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Multiple developmental defects in *Engrailed-1* mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum

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SUMMARY

During mouse development, the homeobox-containing gene *En-1* is specifically expressed across the mid-hindbrain junction, the ventral ectoderm of the limb buds, and in regions of the hindbrain, spinal cord, somites and somite-derived tissues. To address the function of *En-1* during embryogenesis, we have generated mice homozygous for a targeted deletion of the *En-1* homeobox. *En-1* mutant mice died shortly after birth and exhibited multiple developmental defects. In the brains of newborn mutants, most of the colliculi and cerebellum were missing and the third and fourth cranial nerves were absent. A deletion of mid-hindbrain tissue was observed as early as 9.5 days of embryonic development and the phenotype resembles that previously reported for *Wnt-1* mutant mice. In addition, patterning of the forelimb paws and sternum was disrupted, and the 13th ribs were truncated. The results of these studies suggest a cell autonomous role for *En-1* in generation and/or survival of mid-hindbrain precursor cells and also a non-cell autonomous role in signaling normal development of the limbs and possibly sternum.

Key words: engrailed genes, mid-hindbrain, cerebellum, sternum, limb, mouse

INTRODUCTION

Establishment of the basic body plan is a key process during vertebrate development. During patterning of the central nervous system, regional identities appear to be defined by signals from the mesoderm combined with intrinsic properties of the neuroectoderm (reviewed in Gurdon, 1992). A number of vertebrate genes are expressed early in embryogenesis in spatially restricted patterns along the anterior-posterior (A/P) and dorsal-ventral (D/V) axis of the developing neural tube, suggesting that they play key roles in pattern formation by setting up, or responding to, local positional cues. For example, the *Hox* family of homeobox-containing genes are expressed in spatially defined regions of the spinal cord and hindbrain, and many have sharp borders of expression that correlate with rhombomere boundaries in the hindbrain (reviewed in McGinnis and Krumlauf, 1992). Recent analysis of the phenotypes of mice carrying targeted mutations in *Hoxa-1* have demonstrated that this gene is indeed required for regional development of the hindbrain (Carpenter et al., 1993; Mark et al., 1993; Dollé et al., 1993a). The two homeobox-containing *Engrailed* genes, *En-1* and *En-2*, are expressed in a spatially defined domain at the presumptive mid-hindbrain junction, indicating that they may regulate development of this brain region.

The mouse *En* genes were identified on the basis of their sequence similarity to the *Drosophila engrailed* gene (*en*), which belongs to the segment polarity class of genes (Joyner et al., 1985; Joyner and Martin, 1987). Most *Drosophila en* mutants die as larvae and show severely affected segmentation patterns (Kornberg, 1981). In such mutants, anterior transformations and cell death occurs in the posterior compartment of each segment, suggesting that these are the cellular mechanisms leading to the mutant phenotype (Kornberg, 1981; Klingensmith et al., 1989, reviewed in Hooper and Scott, 1992). *Drosophila en*, and a structurally related gene *invected* (*inv*), are co-expressed in cells of the posterior compartment of each segment at the blastoderm stage and later of the imaginal discs, as well as in reiterated subsets of neuroblasts and neurons and in the head neuromeres (Coleman et al., 1987; Brower, 1986; Kornberg et al., 1985; DiNardo et al., 1985; Schmidt-Ott and Technau, 1992). The *Drosophila en* and *inv* genes therefore may play multiple functions, including organization of the segments and development of the nervous system.

In the mouse, *En-1* expression begins at the 1-somite stage in two dorsolateral patches of cells in the anterior neuroepithelium (Davis et al., 1988, 1991; Davidson et al., 1988; Davis and Joyner, 1988; McMahon et al., 1992). The patches subsequently fuse ventrally to mark a band of cells that will give rise to the mid-hindbrain junction region. *En-2* expression begins in a similar manner at about the 5-somite stage. At 9.5 dpc, *En-1* also begins to be expressed in a rostral-to-caudal progression in two lateral stripes along the hindbrain and spinal cord and in the dermamyotome of the somites, as well as in the ventral ectoderm of the limb buds. Later, expression is also found in the compact cells of the somite-derived sclerotome.

In the adult brain, both genes are coordinately expressed in groups of motor nuclei in the pons region and substantia nigra and *En-2* is found in cerebellar cells.

Assuming that expression patterns are indicative of domains of gene function and that the function of *Drosophila en* in the specification of posterior compartments has been conserved in mammals, we speculated that the first function of the mouse *En* genes would be in regionalization of the brain (Davis et al., 1988). In addition, *En-1* might be involved later in development of the limbs, spinal cord and somite-derived tissue. To investigate the role of the *En* genes in mammalian development, we have undertaken a mutational analysis using gene targeting in mouse embryonic stem (ES) cells. Mice homozygous for either exon 1 (Joyner et al., 1991) or exon 2 (Millen et al., 1994) *En-2* mutations are viable but exhibit a distinct cerebellar phenotype that includes a one-third reduction in size and a specific alteration in the folding pattern. Detailed analysis of cerebellar development has shown that *En-2* mutants can be distinguished at 15.5 dpc of embryogenesis by a reduction in the size of the cerebellum and colliculi (Millen et al., 1994). During postnatal cerebellar development, the timing and/or placement of two fissures is altered in the *En-2* mutants and a third fissure does not form. These results suggest that *En-2* is required for production of some of the cerebellar cell precursors and for patterning the fissures.

One possible explanation for the seemingly mild effect that *En-2* mutations have on mid-hindbrain development is that the two structurally related *En* genes have partially redundant functions (Joyner et al., 1991). To test this hypothesis and to determine the unique roles of *En-1* during development, we generated an exon two mutant allele of *En-1* by homologous recombination in ES cells. We found that mice homozygous for this mutation die within a day of birth and have multiple abnormalities. The most obvious mutant phenotypes are abnormally shaped forelimbs and sternum, and a deletion of mid-hindbrain tissue that includes part of the cerebellum and colliculi. These results demonstrate that *En-1* is critical for normal development of mid-hindbrain structures. Furthermore, the brain defect provides additional evidence that patterning of the nervous system in mammals involves a phase of regionally controlled proliferation of cell precursors.

