binding to other proteins or some degradation of the En proteins.

In the mouse, we observed binding of αEnhb-1 to a 41×10^3 Mr band in protein isolated from cerebellum, 12.5 day embryonic mid/hind brain, and embryonic stem cells overexpressing a mouse En-2 cDNA (Fig. 1). This band was absent from the embryonic and adult forebrain and embryonic spinal cord (Fig. 1) and limb buds (data not shown). We also observed binding to a 55×10^3 Mr band in protein isolated from embryonic mid/hindbrain, spinal cord and an embryonic stem cell line overexpressing the mouse En-1 gene (Fig. 1) as well as to limb buds (data not shown, M. Hanks and A. Joyner, unpublished data). This 55×10^3 Mr band was absent in the embryonic and adult forebrain and cerebellum and in embryonic stem cells (Fig. 1). The tissue localizations of the 41 and 55×10^3 Mr bands are entirely consistent with the distributions of En-2 and En-1 mRNAs respectively, as described in previous Northern blot (Joyner and Martin, 1987), and mRNA in situ analyses (Davis et al. 1988; Davidson et al. 1988; Davis and Joyner, 1988). We conclude that the 41×10^3 Mr and 55×10^3 Mr bands of the mouse En-2 and En-1 proteins, respectively. The sizes of the En-2 and En-1 proteins predicted from their coding sequences are 33.8 and 41×10^3 Mr, (M. Hanks, S. Noble-Topham, A. Joyner, unpublished data). The differences between the predicted and observed molecular weights may be due to post-translational modification and/or anomalous mobility during SDS-PAGE analysis.

In the chick, we observed binding of αEnhb-1 to bands at 44 and 39×10^3 Mr in embryonic protein extracts with the same distribution as the mouse proteins of 55 and 41×10^3 Mr, respectively (Fig. 1), except a trace of the smaller protein was detected in chick hindbrain and spinal cord, possibly due to the rhombomere 4 En expression (see below). A cDNA for a gene designated ChickEn, has been cloned (Gardner et al. 1988) and was shown to be the chicken En-2 gene by comparing its sequence with that of the mouse En genes (D. Darnell and C. Ordahl, personal communication). RNA in situ analysis has shown that this gene is expressed in the embryonic chick in the same region of the brain as the mouse En-2 gene (Gardner et al. 1988). The monoclonal antibody inv-4D9D4, which was raised against the Drosophila invaded homeodomain (Patel et al. 1989), also binds to only this embryonic region in whole-mount immunohistochemical analysis of chick embryos (Patel et al. 1989; Gardner et al. 1988; C. Davis, K. Millen and A. Joyner, unpublished data), suggesting that inv-4D9D4 recognizes only the chick En-2 protein. We have recently cloned two chicken
Accumulation of En proteins in head neuroepithelium

In all three species, En proteins accumulated in a similarly positioned band of the anterior neuroepithelium, although the developmental stage at which the proteins were first detected varied. In both the mouse and the frog, staining was first observed in the open neural plate. In the mouse, this occurred as the first somites were condensing (Fig. 2A) and in the frog, at stage 13 (Xenopus staging according to Nieuwkoop and Faber, 1967), prior to somite condensation (Fig. 2E). In both species, the morphology of the neuroepithelium at this time was largely undeveloped. In the chick, staining was not detected until stage HH8 (chick staging according to Hamburger and Hamilton, 1951), just before neural tube closure and during the condensation of somites three and four (Fig. 2C). By this time, the morphology of the neuroepithelium was considerably more developed than in Xenopus and mouse.

The first murine staining, although weak, occurred relatively uniformly in two patches on either side of the midline. The medial and lateral neuroepithelium did not stain (Figs 2A, B, 3A). The borders of the patches were somewhat diffuse and occasionally stained nuclei were observed in cells not adjacent to the patches. The earliest staining in the frog appeared similar (Fig. 2E).

