

Expression patterns of the homeo box-containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development

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We have compared the expression of the murine genes *En-1*, *En-2*, and *int-1* during development by in situ hybridization. Expression of all three genes was first detected at 8.0 days in overlapping bands of the anterior neural folds. By 12.0 days the expression patterns diverged. *En-1* and *En-2* were expressed in a similar ring of cells in the central nervous system (CNS) at the midbrain/hindbrain junction. *En-1* was also expressed de novo in two lateral stripes extending the length of the hindbrain and spinal cord, in the developing vertebral column, in two lateral stripes of dermatome-derived cells, and in the tail and limb buds. By 12.0 days *int-1* expression showed little overlap with the *En* genes and could not be detected at later stages. At 15.5 days *En* gene expression was primarily limited to the midbrain/hindbrain in overlapping but nonidentical sets of differentiated cells. In the adult, *En-1* and *En-2* marked the same sets of cells in the pons, but *En-2* alone was detected in the granular layer of the cerebellum. The results are consistent with *int-1* and the *En* genes playing a role early in development in defining spatial domains in the CNS. Later in development the *En* genes may have an additional function in neurogenesis. *En-1* expression in the developing pericardial tube suggests that it may also be involved in vertebral assembly.

[Key Words: Homeo box; *engrailed*; *int-1*; RNA in situ; mouse development; brain; vertebrae]

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The ease with which mutants may be isolated and characterized in *Drosophila melanogaster* has led to the isolation and study of a large number of genes controlling pattern formation in development. One conserved sequence motif that is both unique to these genes and prevalent among them is the homeobox. Compared with *Drosophila*, knowledge of the genetic control of development in vertebrates has been slow to accumulate due to the difficulty of directly identifying and mutating the genes involved. However, many genes potentially important in vertebrate development, for instance, the homeo box-containing genes (e.g., the *Hox* genes; for review, see Fienberg et al. 1987; Holland and Hogan 1988a), have been isolated by using *Drosophila* developmental genes as probes.

We have been studying the two murine genes, *En-1* and *En-2*, which were isolated on the basis of their extensive sequence homology to the *Drosophila* segmentation gene *engrailed* (*en*) (Joyner et al. 1985; Joyner and Martin 1987). Our in situ hybridization analysis of the expression of *En-2* during development has shown that it is expressed early in embryogenesis in a band of cells across the anterior neural folds and continues to be ex-

pressed in the same region of the developing and adult brain (Davis et al. 1988). Northern blot analysis of expression of *En-1* in embryo tissues showed that its expression pattern is not restricted to the brain but can also be detected in the spinal cord, limbs, and carcass (Joyner and Martin 1987).

In *Drosophila*, mutational analysis has shown that *en* is crucial for generation of the posterior compartments of each segment (Garcia-Bellido and Santamaria 1972; Lawrence and Morata 1976; Kornberg 1981). *en* and a related gene, *invected* (*inv*), are co-expressed early in embryogenesis in the cells of the posterior compartments of the segments (DiNardo et al. 1985; Fjose et al. 1985; Kornberg et al. 1985; Coleman et al. 1987) and later in development in the developing central nervous system (CNS) (DiNardo et al. 1985; Coleman et al. 1987). The regulation of expression of *en* in *Drosophila* is complex. One of the genes involved in maintenance of expression of *en* and which also may be capable of initiating its de novo expression is the segment polarity gene *wingless* (*wg*) (DiNardo et al. 1988). *wg* is expressed in a stripe of cells anterior to the *en/inv*-expressing posterior compartment cells in the cellular blastoderm (Baker 1987;

Rijsewijk et al. 1987). Recently it has been shown that *wg* is the *Drosophila* homolog of the murine oncogene *int-1* (Rijsewijk et al. 1987). In the mouse, the expression of *int-1* has been shown by in situ analysis to be limited to the CNS during embryogenesis (Wilkinson et al. 1987) and the testes in the adult (Jackobovits et al. 1986; Shackelford and Varmus 1987).

Considering that the two related genes *en* and *inv* are co-expressed in the same cells during embryogenesis, it was of interest to determine the pattern of expression of mouse *En-1* during development and to compare it to that of the related gene *En-2*. Furthermore, because in *Drosophila* *wg* plays a role in the regulation of expression of at least *en* and is expressed at the cellular blastoderm stage adjacent to the *en/inv*-expressing cells, it was important to determine whether the expression patterns of *En-1*, *En-2*, and *int-1* suggest a similar interaction between these genes. In this paper, we have used in situ hybridization to examine the pattern of expression of *En-1* at stages throughout development and to compare the expression patterns of *En-1*, *En-2*, and *int-1*. These studies demonstrate that expression of the three genes begins within the same 6-hr period of early somite formation and is restricted to similar overlapping domains within the neural folds. By 12 days of embryonic development, however, the patterns of expression of these three genes have greatly diverged and any overlap in expression is restricted to a small region of the metencephalon. Also, although both *en* genes are expressed in similar regions of the developing brain, *En-1* is also expressed transiently at midgestation in the spinal cord, in the limb and tail buds, and in two somite-derived tissues.