MATERIALS AND METHODS

Targeting vectors

Six different non-isogenic targeting vectors were constructed using genomic DNA derived from C3H or Balb/c mouse strains (Table 1). Part or all of the homeobox, or the entire *En-1* gene, were deleted and replaced by a *neo* cassette in which the promoter was derived from the *β-actin* (Joyner et al., 1989) or *PGK-1* (Boer et al., 1990) gene. The *neo* and *En-1* genes were in the same transcriptional orientation in all vectors except F. An *HSV tk* cassette derived from pMC1tkpa (Mansour et al., 1988) was cloned adjacent to either the long or short arm. The long arm of vector A is a 5 kb *Bam*HI-*Bgl*II C3H genomic fragment from clone Mo-en.1 (Joyner et al., 1985) consisting of sequences immediately 5′ to the homeobox. Vector B is the same as A except that the *tk* cassette is at the opposite end. The long arm of vector C is a 3.2 kb *Cla*I-*Bgl*II C3H genomic fragment from clone Mo-en.1 (Joyner et al., 1985) consisting of sequences immediately 5′ to the homeobox. The long arm of vector D is a 12.2 kb *Sal*I-*Bgl*II C3H-Balb/c genomic fragment from clone SC1 (Hanks, unpublished) consisting of sequences immediately 5′ to the homeobox. The long arm of vector E is a 5 kb *Sal*I-*Bgl*II C3H-Balb/c genomic fragment from clone SC1 consisting of sequences 3 kb 5′ to the start of transcription. Vector F is the same as E except that *neo* is in the opposite orientation. The short arm of vectors A,B,C is a 1.2 kb *Bgl*II-*Hin*dIII C3H genomic fragment consisting of 3′ coding and untranslated sequences. The short arm of vectors D,E,F is a 0.85 kb *Eco*RI-*Hin*dIII C3H genomic fragment consisting of 3′ untranslated sequences. For the targeting vector G, *En-1* genomic sequences from the129 mouse strain were used. The long arm consists of a 5.4 kb *Eco*RI-*Bgl*II genomic fragment immediately 5′ to the homeobox and the short arm consists of a 0.85 kb *Eco*RI-*Hin*dIII fragment 3′ to the homeobox. These two genomic fragments were cloned on either side of the PGK-neo cassette of the pPNT vector (Tybulewicz et al., 1991) with *neo* in the same transcriptional orientation as *En-1*. In this construct, a 0.85 kb fragment was deleted that included the entire coding region of the homeobox exon and 0.28 kb of 3′ untranslated sequences.

ES cell culture, screening for homologous recombinants and blastocyst injection

D3 (Doetschman et al., 1985) and R1 (Nagy et al., 1993) ES cell lines were propagated, electroporated and selected as described (Wurst and Joyner, 1993). The Southern blot and PCR screening procedures to detect homologous recombinants were performed as described (Wurst and Joyner, 1993). The PCR reaction was carried out for 40 cycles of 1 minute at 94°C, 2 minutes at 58°C and 4 minutes at 72°C in the presence of 1.75 mM $MgCl_2$. The primers for PCR (Fig. 1) were derived from the 3′ region of PGK-1 (GCCAGTCATTCCTC-CACTCA) and the 3′ flanking sequence of *En-1* (CACGGTCG-TAAGCAGTTTGG). PCR-positive fragments were confirmed by Southern blot analysis using the 0.85 kb *Eco*RI-*Hin*dIII genomic fragment as a probe. PCR-positive recombinant ES cell clones were further analyzed by genomic Southern blot analysis using two external probes (Fig. 1); a 3′ 0.85 kb *Hin*dIII-*Xba*I fragment and a 5′ 1.2 kb *Sac*I fragment. Genomic DNA digested with *Bgl*II and probed with the 5′ probe gave a 7 kb wild-type and 12 kb mutant band. Genomic DNA digested with *Xba*I and probed with the 3′ probe gave a 6.5 kb wild-type and 2 kb mutant band. Three independent targeted ES cell clones were injected into C57Bl/6J blastocysts as described (Papaioannou and Johnson, 1993).

Germline transmission and genotyping of wild-type and mutant En-1 alleles

Three chimeric males with approximately 90% ES cell contribution to the coat were bred with C57Bl/6J and 129/Sv females and germline transmission of the ES cell genome was obtained with all three lines. Tail DNA's from agouti offspring were digested with *XbaI* and hybridized with the 3′ 0.85 kb *Eco*RI-*Hin*dIII genomic fragment. This probe detects a 6.5 kb wild-type and a 2.0 kb *En-1* mutant genomic DNA fragment.

Histology, immunohistochemistry, RNA whole-mount hybridization, β-galactosidase staining and skeletal preparations

For histological analysis newborn brain tissue and embryos were dissected, fixed in 4% paraformaldehyde overnight at 4°C, processed for wax embedding, sectioned at 4 μm and stained with hematoxylin-eosin. Whole-mount immunohistochemistry using αEnhb-1 antiserum (Davis et al., 1991) and the neurofilament-specific monoclonal antibody 2H3 (ATCC) was performed as described by Davis et al. (1991). Whole-mount RNA in situ procedures were carried out according to Conlon and Rossant (1992). Embryos carrying the Tg 4.35 transgene (Logan et al., 1993) that express *lacZ* from the *En-2* promoter were stained as described by Logan et al. (1993). Alizarin red-staining of bones and alcian blue-staining of cartilage was carried out according to the protocol described by Lufkin et al. (1992).

Table 1. Targeting frequencies for the *En-1* locus using non-isogenic and isogenic vectors

Targeting vector	Neo promoter	Length of homology (kb)	Mouse strain DNA of vector	Length of deletion in Kb	ES cell line	No. of cells electroporated $\times 10^7$	No. of G418^r clones	No. of G418^r+ Ganc^r clones	No. of targeted Clones (by PCR)	Targeting* frequency
A	beta actin	6.2	C3H	0.5	D3	15	5,200	642	0	0
B	beta actin	6.2	C3H	0.5	D3	2.75	700	62	0	0
C	beta actin	4.4	C3H	0.5	D3	5	1,400	126	0	0
D	PGK	13	C3H,Balb/c	0.85	D3	20	41,000	7,100	1	0
E	beta actin	6	C3H,Balb/c	6.5	D3	20	38,000	2,100	0	0
F	beta actin (reverse neo)	6	C3H,Balb/c	6.5	D3	20	28,000	2,000	0	0
G	PKGneo	6.2	129Sv/J	0.85	R1	2.5	9,000	450	18	1/37.5

*Homologous recombinants confirmed by Southern blot analysis.

RESULTS

En-1^{hd} homozygous mutant mice die at birth

Homologous targeting events were obtained only by using a targeting vector containing isogenic DNA (Table 1, Fig. 1A-C and Materials and Methods). Three of the *En-1* targeted embryonic stem (ES) cell lines which showed the predicted En-1 mutation by Southern blot (Fig. 1D) were injected into C57Bl/6J blastocysts to generate chimeras. Three chimeric males derived from three independent cell lines were found to transmit the *En-1^{hd}* (hd, homeobox deletion) allele through the germline. All three mutant alleles produced the same postnatal phenotype; therefore only one allele was studied in detail.

Fifty per cent (25/52) of the ES cell-derived offspring of the three chimeras were heterozygous for the *En-1^{hd}* mutation, had no obvious abnormalities and were fertile. Intercrosses were set up between heterozygous mice (*En-1$^{hd/+}$*) either on an F_1 (C57Bl/6J×129/Sv) or inbred (129/Sv) background to analyze the phenotype of homozygous (*En-1$^{hd/hd}$*) mice on two different genetic backgrounds. Genotyping of newborn offspring revealed that 24% (22/93) were *En-1$^{hd/hd}$*, 50% (46/93) were *En-1$^{hd/+}$* and 26% (25/93) were *En-1$^{+/+}$* (Fig. 1E), indicating that the mutation was inherited in the expected ratio and that homozygous mutants could develop to term. However, at birth the *En-1$^{hd/hd}$* mice were readily distinguishable due to forelimb abnormalities and, by 12 hours after birth, it was obvious that the *En-1$^{hd/hd}$* animals were not feeding, since no milk was present in their stomach. The phenotype appeared the same on either genetic background. Of approximately 30 homozygotes observed, only one fed and survived beyond 24 hours of birth, although it also died at 7 days (see below). The cause of death of newborn *En-1$^{hd/hd}$* mutants has not been determined. These animals are able to move their limbs and jaws and thus should be physically able to suckle; nevertheless, they do not feed. They may, however, lack appropriate innervation from the CNS for feeding as a result of a deletion of brain tissue (see below). The brains, limbs, sternum and vertebral column of mutants were analyzed in detail in newborn mice and embryos to determine the effect of loss of *En-1* function on development of these tissues.