By the time the first three to five somites condensed in the mouse embryo, every nucleus in the region stained intensely and, during this time interval, several hours at most, the area of staining increased almost threefold (Figs 2A, 4A). In the frog there was no observed increase in the size of the band, which remained considerably narrower than in the other two species (Figs 2F, 5E). As in the mouse, the midline and lateral edges did not initially stain (Fig. 3E). Following neural tube closure in the mouse and frog, the staining extended to include the ventral and dorsal midlines, such that the entire circumference of the neural tube at the mid/hindbrain junction was stained. In the chick, although the pattern was similar once the neural tube closed, intense staining first appeared dorsally in scattered cells of the neural folds, i.e. presumptive neural crest (Figs 2C, 3C), and then extended ventrally to include the entire neural tube. Staining unique to the chicken was observed between stages HH11 and HH14 in the lateral neuroepithelium of rhombomere four (Fig. 2D).

Since aEnhb-1 detects both the En-1 and En-2 proteins, the immunohistochemical results do not indicate whether the staining band in the brain neuroepithelium was due to one or both of the proteins. Western blot analysis of 20-somite embryos showed that both proteins are present (data not shown) and our previous mRNA in situ data in the mouse indicated that the two genes are expressed in the same band of cells at the 5- to 6-somite stage (Davis and Joyner, 1988). However, it is possible that there are slight temporal or spatial differences between the expression of the two En genes that were undetected, or that the distribution of the proteins do not match exactly that of their RNAs.

Localization of En proteins in the developing neuroepithelium

We followed the pattern of protein accumulation in the murine neural plate more closely than in the chick or Xenopus. Although the whole-mount immunohistochemistry precisely marked the cells containing the En proteins, it was not always possible to compare the pattern to the surface morphology by light microscopy. However, one marker which was readily apparent in the mouse from the earliest time that we observed En proteins was the pre-otic sulcus. It was clear that the early staining band lay just rostral to this (Fig. 2A) and that the rapid expansion of the band was due largely to the rostral extension of the anterior edge relative to the pre-otic sulcus (Figs 2A, 2C). By 9.0 and 10.0 days in the mouse, by which time the rhombomeres and the constriction at the cephalic flexure dividing the mesencephalon and metencephalon had developed, it was apparent that the staining straddled this constriction and included a large portion of the mesencephalon and most of the metencephalon (Fig. 5A), ending rostral to the boundary between rhombomeres one and two. In the chick, the staining appeared to cover a larger portion of the neural tube than in the mouse at this stage, from the pros/mesencephalon junction into the metencephalon (Figs 2D, 5C) and ending rostral to...
En proteins in mouse, chick and frog embryos 291

Fig. 2. Localization of the En proteins in the early neuroepithelium of mouse, chick, and Xenopus embryos by whole-mount immunohistochemistry using αEnhb-1. (A,B) Dorsal views of 8.0d mouse embryos at the time of condensation of (A) the first somites and (B) after the formation of 3 somite pairs. (pos) pre-otic sulcus. (C,D) Dorsal views of chick embryos at (C) stage HH8 (3–4 somites) and (D) stage HH11 (14 somites). Arrows indicate the junctions between the prosencephalon and mesencephalon and the mesencephalon and metencephalon. rh4 marks the fourth rhombomere. (E) Dorsal view of a stage 13 Xenopus embryo. Arrow marks the location of the En proteins within the neural plate. (F) Lateral view of a stage 14 Xenopus embryo. Scale bars represent 0.3 mm.

the boundary between rhombomeres one and two. In Xenopus, we did not position the first expression with respect to the neural plate morphology, but by the time the neural tube closed and the constriction dividing the mesencephalon and metencephalon developed, the staining occupied a narrow ring of the neural tube centered at the constriction (Fig. 5E).