Results

Experimental strategy

To analyze the expression of *En-1*, *En-2*, and *int-1* in situ during mouse embryogenesis, we used SP6 transcripts of small regions of the three genes. The *En-2* probe was the same 250-bp fragment of 3' untranslated cDNA that was used previously (Davis et al. 1988). For *En-1*, a 180-bp 3' untranslated cDNA fragment was used. This probe hybridized to a single copy sequence on Southern blots of mouse genomic DNA. In a Northern blot analysis of mouse embryo total RNA, it hybridized to only the major 2.8-kb *En-1* transcript in all of the tissues examined including RNA from 9.5- and 12.5-day embryos and 16.5-day brain or body without the head (C. Logan and A.L. Joyner, data not shown). The signal was reduced by at least 10-fold in the 16.5-day body RNA compared to the brain. Thus, this probe appears to detect a single *En-1* transcript. For *int-1* a 230-bp *EcoRI*-*Apal* fragment derived from the *int-1* probe, used previously by Wilkinson et al. (1987), was used. The negative sense strand probes were made from the same fragments cloned in the complementary vector so that in all cases the probes were generated by transcription from the SP6 promoter. Wherever possible, adjacent sections were probed with

each of the three antisense probes. Sense-strand negative control probes were included in each series.

Whole embryos for four different stages of gestation, from 7.7 to 15.5 days, as well as 17.5-day and adult brain were examined. When the sense strand-negative control probes were used, occasional artifactual binding occurred near the borders of the sections or lumens within the embryos. Nonspecific binding was also consistently observed around the edges of the cerebellum as well as to the dentate gyrus and pyramidal cell layer of the adult hippocampus.

Expression of En-1, En-2, and int-1 was first detected in overlapping domains during formation of the early somites

In our previous in situ study we were first able to detect *En-2* transcripts in outbred CD-1 mice during a 6-hr period covering the formation of the first six somites around day 8.0 (Davis et al. 1988). Similarly, the earliest detectable expression of *int-1* by in situ analysis was between days 8 and 9 in CBA mice (Wilkinson et al. 1987). This time corresponds to 7.5–8.5 days in other mouse strains, including CD-1.

To determine the degree of coincidence of expression of *En-1*, *En-2*, and *int-1*, 7.7-day (CD-1 outbred mice) embryos which possessed a neural plate and foregut pocket, but lacked any somites, and 8.0-day embryos which had between 4 and 6 somites were hybridized in situ to the probes specific for each of the genes. The 7.7-day embryos showed no hybridization specific to any of the probes (C.A. Davis, data not shown). However, by 8.0 days all three probes hybridized to the embryos (Fig. 1). *En-1* and *En-2* transcripts were only detected in a band of the neural folds at the level of the foregut pocket. Although it is not possible from this analysis to be certain that the two expression patterns are identical, they are very similar and the slight variations we observed could be due to slight differences between neighboring sections. The *int-1* probe gave a higher background than the *En* probes in all tissues from all stages examined. Nevertheless, from examining six embryos in sagittal or cross sections, it was clear that the same region of the neural folds that hybridized to the *En* probes was also the major site of *int-1* expression in the 8.0-day embryos (Fig. 1b). However, the band of *int-1* expression was not as broad as that of the *en* genes and appeared to be contained within the band of *en* expression at the rostral side. In addition, the lateral edges of the neural plate caudal to the *int-1* band of expression in the neural folds also hybridized (data not shown). The *int-1* expression was the same as that observed by Wilkinson et al. (1987).

The expression patterns of En-1, En-2 and int-1 have diverged by 12.0 days

Expression within the CNS By 12.0 days the neural folds have joined to form the neural tube, and large morphological changes in the brain make it possible to identify the regions from which adult brain structures will

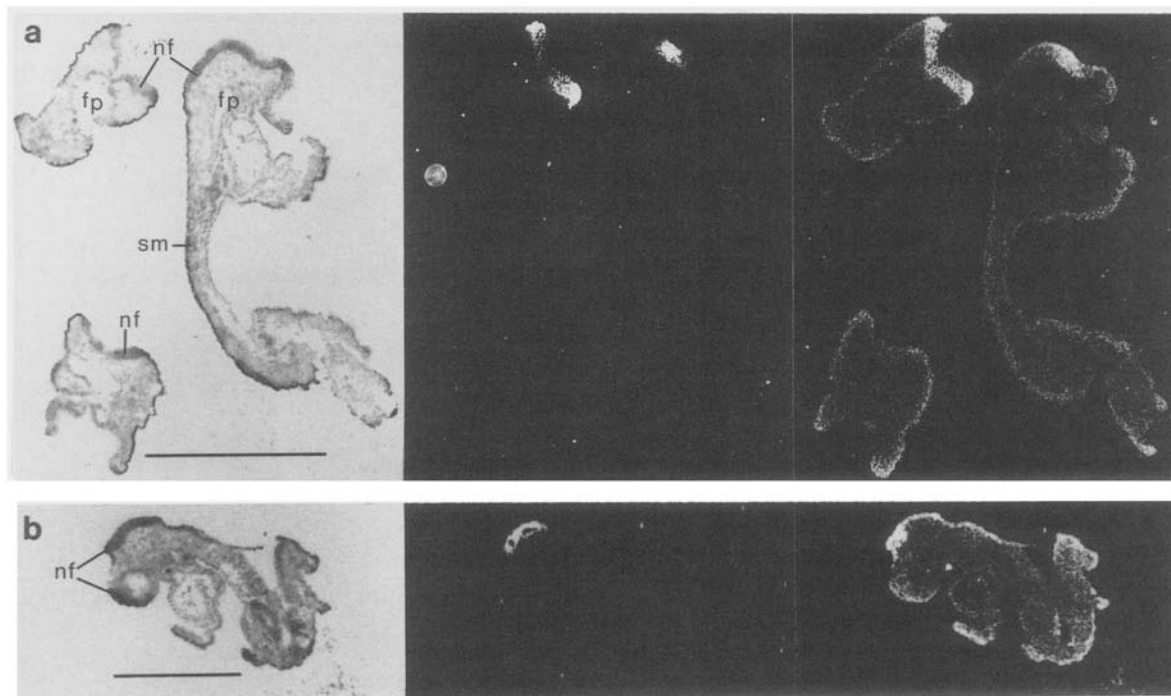


Figure 1. Localization of *En-1*, *En-2*, and *int-1* transcripts in sagittal and transverse sections of 8.0-day embryos. (Left) Bright-field illumination to show morphology; (right) dark-field illumination to show the hybridization to the different probes. (a) Sagittal (right) and two transverse sections: one at the level of the foregut pocket (top) and the other just caudal to the pocket (bottom). Dark-field pictures show *En-2* (left) and *En-1* (right) hybridization. (b) Parasagittal section. Dark-field pictures show *En-2* (left) and *int-1* (right) hybridization. (fp) Foregut pocket; (nf) neural fold; (sm) somite. Scale bars represent 1 mm.