En-1$^{hd/hd}$ lack cerebellar and collicular tissues

Brains of newborn animals derived from *En-1^{hd}* heterozygous intercrosses were examined superficially and then sectioned. A total of 12 homozygotes, 8 on the outbred and 4 on the inbred background were analyzed. The phenotypes were similar on the two backgrounds. Gross morphological examination of newborn *En-1$^{hd/hd}$* mice revealed that most of the cerebellum and colliculi were missing (data not shown). Histological analysis of homozygous mutants (n=4) compared to wildtype (*En-1$^{+/-}$* or *En-1$^{+/+}$*) newborn littermates (n=4) confirmed that, in medial sections of *En-1$^{hd/hd}$* animals, the cerebellum was gone and the colliculi were truncated, with the choroid plexus being present but fused to what remained of the colliculi (Fig. 2A-C). In lateral sections of *En-1$^{hd/hd}$* mutant brains, some cerebellar tissue was present and was fused to the colliculi (Fig. 2D-F). The amount of cerebellar and collicular tissue present varied slightly between mutants (Fig. 2B,C) and in the cerebellum from the left to right side of the same individual (Fig. 2E,F). The pons and substantia nigra, which are also derived from *En*-expressing cells in the mesencephalon and metencephalon, were not grossly affected in the mutants. The one animal that survived to 7 days had a less severe phenotype, with limited cerebellar tissue extending to the midline (data not shown).

15.5 dpc and 12.5 dpc embryos from heterozygous intercrosses also were examined. The *En-1$^{hd/hd}$* mutant embryos could be readily identified by the absence of mid-hindbrain tissue and the presence of abnormal limbs. Histological sections of 15.5 dpc and 12.5 dpc *En-1$^{hd/hd}$* (n=5) compared to wild-type (n=4) embryos showed a similar extent of loss of cerebellar tissue and colliculi as was observed in newborn mutants (Fig. 3A-F). This observation suggests that the loss of mid-hindbrain tissue occurs before the cerebellar and collicular primordia develop.

En-1^{hd} mutant embryos are missing cranial nerves III and IV

In order to analyze the embryonic phenotype in more detail, 10.5 and 12.5 dpc littermates from heterozygous intercrosses were stained with a neurofilament-specific monoclonal antibody (2H3). This antibody exclusively stains axons, thus allowing visualization of the developing cranial nerves and ganglia. Cranial nerves III (oculomotor) and IV (trochlear) have been shown to develop from the midbrain and mid-hindbrain junction, respectively, in chick, a region that expresses En-1. Cranial nerves and ganglia V, VI, VII, VIII, IX and X develop more caudally from hindbrain neural crest-derived cells and ectodermal placode cells (Lumsden and Keynes, 1989). In 10.5 and 12.5 dpc *En-1$^{hd/hd}$* embryos (n=5),

the axons of the third cranial nerves, which normally extend from either side of the cephalic flexure to the eyes, were absent, whereas they were well developed in wild-type (n=15) littermates (Fig. 4A-E and data not shown). Cranial nerve IV, which was just beginning to form at 10.5 dpc in the normal animals, was also not detectable in homozygous mutant embryos (Fig. 4A-E). In addition, there were fewer longitudinal axon projections through the midbrain in the *En-1*$^{hd/hd}$ mutants. Cranial nerves and ganglia V, VI, VII, VIII, IX and X in the hindbrain appeared normal in mutant embryos.

En-2 expression is altered early in *En-1*^{hd} mutant mice

To examine further the extent of the early brain deletion in *En-1* mutants, we used *En-2* expression as a marker for the mid-hindbrain cells that normally express *En-1*. Embryos from heterozygous intercrosses were analyzed in whole mounts with *En-2*-specific cRNA probes and with an antiserum (αEnhb-1) that detects En-2 and En-1 protein (Davis et al., 1991). As expected, no En protein expression was detected in 9.5 dpc *En-1*$^{hd/hd}$ embryos outside the brain where *En-1* is normally expressed (Fig. 4I). This observation confirmed that a homeodomain-containing *En-1* protein was not made by *En-1*$^{hd/hd}$ mice and, furthermore, showed that *En-2* was not ectopically expressed in the trunk. Instead of the characteristic ring of *En-2* expression in *En-1*$^{hd/hd}$ embryos (n=4), there was a dorsal patch at the mid-hindbrain junction and a ventral one at the cephalic flexure (Fig. 4I). A similar result was obtained using whole-mount RNA in situ analysis (data not shown). Furthermore, the midbrain of *En-1*$^{hd/hd}$ mutant embryos was smaller than that of wild types (Fig. 4H,I). These results were further supported by histological analysis that showed that in 9.5 dpc mutant embryos mid-hindbrain cells were missing (Fig. 3G,H and data not shown).

To investigate whether *En-2*-expressing cells were affected earlier, 8.5 dpc (10-somite stage) embryos were examined by whole-mount RNA in situ analysis. In the two homozygous mutants analyzed, the *En-2*-expressing domain was considerably reduced in size compared to wild-type embryos (Fig. 4F,G). At this stage, however, the deletion of cells was not obvious by gross morphological examination.

To study the expression of *En-2* easily later in gestation, we transmitted onto the *En-1*^{hd} mutant background an *En-2* promoter/*lacZ* transgene (Tg 4.35; Logan et al., 1993) that expresses *lacZ* in an *En-2*-like manner in the dorsal and lateral regions of the mid-hindbrain junction. Consistent with the results obtained by whole-mount in situ and immunohistochemical analysis at 9.5 dpc, *lacZ* expression in mutant embryos was restricted to a dorsal patch in the mid-hindbrain region (Fig. 4 J,K). In 12.5 dpc transgenic embryos, *lacZ* expression in the brains of *En-1*$^{hd/hd}$ embryos also was greatly reduced compared to wild-type embryos (Fig. 4L-O). Expression of *lacZ* in the jaw muscles of the mutants appeared to be normal.

En-1^{hd} mutant mice have distinct limb defects

All of the 30 outbred homozygous newborn mutants analyzed had obviously deformed forepaws (Fig. 5B, data not shown). The most frequent abnormalities included: mild truncation of digits, fusion of digits, splaying outwards of the digits, large wrinkles in the skin and a sixth postaxial digit. In most animals the polydactyly was seen in only one of the forelimbs. One animal had seven digits on one forelimb, one supernumerary postaxial and one preaxial digit, and another was missing the fifth digit. In all the *En-1*$^{hd/hd}$ animals studied, the hindlimbs showed no gross abnormalities.