To position the En expression in the mouse neural plate more accurately, we used a combination of wholemount immunohistochemistry and scanning electron microscopy. Three embryos whose neuroepithelial morphologies were representative of the 5, 7, and 9 somite stages as described by Jacobson and Tam (1982), and one embryo at 9.5 days of gestation were examined. The neural tube of the oldest embryo was opened at the dorsal midline to expose the inner surface. By the 5 somite stage, the En expression occupied most of the broad plate that extends from just rostral to the preotic
sulcus to the prosencephalon–mesencephalon junction. Some expression could also be seen extending rostrally across the prosencephalon–mesencephalon junction (Fig. 4A). By the 7 somite stage, the caudal limit of staining remained anterior to the pre-otic sulcus but the rostral limit shifted back into the mesencephalon (Fig. 4B). At the nine somite stage, the expression spanned mesencephalic neuromere 2 (Jacobson and Tam, 1982). Forming rhombomeres were also visible caudal to the region of expression (Fig. 4C). By 9.5 days of gestation, the constriction at the mesencephalon–metencephalon boundary had formed and the band of En expression clearly spanned it, with a rostral boundary in the mesencephalon. SEM analysis
of a 9.5 day embryo confirmed that the caudal border lay anterior to the rhombomere one–rhombomere two border (data not shown).

The preotic sulcus is transient and has been described in different reports as marking the later boundary between the metencephalon and myelencephalon (Meier and Tam, 1982), or between the mesencephalon and metencephalon (Jacobson and Tam, 1982), or within the metencephalon (Morriss-Kay and Tan, 1987). The first location is consistent with an unmoving band of *En* expression in the neuroepithelium. However, if either of the latter positions are correct then a caudal shift in the staining band must be occurring since following neural tube closure the band clearly spans the mesencephalon–metencephalon junction and covers much, if not all, of the metencephalon. A lineage analysis of cells in the developing neuroepithelium will be required to resolve this.

**En proteins in non-neuroepithelial cells of the head**

While the most prominent accumulation of *En* proteins in the early embryos was in the brain neuroepithelium, in the mouse and chick a small number of scattered staining cells, most likely neural crest, were observed at the same rostral–caudal level within the head mesenchyme. Although such cells were not previously identified by RNA in situ analysis in mouse, *En* mRNA and protein were previously observed in cultured chick neural crest cells (Gardner et al., 1988). In our analysis of chick embryos prior to neural crest migration, *En* protein was clearly present in scattered cells within the presumptive neural crest (Figs 2C, 3C). In whole-mount preparations after neural tube closure, the staining was clearest in the chick embryos, where a halo of dispersed labelled cells surrounded the *En*-expressing neuroepithelium (Fig. 2D). In the mouse embryo, staining was restricted to the neuroepithelium until the first 4 somites developed. At this time a small number of positive cells could be distinguished dispersed in the mesenchyme adjacent to the edge of the neural plate (Fig. 3B). By the 12 somite stage in the mouse, *En* expressing cells were detected in the mesenchyme forming a stream extending ventrally and caudally from the dorsal midline of the neural tube at the caudal edge of the *En*-expressing neuroepithelium (data not shown). The time and location of the appearance of these cells in the mouse embryo suggests that there may be a scattered subpopulation of neural crest cells arising from this level of the neural tube which contain one or both of the *En* proteins. Although it was not clear what the eventual fate of these cells was, by 9.5 days of development weak staining was observed in the core of the mandibular arch (Fig. 3D) and at 15.5 days staining was observed in the cells ventral to the pituitary (Fig. 3F). Staining was also observed in the core of the developing Xenopus mandible (data not shown). These tissues are all likely neural crest derived (Le Douarin, 1982; Sadaghiani and Theibaud, 1987). The staining around the developing mouse pituitary was likely due to *En-2* since we observed hybridization in situ to this region with an *En-2* mRNA-specific probe but not with an *En-1* probe (Davis and Joyner, 1988). It is intriguing that the later staining in the head mesenchyme was identified in only a few of the tissues which probably arise from midbrain/hindbrain neural crest.