develop. Neurogenesis has also begun and differentiated neurons and glioblasts have started to migrate out of the germinal zone. By day 12.0, this process is well under way in the spinal cord and hindbrain and, to a lesser extent, in the midbrain.

As was shown previously (Davis et al. 1988), *En-2* was expressed at high levels throughout a ring of the neural tube at the midbrain/hindbrain junction (Figs. 2a,b and 3c,d), a region that will later form the pons, cerebellum, and part of the colliculi and periaqueductal gray. This region is at the same level of the CNS as the band of expressing cells observed at 8.0 days and probably consists of their descendants. *En-1* was expressed in the same region of the developing brain (Figs. 2a,b and 3b–d), although there were some differences. Within the germinal zone *En-1* expression was diminished compared to *En-2* in the caudal and dorsal regions where the cerebellum, pons, and colliculi later develop (Figs. 2a,b and 3c). However, in the overlying regions of differentiated cells, expression of *En-1* was more similar to that of *En-2*, both in its pattern and intensity (Figs. 2a and 3c).

In addition to the domain of *En-1* expression in the developing midbrain/hindbrain, it was also expressed in two ventrolateral stripes extending from the rostral hindbrain down the length of the spinal cord (Figs. 2b,c and 3b–d). Here, both germinal zone and differentiated cells expressed the gene. However, in contrast with the germinal zone expression of *En-1* in the midbrain, where

the entire thickness of the layer predominantly hybridized, the cells nearest the lumen in the stripe did not express *En-1* (Fig. 3d). Since we did not detect hybridization of the *En-1* probe to the 8.0-day neural plate anywhere other than the headfold band, the expression in the 12-day spinal cord almost certainly represents a second initiation of *En-1* expression in the CNS.

Although *int-1* was expressed in the same domain of the CNS as *En-1* and *En-2* at 8.0 days, the hybridization pattern we observed at 12.0 days was very different. Hybridization occurred primarily in a thin stripe of cells along the dorsal midline of the spinal cord and brain (Figs. 2c and 3c) and outlined the edge of the membranous roof of the hindbrain (C.A. Davis, data not shown). In the study by Wilkinson et al. (1987), *int-1* expression at 12.5 days was also observed in several small regions of the midbrain, which we did not see. Perhaps *int-1* expression in the midbrain is punctate and the slides we examined did not contain the expressing cells. Therefore, the only overlap in expression of the *en* and *int-1* genes that we observed at 12.0 days was where the expression of *int-1* traversed the dorsal region of *en* expression in the brain (Fig. 3c).

Expression outside the CNS *En-2* showed very limited expression outside the CNS. Previously, we described faint hybridization in the region of Rathke's pouch (Davis et al. 1988). This second analysis confirmed that cells around Rathke's pouch expressed *En-2* (Fig. 3d). We