To investigate the forelimb defects further, skeletal preparations were made of ten newborn mutant and five wild-type littermates and stained with alcian blue and alizarin red to visualize the cartilage and bone, respectively (Fig. 5C-F). The sizes of condensing bones were measured in both forelimbs

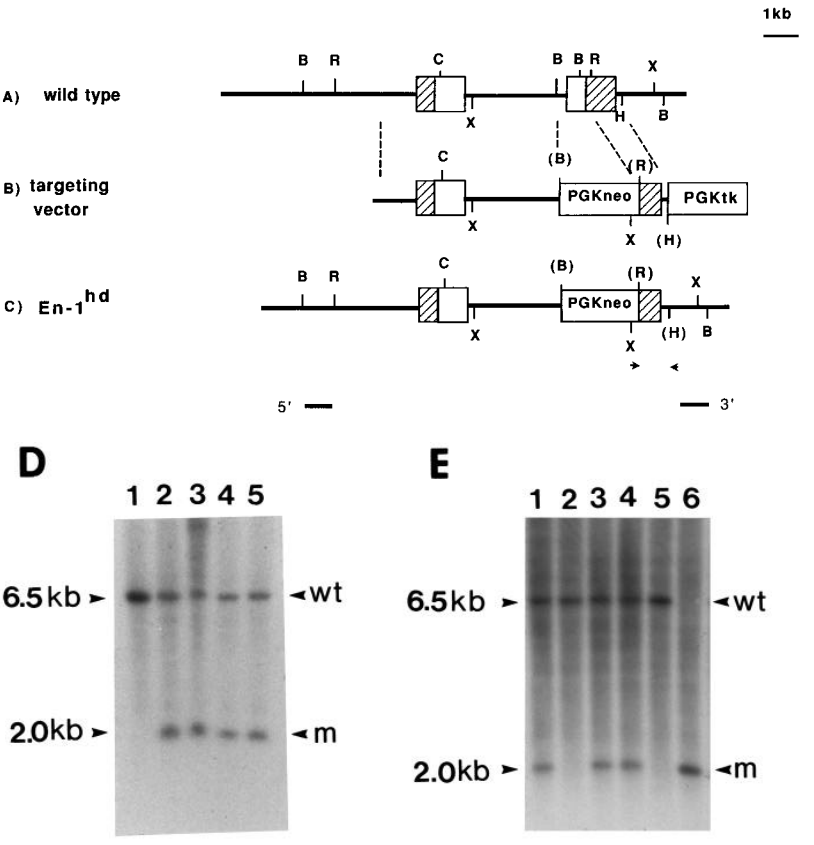

Fig. 1. Schematic representation of the *En-1* targeting vector and recombination at the *En-1* locus. (A) Restriction map of the wild-type *En-1* locus. The homeobox is located in the second exon. Hatched boxes indicate the 5′ and 3′ untranslated region and open boxes the coding sequence. Dashed lines indicate homology in the targeting vector. (B) Restriction map of the *En-1* targeting construct. PGK-neo indicates the PGK-neo cassette and the PGK-tk indicates the PGK-tk cassette. (C) The predicted structure of a mutated *En-1* allele following homologous recombination. Filled arrowheads indicate the primers used to identify homologous targeting events by PCR. The bars (5′ and 3′) indicate the probes used for Southern blot analysis. (D) Southern blot analysis of restriction enzyme digested DNA from targeted *En-1*^{hd} ES cell clones. DNA from one wild-type (lane 1, control) and four (lanes 2-5) PCR-positive targeted cell clones digested with *Xba*I and hybridized with the 3′ probe. Wt and m indicate the position of the wild-type (6.5 kb) and the mutated (2.0 kb) allele, respectively.
(E) Southern blot analysis of *Xba*I-digested DNA from littermates of a heterozygous intercross. B, *Bgl*II; C, *Cla*I; R, *Eco*RI; H, *Hin*dIII; X, *Xba*I

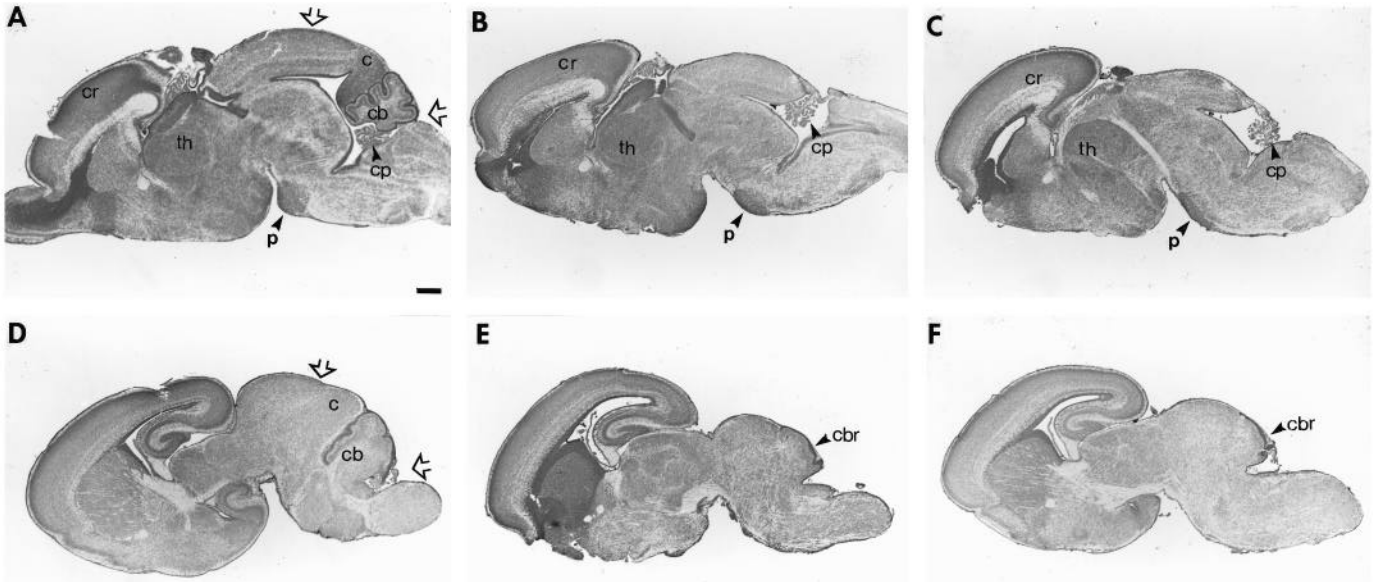

Fig. 2. Sagittal sections of newborn brains from intercrosses of *En-1*^{hd} heterozygotes. (A-C) Midsagittal and (D-F) lateral sections through newborn brains. (A,D) Wild-type brains; (B,C,E,F) brains of *En-1*^{hd} homozygotes. (A,D) The area affected in the mutant is marked by open arrows. (B,C) In homozygous mutants, the cerebellum and parts of the colliculi are deleted as shown in two different animals and the choroid plexus is fused to the colliculi. (E,F) In similar left and right lateral sections of one mutant animal the cerebellar rudiments vary in size. cr, cerebral hemisphere; th, thalamus; p, pontine nuclei; c, colliculi; cb, cerebellum; cbr, cerebellar rudiment; cp, choroid plexus