**Ventral-lateral stripes of *En* protein in the spinal cord and hindbrain**

We followed the appearance of *En* proteins in the early mouse spinal cord and hindbrain by whole-mount immunohistochemistry. Although *αEnhb-1* detects both *En* proteins, our present Western blot and previous Northern blot data (Joyner and Martin, 1987) detected only *En-1* expression in the spinal cord. Furthermore, RNA in situ analysis of 12.5 day embryos indicated that *En-1* alone was expressed in two ventrolateral stripes running the length of much of the spinal cord and hindbrain (Davis and Joyner, 1988; Davidson et al. 1988). By 10.0 days of development the protein was distributed in the same pattern in nuclei outside of the germinal zone, extending from the junction of rhombomeres one and two in the hindbrain to the caudal spinal cord (Figs 5A, B, 6A). Although the final pattern was simple, its rostral–caudal development was not (shown schematically, Fig. 6B). Staining was first seen at approximately the 17 somite stage within the area bounded anteriorly by the otic vesicle and posteriorly by the first somite. The position was somewhat variable from one embryo to another and between the two sides of the neural tube in the same embryo. Staining nuclei then accumulated rostrally and caudally. The caudal expression progressed uniformly down the length of the spinal cord. However, rostral progression halted just behind the otic vesicle at the junction of rhombomeres six and seven. Positive nuclei then appeared in rhombomeres three and four. Finally, positive nuclei appeared in rhombomeres five and six, and the final anterior limit of expression at the junction between rhombomeres one and two was defined. In the 15.5 day spinal cord, these cells are widely spread in the ventral portion (Fig. 6E). We were unable to identify these cells positively on the basis of their position.
In the chick and Xenopus, αEnhb-1 marked cells in the same relative position in the spinal cord and hindbrain as in the mouse (Figs 5C–F, 6C), although we did not follow the generation of the pattern in these species in the same detail. For the reasons outlined previously, it is likely that this pattern is due to expression of the En-1 homologs. In the chick, expressing nuclei were seen in the spinal cord by the time 17 somites had formed and in approximately the same position as in the mouse. The final pattern appeared identical to the mouse (data not shown). In the frog, staining nuclei were seen by the time 5 somites had developed (stage 19) and were absent prior to somite formation. The first staining nuclei appeared at the level of the first and second somites, followed by a rostral-caudal extension, as observed in the mouse and chick. However, the rostral progression differed from the mouse both in its kinetics and in its final pattern. Staining nuclei accumulated rostrally to a point just caudal to the otic vesicle (Fig. 5F) and, after a pause, continued rostrally all the way to the band of En expressing cells at the mid/hindbrain junction (Figs 5E, 6C).

En protein in the somites
In the 9.5d embryonic mouse (19–20 somites) En protein started to accumulate in a stripe covering the lateral middle third of the dermatome portion of the somites (Figs 5A,B, 6A,D). The expression was at approximately the same dorsal-ventral level as the En expressing neurons in the spinal cord (Figs 5B, 6A,D). Staining started in the second somite just after the appearance of the first positive nuclei in the spinal cord and progressed caudally and rostrally to the first somite. In the chick, an identical somite pattern developed, starting by the time approximately 19 somites had formed (HH13). Based on our Western blot analysis and previous studies of mouse En-1 expression (Davis and Joyner, 1988; Davidson et al. 1988), it is likely that the staining in the somites in these two species was due to expression of the En-1 gene.

In Xenopus somites, αEnhb-1 staining was observed in the myotome (Fig. 5E,F), starting, as in the other species, just after the appearance of the first staining nuclei in the spinal cord and hindbrain, and extended caudally with time. The staining appeared as stripes due to the fact that the myotome is only one cell long and the nuclei lie at the center. The pattern was different and the staining much fainter than that observed in the mouse and chick somites. We cannot be sure from this analysis that the staining is due to an En protein since we were unable to confirm the presence of the protein by Western analysis and it has been reported that some rabbit polyclonal sera bind non-specifically to Xenopus somite nuclei (Wright et al. 1990).