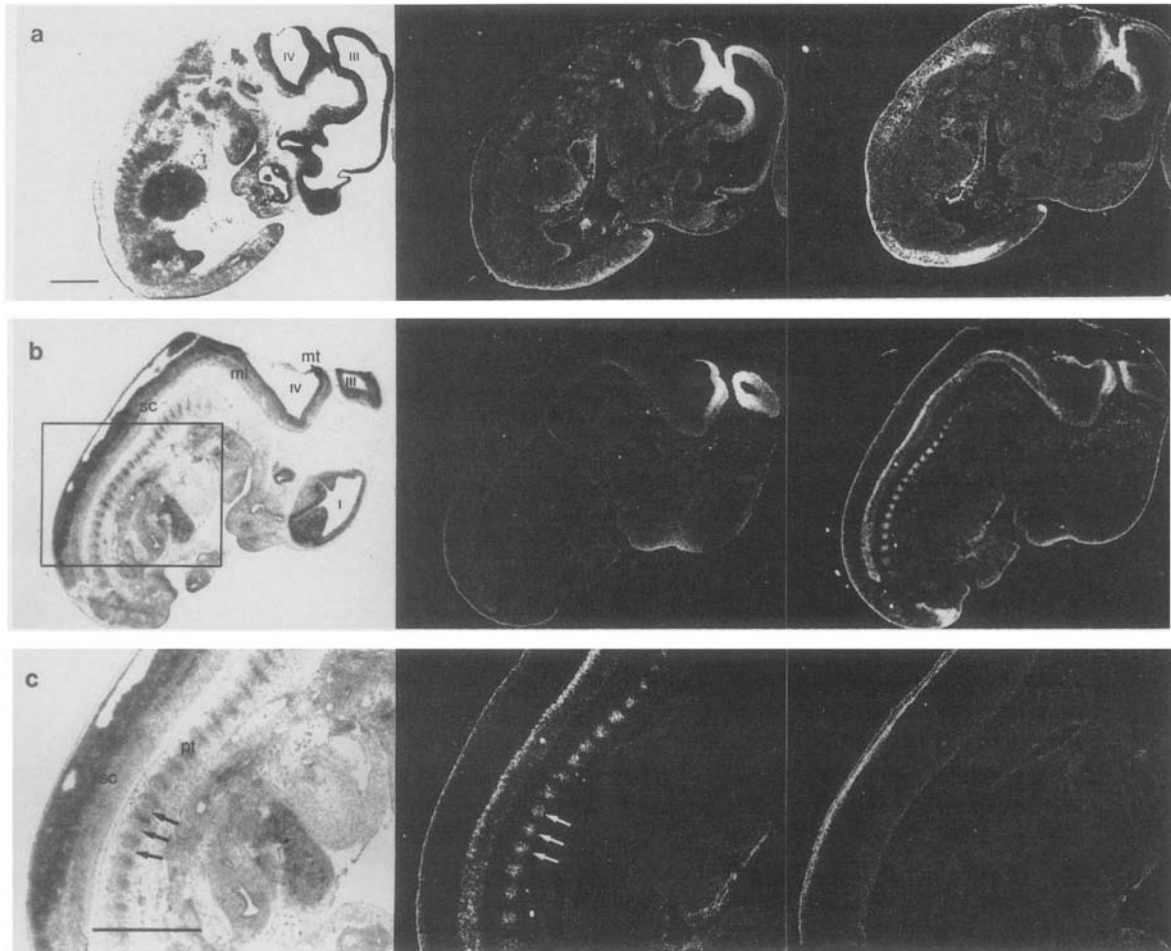


Figure 2. Localization of *En-1*, *En-2*, and *int-1* transcripts in sagittal sections of a 12.0-day embryo. (a,b) Parasagittal sections. Dark-field pictures show *En-2* (left) and *En-1* (right) hybridization. (c) Higher magnification of the region in the box in b. Dark-field pictures show *En-1* (left) and *int-1* (right) hybridization. Arrows point to the loose-packed sclerotome-derived cells of the pericordal tube. (ml) Myelencephalon (hindbrain); (mt) metencephalon; (pt) pericordal tube; (sc) spinal cord; (I) first ventricle; (III) third ventricle; (IV) fourth ventricle. Scale bars represent 1 mm.

do not know the origin or fate of this tissue. In contrast, *En-1* was expressed in a number of other tissues, two of which were of somite origin. *En-1* was expressed in a swath of dermatome-derived cells extending from the top of the head to the tip of the tail on both sides of the embryo (Figs. 2a and 3b–d). The second tissue of somite origin that expressed *En-1* was the sclerotome-derived loosely packed cells of the pericordal tube (Fig. 2b,c), which later gives rise to the vertebrae. As these cells alternate with the densely packed cells from which the intervertebral disks form (Fig 2c), the developing backbone showed a periodic or striped pattern of expression of *En-1* throughout the length present on the sections we examined, extending from the thoracic to tail prevertebrae. Additional *En-1* expression was detected in the tail bud (Fig. 2a,b) and regions of the limb buds (Fig. 3c,d).

Following neurogenesis En-1 and En-2 mark overlapping but nonidentical groups of cells in the brain

By 15.5 days it was apparent that *En-1* and *En-2* expression was mainly limited to the brain. We could not de-

tect any expression of *int-1* at 15.5 days or later, although lower levels could have been masked by background. Outside of the brain no hybridization of the *En-2* probe could be seen to the region around Rathke's pouch. Some expression of *En-1* could be detected in the 15.5-day spinal cord (Fig. 4b) although the hybridization was faint and confined to two narrow stripes. Similarly, faint hybridization could be seen in the vertebrae (Fig. 4b) and under the skin (Fig. 4c,d).

In the 15.5-day brain, the *En* genes were still strongly expressed in the midbrain/hindbrain region (Fig. 4c,d). By this time, neurogenesis is well underway and by 17.5 days will be almost completely finished in this region. An exception to this is the external granular layer of the cerebellum that forms from migrating germinal zone cells from the rhombic lip at the junction of the hindbrain and midbrain. These cells form a layer coating the developing cerebellum and continue to divide until approximately postnatal day 10, when they migrate into the interior of the cerebellum to form the granular layer of neurons.