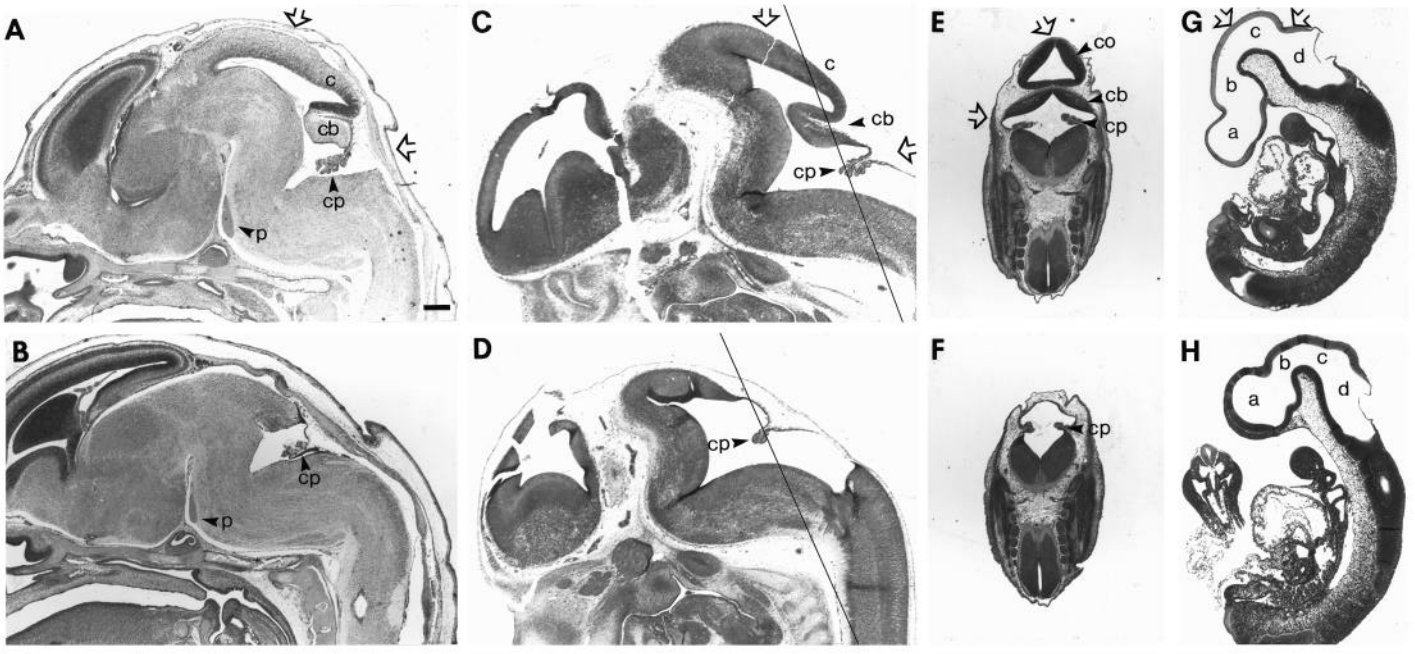

Fig. 3. Histological sections through 15.5, 12.5 and 9.5 dpc embryos from *En-1*^{hd} heterozygous intercrosses. (A,C,E,G) Sections through wild-type and (B,D,F,H) sections through mutant embryos. (A-D,G,H) Sagittal sections and (E,F) coronal sections. (A,B) 15.5 dpc embryos; (C-F) 12.5 dpc embryos; (G,H) 9.5 dpc embryos. (A) The region affected in the mutant is marked by open arrows. (B) In the mutant the cerebellum and areas of the midbrain, including part of the colliculi (c), are absent and the choroid plexus is fused to the colliculi. (C,E) By 12.5 dpc, the cerebellar anlage and the colliculi (c) become distinguishable on histological sections. (D,F) Mutants lack the cerebellar anlage and also part of the colliculi. The choroid plexus is present and fused anteriorly. (G) By 9.5 dpc, the midbrain and hindbrain are distinguishable on histological sections. The region affected in the mutant is marked by open arrows. (H) Mutants lack part of the midbrain and mid-hindbrain region. Photograghs of A-D and E,F and G,H are at the same magnification. a, telencephalon; b, diencephalon; c, midbrain; cb, cerebellum; cp, choroid plexus; co, colliculi; d, metencephalon; p, pontine nuclei