En proteins in the mouse and chick limb buds
Binding of αEnhb-1 was seen in the developing mouse and chick limb buds, beginning with the earliest visible swellings, which is also likely due to expression of the En-1 gene. Xenopus embryos were not examined at the postembryonic stages when limb buds form. The first expression in the mouse was evident as a thin crescent of staining nuclei along the ventral edge of the developing limb bud. The expression preceded the development of the apical ectodermal ridge (Fig. 6A), which develops approximately a half day after the initial swelling (Waneck et al. 1989). By 10 days, (limb bud stage 3 (Waneck et al. 1989)), the entire ventral surface of the limb buds stained, as did the adjacent ventral trunk epidermis. Staining within the apical ridge always included the ventral portion and occasionally extended without any apparent pattern to include the dorsal ridge. By 11.5 days (limb bud stage 4), staining had faded uniformly in the ventral ectoderm of the limb, but was still strong in a stripe approximately six cells wide traversing the midline of the tips of the developing digits (Fig. 6F).

Discussion
In this study, we describe the production of a polyclonal antiserum, designated αEnhb-1, and demonstrate its specificity for the En-1 and En-2 proteins of several vertebrates by Western blot analysis and by comparing whole-mount immunohistochemical staining patterns with previous RNA in situ analyses (Davis et al. 1988; Davidson et al. 1988; Davis and Joyner, 1988; Gardner et al. 1988). We used αEnhb-1 to follow the early accumulation patterns of the En proteins in mouse, chick and Xenopus embryos. In doing so, we were able to explore the developmental processes involving En gene expression and to contrast these processes in vertebrate species of different classes. The monoclonal antibody inv-4D9D4 (Patel et al. 1989), which was raised against the protein encoded by the Drosophila engrailed-like gene invected, binds to chick and Xenopus embryos. In the mouse and chick, staining which is likely inv-4D9D4 recognizes only En-2 in these species, whereas αEnhb-1 most likely detects both En-1 and En-2 in the chick and frog as well as in mouse.

We first observed αEnhb-1 staining in the anterior neuroepithelium of all three species and in neural crest-derived cells in the same region of the chick and mouse head. The neuroepithelial staining represents a mixture of expression of En-1 and En-2. Staining which is likely to be due to En-1 expression alone appeared later during development in a number of different body tissues. In all three species, a similar set of differentiating neurons in the spinal cord and hindbrain stained. Just after the first of these appeared, En protein also accumulated in a lateral stripe in the dermatomes of the mouse and chick somites and possibly in all the myotome cells of the Xenopus somites. In the mouse and chick, we observed staining in the ventral ectoderm of the limb buds as soon as a visible swelling could be distinguished. These results indicate that En gene...
Fig. 4. Localization of En proteins in the embryonic mouse neural plate using scanning electron microscopy and elemental analysis following whole-mount immunohistochemistry with aEnhb-1. These embryos are staged according to the neural plate morphology described by Jacobson and Tam (1982). All views are of the dorsal surface showing the open neural plate of the developing brain. Rostral is to the right. Panels on the left are digital maps (purple) of the location of the nickel accumulated during the immunohistochemistry enhancement step superimposed on digital black and white images of the sample. Panels on the right are SEM images of the samples taken at the same time as the mapping was done. There is an approximate 10% compression of the length of the embryos in the digital maps due to the pixel dimensions. (A) 5 somite stage. (B) 7 somite stage. (C) 9 somite stage. (pm) prosencephalon-mesencephalon border; (pos) pre-otic sulcus; (ms1) and (ms2) mesencephalic neuromeres 1 and 2. Scale bars represent 0.1 mm.
Fig. 5. Distribution of En proteins in the bodies and hindbrains of mouse, chick and Xenopus embryos visualized by whole-mount immunohistochemistry using aEnhb-1. (A) Lateral view of a 10.0d mouse embryo. The arrow indicates the mesencephalon–metencephalon border. The otic vesicle staining is probably artifactual, since the staining is non-nuclear and secondary antibody alone binds as well. (B) Magnification of the hindbrain region in A. The arrows indicate the rostral limit of the stripe of En-1 protein in the hindbrain. (C) HH15 chick embryo. The arrows indicate the mesencephalon–metencephalon and prosencephalon–mesencephalon border. (D) Magnification of the trunk region in C showing the developing staining patterns in the somites and spinal cord. (E) Lateral view of a stage 31–32 Xenopus embryo. The large arrow indicates the mesencephalon–metencephalon border. The small arrow indicates staining myotome nuclei. (F) Trunk region of a stage 28 Xenopus embryo. The large arrow indicates the location where the rostral progression of the spinal cord and hindbrain staining pauses. The small arrow indicates staining myotome nuclei. For all panels rostral is to the right, (ov) otic vesicle; (lb) limb bud. Scale bars represent 0.5 mm.
expression is a feature of at least four different developmental processes involving pattern formation: the spatial subdivision of the neuroepithelium, neurogenesis of a spatially defined group of cells in the spinal cord and hindbrain, the spatial subdivision of the somites, and the dorsal–ventral polarization of the developing limb buds. The patterns of En protein distribution have been largely conserved in the three species, and thus the function of these genes in development may have been conserved. However, some differences in the accumulation patterns were apparent, suggesting variations in the underlying processes responsible for En expression.