By 15.5 days, it was clear that *En-1* and *En-2* marked

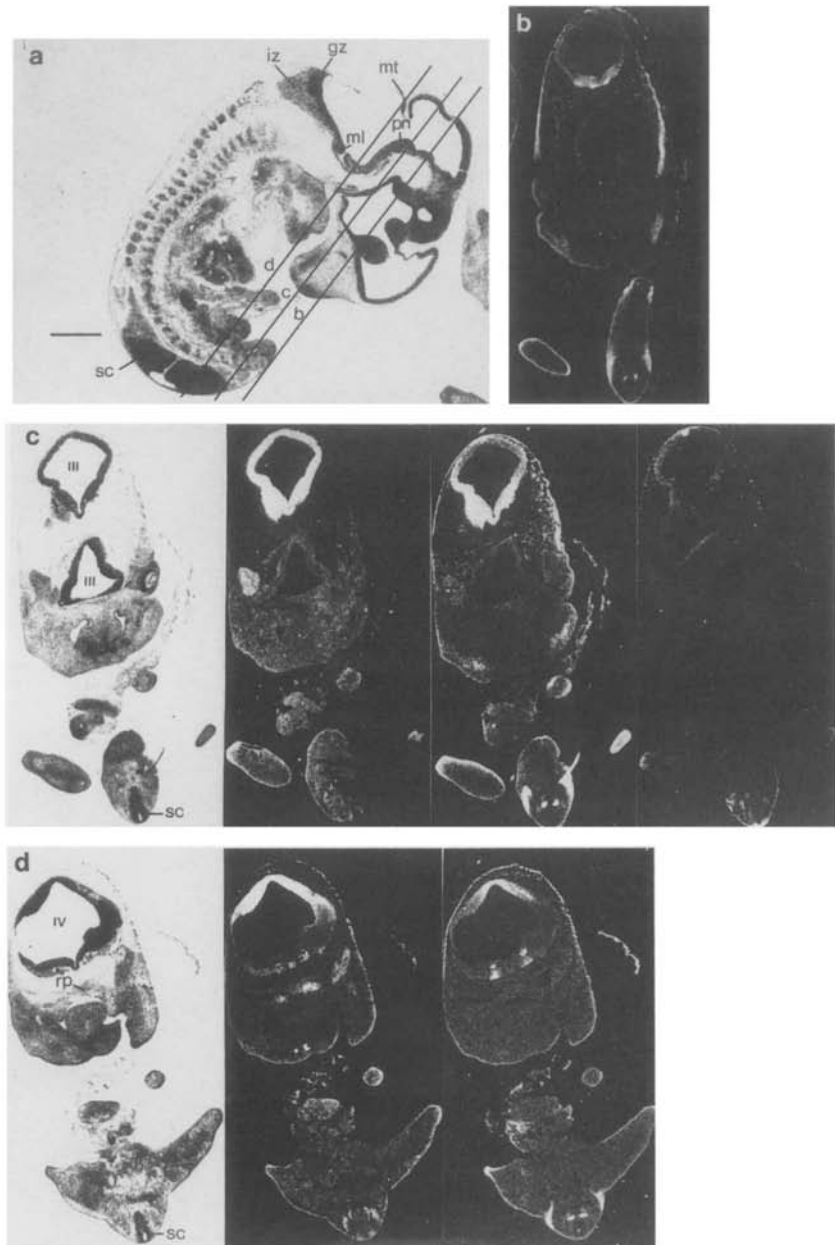


Figure 3. Localization of *En-1*, *En-2*, and *int-1* transcripts in frontal sections of a 12.0-day embryo. (a) Bright-field photograph showing structures of the brain. Lines b,c,d indicate the levels of the frontal sections shown in b,c,d, respectively. (b) Dark-field photograph of a frontal section at the level b, showing *En-1* hybridization. (c) Frontal sections at the level c. Dark-field pictures show *En-2* (left), *En-1* (middle), and *int-1* (right) hybridization. (d) Frontal sections at the level d. Dark-field pictures show *En-2* (left) and *En-1* (right) hybridization. (gz) Germinal zone—dividing undifferentiated neuroepithelium; (iz) intermediate zone—postmitotic neurons and glioblasts; (ml) myelencephalon; (mt) metencephalon; (pn) pons; (rp) Rathke's pouch; (sc) spinal cord; (III) third ventricle; (IV) fourth ventricle. Scale bars represent 1 mm.

different populations of differentiated cells in the brain outside the germinal zone (Fig. 4c,d). *En-1* was expressed in only a few patches of cells in the cerebellum and was weakly expressed in the lateral colliculi, whereas *En-2* was expressed extensively in both. In the pons and periaqueductal gray, *En-1* and *En-2* showed the same hybridization pattern, marking cells extending forward from just under the cerebellum to the midbrain (Fig. 4c). In the hindbrain, the expression of *En-1* which appeared at 12.0 days to be contiguous with the spinal cord expression continued in the 15.5-day samples, although it was limited to a ventral patch of differentiated cells (Fig. 4d).

In the 17.5-day brain sections, *En-2* was expressed extensively in the cerebellum, including the external granular layer, whereas *En-1* was expressed to a lesser degree (Fig. 5). In the pons, *En-2*, but not *En-1*, expression was detected in a group of cells just under the caudal edge of the cerebellum (Fig. 5b). This was tentatively identified as the trigeminal motor nucleus. The rest of the pontine hybridization was similar for the two genes and extended from the junction of the cerebellum and pons rostrally into the midbrain. In the hindbrain *En-1* was expressed in the same cells described in the 12.0-day and 15.5-day embryos (Fig. 5b).