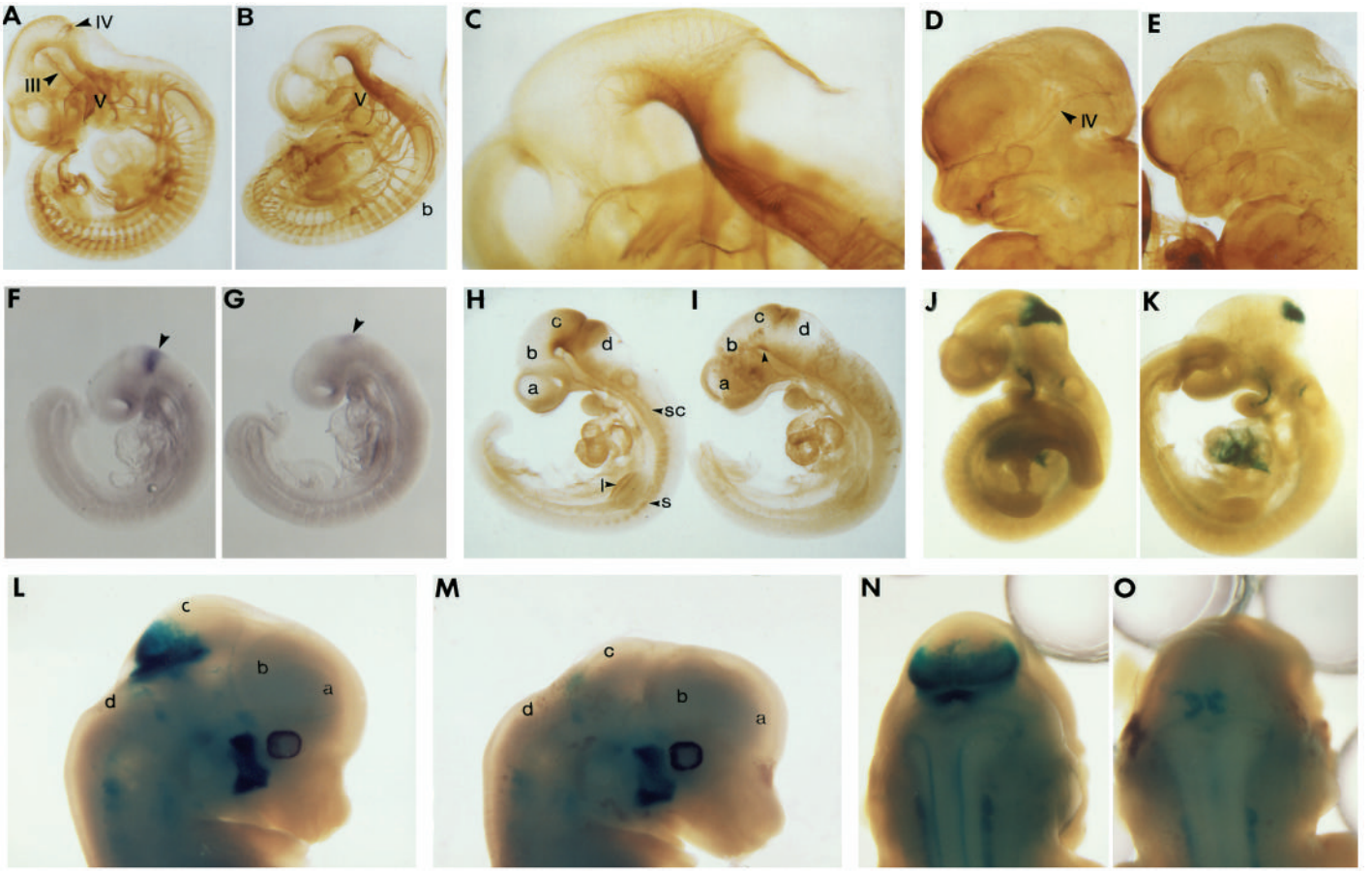

Fig. 4. Whole-mount mRNA in situ hybridization, antibody staining and β-galactosidase analysis of expression of *En-2* and neurofilament. (A,D) Normal and (B,C,E) mutant embryos stained with neurofilament-specific antibody. (A) At 10.5 dpc of development, cranial nerve III is clearly visible and cranial nerve IV is just being formed in wild-type embryos. (B) In the mutant, cranial nerves III and IV are absent. (C) Close up of mutant embryo in B showing the absence of cranial nerve III. (D) Normal 12.5 dpc embryo stained with neurofilament-specific antibody clearly shows the absence of cranial nerves IV in mutant littermates (E). (F,G) Whole-mount mRNA in situ hybridization of 8.5 dpc (about 10-somite stage) with an *En-2*-specific riboprobe. (F) In wild-type embryos, *En-2* is expressed in a wedge across the mid-hindbrain junction. (G) In mutant littermates, only a dorsal patch of *En-2*-expressing cells is present (arrowhead). (H,I) Whole-mount α-Enhb-1 antiserum staining of 9.5 dpc embryos. (H) In the wild-type embryo, the antiserum recognizes *En-1* and *En-2* protein in the mid-hindbrain junction and *En-1* expression in lateral stripes in the hindbrain, spinal cord, somites and the ventral ectoderm of the limb buds. (I) In the mutant, the antibody detects only dorsal and ventral patches (arrowhead) at the mid-hindbrain junction. (J,K) Tg 4.35 transgene (*En-2* promoter driving *lacZ*) expression in 9.5 dpc embryos of littermates from heterozygous matings. (J) In wild-type embryos, *En-2*-specific *lacZ* expression is shown in a wedge in the mid-hindbrain junction and in the first branchial arch. (K) In the mutant embryos, *En-2*-specific expression is restricted to a dorsal patch at the mid-hindbrain junction. Branchial arch expression is unaffected. (L-O) Tg 4.35 transgene expression in 12.5 dpc wild-type (L,N) and mutant (M,O) littermates in a lateral (L,M) and dorsal view (N,O). (L,N) In the wild type, *lacZ* expression is detected in the mid-hindbrain junction and in the jaw muscle. (M,O) In the mutant, *En-2* expression is restricted to two small stripes in the dorsal midline (O). The jaw muscle expression is unaffected. III, cranial nerve III; IV, cranial nerve IV; V, granial ganglia V; a, telencephalon; b, diencephalon; c, midbrain; d, metencephalon; l, limb buds; s, somites; sc, spinal cord.

and hindlimbs of five mutant and wild-type animals (Table 2). The most striking finding was that, in the forelimbs, the condensing bones of the second phalanges were absent in *En-1*$^{hd/hd}$ mutants. In the hindlimbs of mutants, the condensing bone of the second phalanges were also absent or only rudimentary compared to wild-type animals. In addition, the condensing bones of the other forelimb phalanges were reduced in size. In contrast, the whole phalanges (bone and cartilage) were only slightly reduced, indicating that the main defect was a delay in ossification. The postaxial supernumerary digit contained only one phalange (Fig. 5F) and this was also the case for the one preaxial supernumerary digit. The bone or cartilage of two adjacent digits was fused in some limbs, indicating that the digit fusions were due to bone fusions. There was variability in which digits and bones were fused.

En-1^{hd} mutant mice have sternum and rib defects

All of the homozygous *En-1* mutant skeletal preparations examined had obvious sternum and rib abnormalities. In the 7 newborn *En-1* mutants examined, the sternum was reduced in length by about 25% and the ossification centers of the sternebrae was reduced from the most rostral to caudal sternebrae by 40 to 80% as compared to wild type (Fig. 5G,H). The fourth intercostal ossification center, which is normally the last to form, was either not present or only rudimentary in mutants. In addition, the remaining bilateral pairs of ossification centers