En gene expression and the spatial subdivision of the neuroepithelium

The subdivision of the neuroectoderm into regions committed to give rise to the different structures of the CNS has been examined experimentally. While there is no consensus model of the subdivision process, the results consistently indicate that the early neuroepithelium does not exhibit regional determination and that the underlying mesoderm is almost certainly involved in regionalization (reviewed-Jacobson (1978)).

The localization of the En proteins in a similar band of the anterior neuroepithelium of all three species likely reflects the rostral-caudal subdivision of the CNS. In the chick, En expression does not begin until after the process of regionalization can first be detected but prior to regional determination. By explanting portions of the neural plate from different stage embryos, it has been shown that the prospective mid/hindbrain region first begins to develop in culture into mid/hindbrain structures at stage HH14 (before somite formation) (Rao, 1968). However, in an experiment where the mesencephalon and part of the metencephalon were inverted in stage HH10 embryos (after the start of En expression), the region regenerated a normal morphology and a normal En expression pattern (Alvarado-Mallart et al. 1990; Martinez and Alvarado-Mallart, 1990). There is no equivalent data for Xenopus or the mouse. In all three species, the staining eventually occupied a ring of the developing brain spanning the constrictio n between the mid and hindbrain. However, the width of the staining band varied, being narrowest in Xenopus and widest in the chick, where the anterior edge extended to the midbrain-forebrain junction. Since the one invariant feature of expression in all the species was the positioning at the mesencephalon–metencephalon border, it is possible that the critical function of the En genes is to define a border region.

In the mouse, the results of this study show that the earliest band of En expression observed in the neuroepithelium was not static relative to the neuroepithelial morphology. The anterior edge first moved rostrally and then retracted, followed by a possible caudal shift of the entire band. From these results alone it is not possible to determine whether this is due to cell movement or to a shift in expression of the genes. However, Morriss-Kay and Tuckett (1987) have shown that the early expansion of the rat forebrain, at the same stage as the rostral expansion of En staining in the mouse was observed, occurs faster than can be accounted for by cell division and is partially due to the rostral movement of cells from the mesencephalon region, i.e. from the region that is expressing the En genes. A rostral movement of the mouse neuroepithelium with respect to the underlying mesoderm has also been described (Jacobson and Tam, 1982). If an interaction with underlying mesoderm is involved in regulating En expression, then it is possible that after some of the cells that initially express the En genes move forward, away from their mesodermal neighbors, they turn off the En genes.