In the adult brain (Fig. 6), the extent of expression of

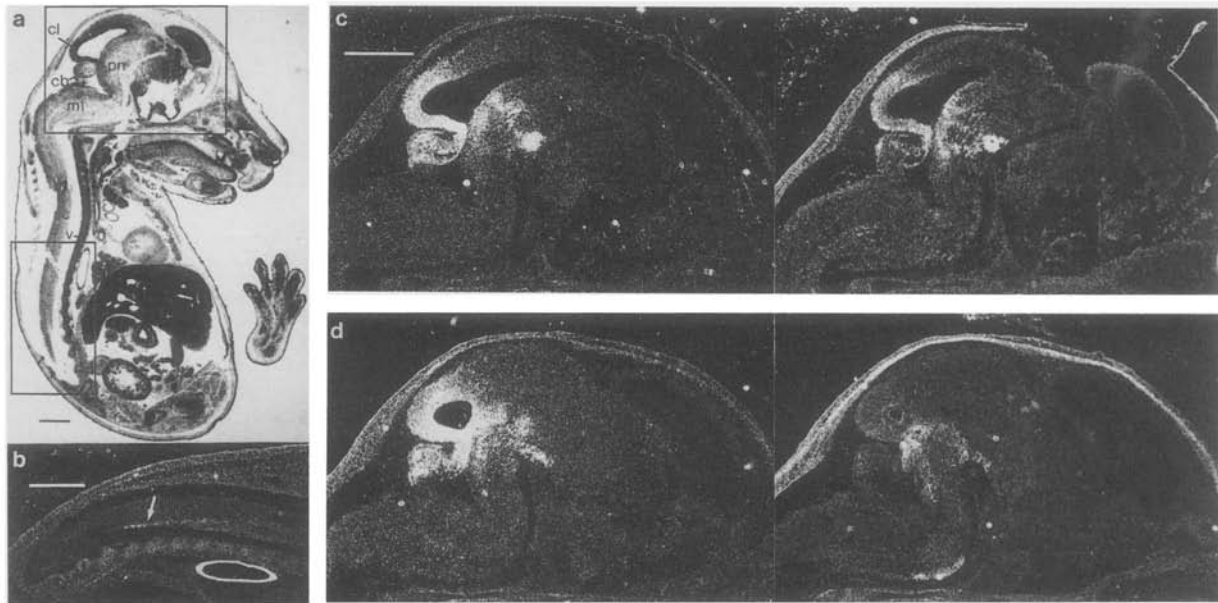


Figure 4. Localization of *En-1* and *En-2* transcripts in sagittal sections of a 15.5-day embryo. (a) Bright-field photograph showing a midsagittal embryo section. Boxes show the regions magnified in b,c,d. (b) Dark-field of the region in the lower box in a showing *En-1* hybridization (photograph rotated 90° clockwise). (c,d) Detail of regions corresponding to the upper box in a. Dark-field pictures show *En-2* (left) and *En-1* (right) hybridization in midsagittal (c) and parasagittal (d) sections of the brain. (cb) Cerebellum; (cl) colliculi and periaqueductal gray; (ml) myelencephalon; (pn) pons; (sc) spinal cord; (v) vertebrae. Scale bars represent 1 mm.

the *En* genes had decreased further. In the cerebellum, strong *En-2* expression was seen in the granular layer, whereas no *En-1* expression was detected (Fig. 6b). The binding of the *En-1* probe to the Purkinje layer and the exterior surface of the cerebellum (Fig. 6b) is probably artifactual, because it was seen with the negative control probes as well. There was no hybridization of either gene to the dorsal periaqueductal gray or the colliculi. In the pons and ventral periaqueductal gray, the pattern of

expression of the two genes appeared to be the same. They were both expressed in groups of cells extending forward from under the rostral portion of the cerebellum to the midbrain. The extent of hybridization diminished rostrally in the same fashion as was seen in the 17.5-day sagittal brain sections. Near the junction of the pons and midbrain, hybridization had diminished to two lateral patches of cells (Fig. 6c). From the caudal to rostral pons we have tentatively identified the hybridizing cells as

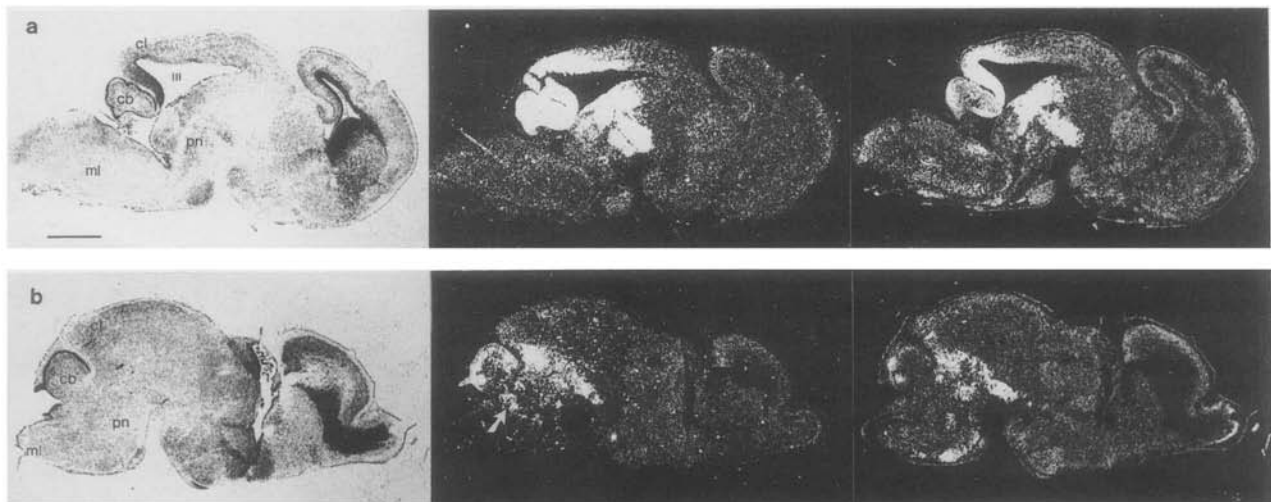


Figure 5. Localization of *En-1* and *En-2* transcripts in sagittal sections of a 17.5-day brain. Dark-field pictures show *En-2* (left) and *En-1* (right) hybridization. (a) Midsagittal sections; (b) parasagittal sections. (cb) Cerebellum; (cl) colliculi and periaqueductal gray; (ml) myelencephalon; (pn) pons; (III) third ventricle. Scale bars represent 1 mm.