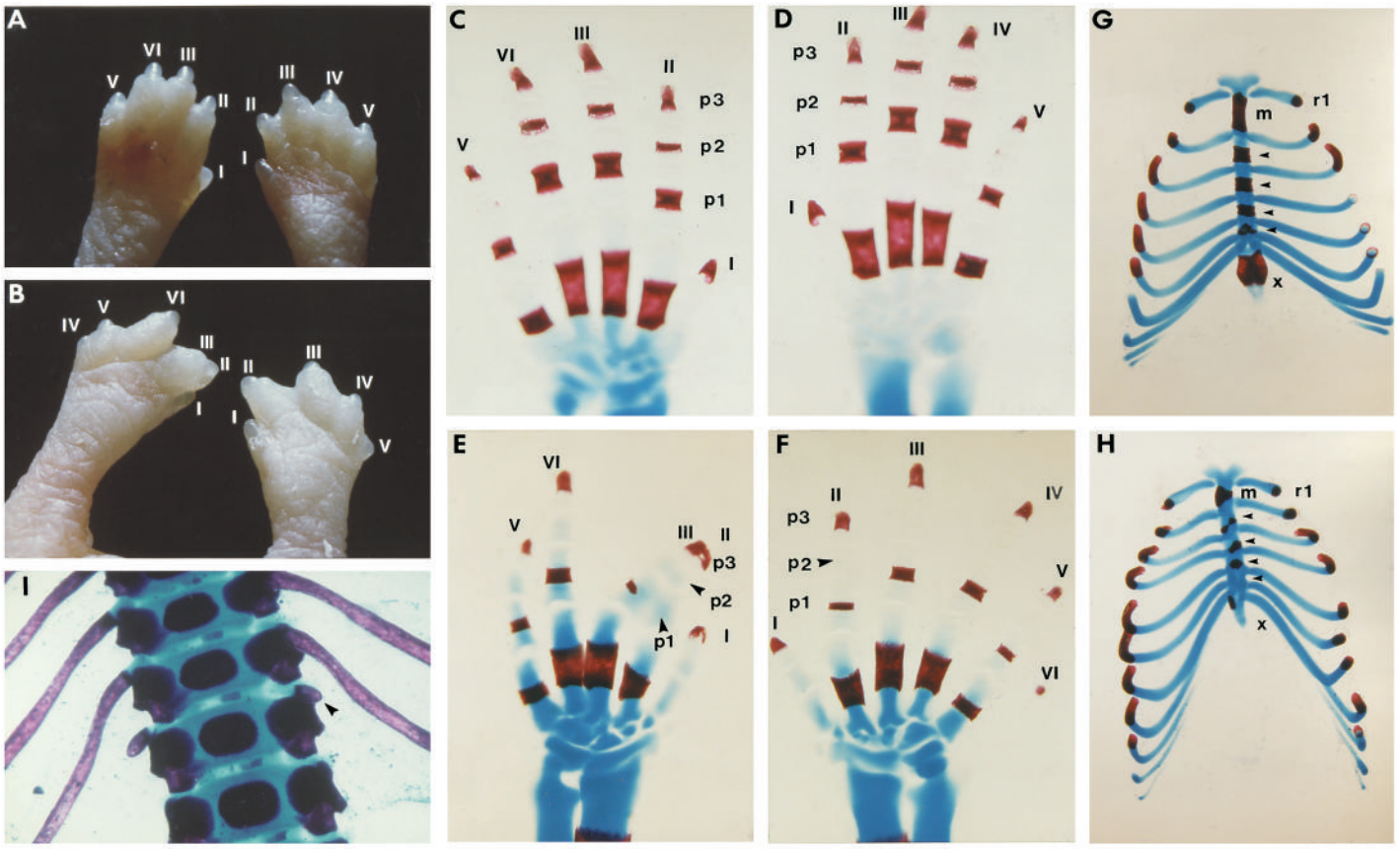

Fig. 5. Forelimb and skeletal preparations of littermates from heterozygous matings. (A,B) Photograph of wild-type (A) and mutant (B) forelimb of newborn littermates taken at the same magnification. (B) Mutant forelimbs show fusion of digit II and III, and an extra sixth digit and truncation of the digits. (C-F) Photographs taken at the same magnification of forelimb cartilage and bone staining with alcian blue and alizarin red of newborn wild-type (C,D) and mutant (E,F) littermates. (C,D) In the wild type, ossification is present in all phalanges. (E,F) In the mutant, ossification of the second phalange is not detectable. (E) Fusion of digit II and III at the level of phalanges 3 and 2 and inhibition of ossification of phalange 1 in digits 2 and 3 is observed. (F) An additional postaxial sixth digit (VI) is present with one phalange. (G) Normal sternum stained with alcian blue and alizarin red. (H) Truncated sternum of newborn mutant and abnormal placement of the ribs. The sternebrae are reduced in size and intercostal ossification is reduced and irregular. G and H were photographed at the same magnification. (I) Truncation of the 13th rib (arrow) in a newborn mutant animal. r1, first rib; m, manubrium; x, processus xiphoideus; arrows, indicate remaining sternebrae; p, phalange

of each sternebrae were not positioned symmetrically and were abnormally fused at the midline. The ribs were not positioned symmetrically where they attached to the sternum.

Examination of the skeletal preparations also revealed that three out of seven *En-1*$^{hd/hd}$ mutant mice had only 12 complete pairs of ribs instead of 13. In the mutants, as in the wild-type mice, the seven rostral ribs were attached to the sternebrae and all but the most caudal pair of ribs seemed to be normal in shape. All the vertebrae also seemed to be normal. It therefore appeared that it was the 13th pair of ribs that was either missing (one case) or dramatically truncated (two cases) (Fig. 5I).

DISCUSSION

In an attempt to gain insight into the cellular and molecular mechanisms that control patterning of the nervous system in mammals, we have undertaken an analysis of mice carrying mutations in the two putative pattern formation genes, *En-1* and *En-2*. The present studies of mice carrying a homeobox deletion mutation in *En-1* demonstrates that *En-1* is required early in development of the brain, limbs, sternum and ribs. *En-1* mutants had a striking deletion in the mid-hindbrain junction region from 9.5 dpc onwards, that is similar to the defects seen in *Wnt-1* mutants. The brain deletion in *En-1* mutants is less severe than that of lethal *Wnt-1* mutants (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), but more severe than viable *Wnt-1* mutants (Thomas et al., 1991). The forelimb paws of *En-1* mutants were grossly deformed showing truncations, fusions, supernumerary digits and a delay in ossification of the digits. In *En-1* mutants, the sternum also showed truncation and a delay in ossification, as well as misalignment of the ribs and abnormal sternum ossification patterns. The hindbrain, spinal cord and dermatome-derived tissues were not analyzed in sufficient detail to determine any effect of loss of *En-1* function.

En-1 function in the CNS

The inferior colliculi and cerebellum in chick normally develop from dorsal mesencephalon and mes-metencephalon rescep-

Table 2. Relative size of digit elements in *En-1^{hd}* mutants (in per cent of normal size)

	digit 1				digit 2				digit 3				digit 4				digit 5			
	p3	p2	p1	m	p3	p2	p1	m	p3	p2	p1	m	p3	p2	p1	m	p3	p2	p1	m
Forelimb*·†																				
Bone and cartilage	104	115	-	-	92	83	99	85	90	87	93	94	100	93	101	99	100	105	103	103
Bone	89	-	-	-	78	0	31	80	85	2	59	75	90	4	63	88	91	-	91	87
Hindlimb†																				
Bone and cartilage	122	100	-	105	98	90	103	103	100	85	101	102	98	81	96	102	100	105	100	97
Bone	104	101	-	112	92	0	89	100	87	0	90	93	87	0	90	93	98	0	78	92

p1, first phalange; p2, second phalange; p3, third phalange; m, metacarpel/metatarsal.
*Forelimb digits are fused in some mutant animals.
†10 forelimbs and 4 hindlimbs have been measured.

tively (Hallonet et al., 1990), the region where *En-1* normally is expressed. To study the spatial and temporal characteristics of the mid-hindbrain deletion in *En-1* mutants, we used *En-2* expression as a marker for *En-1*-expressing mid-hindbrain cells, as *En-2* normally is expressed in a pattern similar to *En-1*. As early as 8.5 dpc the extent of *En-2* expression was decreased and this persisted into later stages. The expression studies, combined with the histological analysis, suggest that there is a continuous loss, or lack of proliferation, of mid-hindbrain cells during 8.5 to 12.5 dpc of development in *En-1* mutants. In chick, cells of the mes-metencephalon region of the neuroepithelium initially are pluripotent, and become determined at the 10-somite stage (Alvarado-Mallart et al., 1990). Since in *En-1* mouse mutants mes-metencephalon cells were probably lost by the 10-somite stage, pluripotent cells are likely affected by the mutation.

The mid-hindbrain phenotype in *En-1* mutant mice suggests several potential functions for the *En-1* gene during its earliest period of expression in the neural tube. One possibility is that *En-1* determines the fate of mid-hindbrain precursor cells. In the absence of *En-1* protein, mes-metencephalon cells may change their identity and develop into different cell types or their development may be compromised resulting in cell death. Another possibility is that *En-1* regulates cell proliferation. This could be achieved through *En-1* protein regulating the expression of growth factors which are necessary for the proliferation and maintenance of mid-hindbrain cells or alternatively by regulating cell cycle genes. There was no evidence from histological examinations and studies using neurofilament as a specific marker that mid-hindbrain cells changed their fate in mutants and caused duplications of forebrain or hindbrain tissues. Further studies of cell death and proliferation, as well as lineage analysis, are required to determine the fate of *En-1$^{hd/hd}$* mutant mid-hindbrain cells.

The *En* and *Wnt* genes are required for generation of mid-hindbrain structures

In *Drosophila*, *en* and *wg* are mutually dependent for maintenance of their expression in adjacent cells at the postblastoderm stage (DiNardo et al., 1988; Martinez-Arias et al., 1988: Bejsovec and Martinez-Aries, 1991; Heemskerk et al., 1991) and this is achieved through a complex genetic pathway involving several segment polarity genes (reviewed in Hooper and Scott, 1992). The fact that the *En* genes and *Wnt-1* are initially expressed in a similar pattern in the midbrain (Wilkinson et al., 1987; Davis and Joyner, 1988; McMahon et al., 1992; Bally-Cuif et al., 1992) and the finding that mice lacking functional En-1 or Wnt-1 protein fail to develop normal colliculi and a cerebellum is consistent with conservation of this genetic pathway from *Drosophila* to mammals. It is interesting that, although *Wnt-1* is not expressed in the rostral hindbrain where the *En* genes are expressed, nevertheless, in mouse *Wnt-1* targeted mutants, a deletion of both midbrain and hindbrain tissue occurs and this correlates with a complete loss of *En-1* and *En-2* expression by 9.5 dpc (McMahon et al., 1992). In our studies of *En-1* mutants, a smaller deletion of mid-hindbrain tissues occurred and this correlated with a decrease, but not a complete loss, of the *En-2* expression domain indicating that *En-1* is only required in a subset of cells that require *Wnt-1*. It will be interesting to analyze *Wnt-1* expression in the midbrain cells that remain in *En-1$^{hd/hd}$* mutant embryos and determine whether *En-2* is required for maintenance of the remaining dorsal cells.