Ventral-lateral stripes of En proteins in the spinal cord and hindbrain

Following the initial appearance of En proteins in the neuroepithelium, there was a second initiation of aEnhb-1 staining, most likely due to En-1, in all three species. It is interesting that whereas the early expression of En-1 and En-2 in the head appeared to reflect anterior–posterior patterning within the CNS, all subsequent expression of En-1 seemed to reflect dorsal–ventral patterning. In all three species, En proteins were found in two ventrolateral stripes outside of the ventricular zone, extending from the rostral hindbrain to the end of the spinal cord. The progressive appearance of staining nuclei in the mouse clearly reflected the rhombomere pattern, providing further evidence for the segmentation of the hindbrain. Neuroepithelial cells generally differentiate as they leave the ventricular zone (Jacobson, 1978), therefore it is most likely that En expression is marking, and may be involved in the development of, a set of spatially defined neurons. According to the staining, these neurons show no periodicity in their pattern of birth or initial placement in the three species, unlike many spinal and hindbrain neurons of the zebrafish (Hanneman et al. 1988). aEnhb-1 exhibits similar staining in the zebrafish (C. Kimmel and K. Hatta, personal communication) and will allow a direct comparison between these neurons in zebrafish and the other vertebrates.

Accumulation of En-1 protein in the somites

There appears to be considerable variation in aEnhb-1 staining in the somites of different developing vertebrate embryos. In the chick and mouse somites, En-1 protein accumulated in a lateral stripe in the middle of the dermatomes which developed from rostral to caudal, suggesting a dorsal–ventral subdivision of this overtly homogenous tissue. In the Xenopus somites, the entire myotome stained faintly. In zebrafish somites, the monoclonal antibody inv-4D9D4 (Patel et al. 1989) and aEnhb-1 both strongly stain a number of cells in the lateral midline of the myotomes (K. Hatta and C. Kimmel, personal communication). Although the staining patterns varied in the different species, all developed with similar kinetics; after the somites were fully formed, and just after the first staining nuclei appeared in the spinal cord. If the patterns in Xenopus
and zebrafish are due to En-1 expression then the variations in the expressing cell types could be due to differences in cis-acting regulatory elements or to different intracellular signalling.

**En protein distribution in the limb buds**

The dorsal–ventral polarity of En-1 gene expression in the developing limb buds of chick and mouse is novel. The growth factor–like gene, BMP-2A, is first expressed in the ventral ectoderm, but becomes limited to the apical ectodermal ridge after its formation (Lyons et al. 1990). While proximal–distal and anterior–posterior determination have been studied experimentally in the chick and Amphibia, and a number of genes have been shown to be expressed differentially along these axes (Oliver et al. 1988b; Dolle et al. 1989), dorsal–ventral differentiation is relatively unexplored. However, there is some experimental evidence indicating that the ectoderm is involved (MacCabe et al. 1974). αEnh-1, as a marker of dorsal–ventral polarity in the limbs, may prove to be a useful probe for studying limb bud development.

In conclusion, our present data with the antisera αEnhb-1, taken together with previous RNA in situ studies and immunohistochemical studies using the monoclonal antibody inv-4D9D4, demonstrate that the expression patterns of En-1 and En-2 have been largely conserved during the evolution of the different vertebrate classes. This strongly suggests a role for these genes in the tissues in which they are expressed. In addition, αEnhb-1 provides a useful probe for a number of developmental processes in many divergent species.

The patterns of protein distribution reflect processes of spatial subdivision in a number of tissues during development. While we have concentrated only on the early developmental stages where wholemount techniques were applicable, extending the analysis later in development will provide further information. All of the processes in which we have followed the accumulation of the En proteins are amenable to experimental manipulation. Thus, En expression could be used as a marker to assess the developmental potential of neural plate, limb bud ectoderm and somite mesoderm following transplantation or culturing.

We thank Dr J. Rossant for her assistance throughout the course of this work and for her review of this manuscript, C. Semard for making the En-2/TrpE fusion construct, Dr N. Patel for the monoclonal antibody inv-4D9D4, Dr W. Wurst for the ES cell line overexpressing En-2, Drs M. Moran and P. Greer for their advice on generating polyclonal antisera, T. Drysdale and Dr R. Ellinson for Xenopus embryos and Dr I. Gitelman for assistance with the figures. C.D. is a recipient of a Canadian Medical Research Council (MRC) studentship and A.J. is an MRC scholar. This work was funded by grants to Dr Joyner from the MRC and the National Cancer Institute of Canada.

**References**


Hanneman, E., Trevorrow, B., Metcalfe, W. K., Kimmel, C. B.


(accepted 22 October 1990)