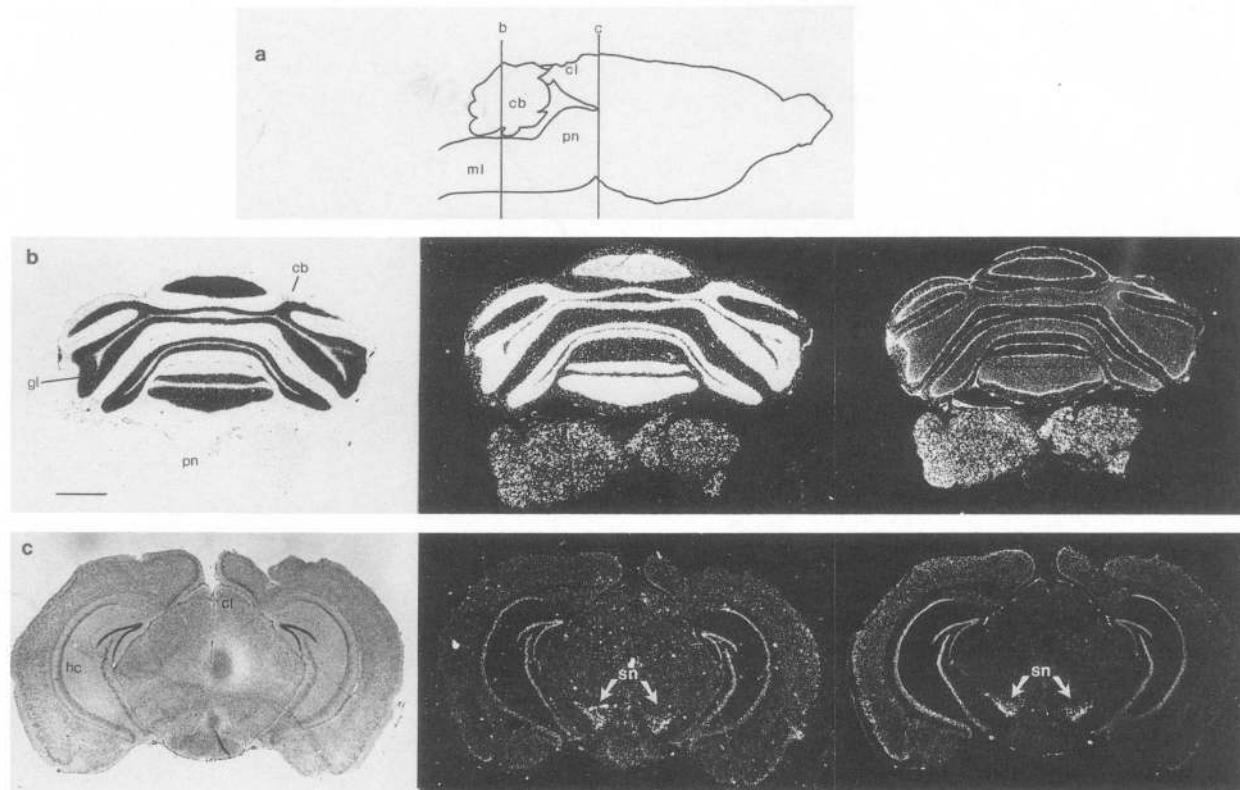


Figure 6. Localization of *En-1* and *En-2* transcripts in coronal sections of an adult brain. (a) Diagram of a midsagittal adult brain. Lines b and c indicate the levels of the coronal sections in b and c. (b,c) Coronal sections at the levels b and c. Dark-field pictures show *En-2* (left) and *En-1* (right) hybridization. (cb) Cerebellum; (cl) colliculi; (gl) granular layer; (hc) hippocampus; (ml) myelencephalon; (pn) pons, (sn) substantia nigra. Scale bars represent 1 mm.

belonging to the ventral periaqueductal gray, raphe linearis, interpeduncular nuclei, and substantia nigra. In the sections examined, we did not locate either the trigeminal motor nucleus, which had expressed *En-2* in the 17.5-day embryonic pons, or the hindbrain cells, which expressed *En-1* earlier.

Discussion

A comparison of the patterns of expression of *En-1*, *En-2*, and *int-1* has shown that all three of the genes initiate expression within the same band of the anterior neural folds during formation of the early somites, with additional *int-1* expression down the lateral edges of the neural folds. However, by 12.0 days, the overlap between *int-1* and the two *En* genes seemed coincidental. Both *En* genes continued to express in a band or ring at the mid-brain/hindbrain junction, but in our analysis *int-1* expression was only observed along the dorsal midline of the spinal cord and brain and was co-expressed with the *En* genes only where this path crossed the ring of expression of the *En* genes in the brain. Wilkinson et al. (1987) observed additional hybridization of *int-1* to several groups of cells in the midbrain, only some of which would likely be within the region of *En* expression. Overall, it therefore seems that unlike the situation

with *en* and *wg* in *Drosophila*, the expression data provide little evidence for the regulation of the murine *En* genes by *int-1*, although the initial extensive overlap in the expression of the genes is consistent with a regulatory interaction.

By 12.0 days, the expression patterns of *En-1* and *En-2* have also partially diverged in the midbrain/hindbrain region. The expression of *En-1* was weaker than *En-2*, especially in the germinal zone, although the two genes marked similar sets of differentiated cells. Later in development, the expression of the two genes occurred in overlapping but nonidentical sets of differentiated cells in the brain. Both genes were expressed in the same sets of cells in the periaqueductal gray and pons into adulthood. However, *En-2* was expressed in the cerebellum throughout its development and in the cells of the granular layer in the adult, whereas *En-1* expression was detected in only a few patches of cells in the cerebellum during development and not at all in the adult.

The most striking difference between *En-1* and *En-2* was the extensive expression of *En-1* outside the mid-brain/hindbrain junction at mid-gestation. At 12.0 days *En-2* expression was observed only in cells surrounding Rathke's pouch, whereas *En-1* was expressed in several other tissues. Within the CNS, *En-1* was expressed in two lateral stripes of cells down the length of the hind-

brain and spinal cord. Outside the CNS a swath of cells running the length of the embryo under the skin and the loosely packed cells of the prevertebral column both expressed *En-1*. The former is derived from the dermatome and the latter from the sclerotome portion of the somites. In addition to these, the limb and tail buds also expressed *En-1*. By 15.5 days expression of *En-1* was barely detectable outside the CNS. The divergent patterns of expression of *En-1* and *En-2* differ from their *Drosophila* counterparts *en* and *inv*, which share the same expression pattern throughout development (Coleman et al. 1987).