Different roles for *En-1* and *En-2* in mid-hindbrain development?

The brain phenotypes of *En-1* and *En-2* mutants are strikingly different, despite their similar expression patterns and structurally related protein products. However, the small reduction in size of the cerebellum and colliculi in *En-2$^{hd/hd}$* mutants may reflect an early deletion of mid-hindbrain cells similar to that in *En-1$^{hd/hd}$* mutants, albeit much less severe. A question that remains is why there is such a difference in severity of the phenotypes. If the two *En* proteins are functionally equivalent, the early *En-1^{hd}* mutant phenotype could be attributed to defects arising at early embryonic stages when *En-1*, but not *En-2*, is expressed. In support of this possibility, a phenotype is already apparent in *En-1* mutants at the 10-somite stage, based on *En-2* expression. Also, our preliminary analysis of mice carrying mutations in both *En* genes indicates that the compound homozygous mutant phenotype is more severe than the *En-1* homozygous phenotype (W. W. and A. J., unpublished data). Alternatively, the two En proteins may not be functionally equivalent. *En-1* would therefore have a unique early function in generation of mid-hindbrain cells and *En-2*, a later function in patterning the cerebellum. In support of this possibility is the finding that *En-2*-expressing cells apparently are lost, or fail to proliferate, between 9.5 and 12.5 dpc in *En-1^{hd}* mutants. To determine whether the two *En* proteins are functionally equivalent, gene targeting in ES cells can be used to replace the *En-1* coding region with that of *En-2* and the phenotype determined.

En-1 and limb development

Considering that *En-1* expression is restricted to the limb ectoderm, the skeletal defects observed in *En-1$^{hd/hd}$* mutant forelimbs are likely due to altered ectodermal-mesenchymal interactions during development. This may be analogous to the effect of the *polydactilous eudiplopodia* mutation in chicken, where the manifestation of the mutant phenotype can be transferred to normal recipients by transplantation of mutant ectoderm (Goetinck, 1964). In *En-1* mutants the primary limb pattern is disrupted by the formation of an additional postaxial digit, followed by digit fusions, reduction in the size of the first digits to be generated (i.e. IV, III and II), and a delay in ossification that is seen most strikingly as a lack of ossification of the second phalanges.

Limb bud outgrowth is dependent on interactions between the apical ectodermal ridge (AER) and the underlying mesenchyme of the progress zone. Mesenchymal cells, upon leaving the progress zone, begin to differentiate and form prechondrogenic condensations, which undergo secondary modifications to form the final adult skeletal pattern (Shubin and Alberch, 1986; Shubin 1991). It has been shown that limb bud ectoderm has antichondrogenic effects on mesenchymal cells (Solursh, 1984; Hurle et al., 1991). For example, removal of interdigital ectoderm in vivo results in ectopic formation of interdigital cartilage. Thus skeleton patterning may be dependent on the appropriate balance between the intrinsic tendency of mesenchyme to condense and form cartilage and the antichondrogenic effect of the adjacent ectoderm (Solursh, 1984; Hurle et al., 1991).

This balance of ectodermal and mesenchymal interaction is likely altered in *En-1* mutants leading to formation of extra digits and interdigital cartilage and bone causing digit fusions. In addition, En-1 protein in the AER might regulate the expression of growth factors needed for normal stimulation of proliferation of mesenchymal cells in the progress zone. This could lead in mutants to the observed truncations and possibly the delay of ossification of the digits. The hindlimb phenotype could reflect a milder version of the forelimb defect.

The *En-1$^{hd/hd}$* forelimb digit skeletal defects have some similarities to the recently reported phenotype of *Hoxd-13* mutants (Dollé et al., 1993b). Both mutants show an early truncation of digits, delay in ossification and an extra postaxial digit. However, the *Hoxd-13* mutants do not have digit fusions and, in contrast to *En-1* mutants, they show complete deletions of the second phalanges in digits II and V. Finally, our analysis would not detect subtle alterations in dorsal/ventral patterning. This will be particularly interesting to clarify, since in chick the ectoderm has been shown to determine dorsal/ventral patterning of the limb (MacCabe, 1974). In this respect, it is interesting to note that *Wnt-7a* is expressed in the dorsal ectoderm and *Wnt-5a* primarily in the ventral ectoderm of the limb buds (Parr et al., 1993).

En-1 and development of the sternum and ribs

The *En-1* mutation resulted in a truncation of the sternum, abnormal positioning of the sternebrae ossification centers and attachment of the ribs, a delay in sternum ossification and truncation of the 13th ribs. *En-1* expression has been detected in the ribs, vertebrae and ventral dermis, but not in the sternum (Davis and Joyner, 1988; Davidson et al., 1988; our unpublished data). The sternum forms from a pair of dorsolateral mesodermal condensations that appear at 12 dpc and move ventromedially to fuse at the midline (Chen, 1952). The sternal condensations elongate caudally from 12 to 14 dpc and at 16 dpc ossification of the intercostal areas begins from rostral to caudal, with the exception of the caudal xiphisternum which forms early. The regions of the sternum that contact the ribs remain as cartilage, seemingly due to an inhibitory effect of the ribs on ossification (Chen 1952, 1953).

One primary skeletal defect in *En-1^{hd}* mutants appears to be a decrease in caudal outgrowth of the sternum. This may result in abnormal spatial attachment of the ribs. Consequently, misplaced ribs would be expected to cause the observed abnormal placement of sternebrae ossification centers and possibly a delay in ossification. Since *En-1* expression has not been detected in the sternum, the phenotype in *En-1^{hd}* mutants may arise from a defect in signaling from the overlying dermis, which normally expresses *En-1*. A similar hypothesis has been proposed for the sternum phenotype seen in *Hoxb-4* mutants (Ramirez-Solis et al., 1993). If this is the case, then in both the limbs and the sternum of *En-1* mutants the abnormalities arise from defects in signaling from *En-1*-expressing cells to adjacent tissues, indicating a non-cell autonomous effect of a transcription factor.

CNS pattern formation in mammals

Similar to the brain phenotype of *En-1* mutants, early regional loss of hindbrain cells has been seen recently in mouse mutants containing targeted mutations in two other genes that are homologs of *Drosophila* pattern formation genes and that encode transcription factors. Mutations in the homeobox-containing gene *Hoxa-1* (Carpenter et al., 1993, Mark et al., 1993, Dollé et al., 1993a) and the Kruppel-like gene, *Krox-20* (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993), result in early reduction of specific hindbrain rhombomeres. Taken together, these mutant studies in mice suggest that transcription factors direct early regional development of the CNS in mammals. A prediction is that mutations in other transcription factors thought to control pattern formation and which are expressed in the presumptive forebrain, such as *otx* and *ems* (see for example Simone et al., 1992) also will result in regional deletions of precursors. In many cases, like with the *En* genes, compound mutants may be required to uncover the full extent of deletion phenotypes.

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