The initial expression of all three genes may be relevant to the question of compartmentalization of the early CNS. The near simultaneous initiation of expression of the genes at 8.0 days in specific regions of the CNS suggests that, despite their uniform appearance, the neural folds are being subdivided into different regions, at least on the basis of gene expression. It is also during this time that *Hox-1.5* has been shown to mark a specific region of the CNS (Gaunt, 1987), although a more widespread hybridization was seen earlier in the entire posterior region of the embryo. As discussed by Nieuwkoop (1985), studies in several amphibian species suggest that there is a subsequent regional commitment in the CNS following the formation of the neural plate. It is conceivable that the *En* and *int-1* genes, as well as other homeo box-containing genes, are involved in this process.

The differences in the later expression of *En-1* and *En-2* in the CNS support the hypothesis that the genes may also be involved in neural differentiation. Although the genes initially appear to delineate similar or identical regions of the CNS, they later mark overlapping but different groups of differentiated cells. *En-2* alone marks what may be the trigeminal motor nucleus in the developing brain, as well as the granular layer of the adult cerebellum. The expression of *En-1* in two ventrolateral stripes running the length of the spinal cord and hindbrain at 12.0 days suggests a second independent induction of expression sometime between 8.0 and 12.0 days. Within these stripes, *En-1* expression was found in only the outer germinal zone and differentiated cells, consistent with postmitotic cells initiating expression of the gene. Mitosis occurs at the luminal surface of the germinal zone, and cell bodies migrate away from the lumen after mitosis. Thus, postmitotic neurons and/or glioblasts may be initiating expression of *En-1* after their last mitosis at the lumen surface.

A dorsal-to-ventral variation in expression within the spinal cord, as is seen with *En-1*, has also been observed for several *Hox* genes. *Hox-1.4* and *Hox-2.5* are expressed primarily in the dorsal spinal cord (Fienberg et al. 1987; Toth et al. 1987), whereas *Hox-3.1* is expressed in the ventral spinal cord (LeMouellic et al. 1988). *En-1* would define an intermediate zone between these two regions. This division into three different dorsal/ventral regions is suggestive because the spinal cord is classically divided into three portions—the dorsal horn, the intermediate gray, and the ventral horn, which primarily

consist of sensory neurons, interneurons, and motor neurons respectively. However, as of yet there is no evidence that the boundaries of gene expression correspond to these functional boundaries, and in the case of *Hox-2.1*, which also shows a lesser degree of dorsal/ventral restriction in expression, the investigators state that the boundary of expression does not correspond to any functional boundary (Holland and Hogan, 1988b).

An interesting feature of the expression of *En-1* is that it is expressed extensively, as are the *Hox* genes, in mesodermal tissue. However, the expression of *En-1* in the somite-derived tissue was quite different than that observed for the *Hox* genes. The *Hox* genes are often expressed in a continuous subset of somites and somite derived tissues, whereas *En-1* was expressed in a periodic fashion throughout the length of the pericardial tube and in two stripes of dermatome-derived cells running the entire length of the embryo. Its expression within the pericardial tube appears to be the exact complement of the paired box-containing gene *Pax1*, which is expressed only in the tightly packed cells of the pericardial tube; cells that will later form the intervertebral disks (Deutsch et al. 1988). Both genes may be expressed during the same period of development, with *En-1* beginning between 8 days and 12 days and decreasing by 15 days and *Pax1* beginning around 9 days and dropping sharply between 15 and 16 days. Thus, it is tempting to speculate that in addition to its role in the CNS, *En-1* may be involved in coordinating vertebrae assembly.

Methods

Sample preparation

Embryos from outbred CD-1 mice (Charles River) were collected between 7.5 and 17.5 days of gestation and removed from the uterus (the midpoint of the dark interval during which plugging occurred was considered day 0). The samples were then frozen, sectioned, and stored, as described previously (Davis et al. 1988).

Preparation of probes

To generate suitable probes for the in situ analysis restriction fragments from cDNA clones of the genes *En-1*, *En-2*, and *int-1* were subcloned into the SP6 transcription vectors pGEM-1 and pGEM-2. For *En-2* the 3' noncoding *BglII-SstI* fragment described previously was used (Davis et al. 1988). For *En-1*, a 180-bp *Sau3A-EcoRI* fragment of 3' untranslated DNA was used. The 5' ~900-bp *EcoRI* fragment from the cDNA clone λ *En-1c* (Joyner and Martin 1987) was gel-purified and digested with *Sau3A*, and the 180-bp 3' *Sau3A-EcoRI* fragment subcloned into *BamH-EcoRI*-cut pGEM1 and pGEM2 plasmids. For *int-1* a 230-bp *EcoRI-ApaI* fragment from the cDNA fragment used in a previous in situ study was used (Wilkinson et al. 1987). Sense and antisense transcripts incorporating ³⁵S were made from the SP6 promoter, as described previously (Davis et al. 1988). The quality of the probes was checked by gel electrophoresis before use.

In situ hybridization to tissue sections

Hybridizations were done as described previously using a final probe concentration of 0.2 μ g/ml (kb of complexity) for 15–24

hr at 39°C. The washed and dipped slides were exposed for ~30 days. The sections were stained with toluidine blue and photographed using a Leitz microscope.

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Notes

Since submitting this manuscript, a paper has appeared describing the early expression of *En-1* in mouse embryos which is in agreement with the results presented here (D. Davidson et al., *Development* **104**: 305–316).

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Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene int-1 diverge during mouse development.

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