En-1 and *En-2*, two mouse genes with sequence homology to the *Drosophila engrailed* gene: expression during embryogenesis

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Two mouse genes, *En-1* and *En-2*, have sequence homology to the *engrailed* (*en*) and *invected* (*inv*) genes of *Drosophila* (Joyner et al. 1985). Partial nucleotide sequence analyses of genomic and cDNA clones show that the homologous sequences can encode a stretch of 107 amino acids, including a centrally located, 60-amino-acid homeo box. Within the homologous regions of the mouse and *Drosophila* genes, 78 (73%) of the amino acids are identical. Such extensive conservation of sequence outside the homeo box between vertebrate and *Drosophila* homeo box-containing genes is thus far unique. *En-1* and *En-2* are expressed during mouse embryogenesis. Transcripts from both genes were detected in RNA samples from teratocarcinoma cells, which serve as in vitro models for the early embryo, and from embryos at 9.5 through 17.5 days of development. For each gene we observed a unique pattern of changes in the number and relative intensities of transcripts detectable during embryonic development. Transcripts from both genes are represented abundantly in RNA extracted from the posterior portion of the fetal brain, and are much less abundant in RNA from other fetal tissues, including the anterior portion of the brain and the spinal cord. The chromosome map positions of *En-1* and *En-2* were determined by recombinant inbred strain analyses. Unlike their *Drosophila* counterparts, they are unlinked: *En-1* is in the central portion of the mouse chromosome 1 and *En-2* is in the proximal portion of mouse chromosome 5. Both genes map in the vicinity of mutations that are known to cause abnormalities during development.

[Key Words: Homeo box; *invected* gene; chromosome mapping; developmental mutants]

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Development in multicellular organisms can be viewed as a two-step process. First, a general body plan is established, and subsequently the various components of the basic body plan diversify and specialize. It has not been obvious how to identify or isolate the genes responsible for the control of these processes in vertebrate species. However, the existence of mutations that perturb these processes in *Drosophila melanogaster* has led to the identification of three groups of genes that control development in that organism: The maternal effect and segmentation genes function to establish a body plan composed of repeating segmental units which are subdivided

into anterior and posterior compartments, and the homeotic genes function to provide each segment with a specific identity (Ouweneel 1976; Lewis 1978; Nusslein-Volhard 1979; Kaufman et al. 1980; Nusslein-Volhard and Weischaus 1980). Many of these genes have now been cloned, and a substantial proportion have been found to contain a common 180-bp sequence known as the homeo box (reviewed by Gehring 1985), which is also conserved in the vertebrate genome (McGinnis et al. 1984).

The *Drosophila engrailed* gene (*en*) is known to play an important role in segmentation (Lawrence and Morata 1976; Kornberg 1981; Lawrence and Struhl 1982), and genetic analysis has led to the suggestion that *en* functions as a binary developmental switch to "select" the cells in the posterior compartment (Garcia-Bellido 1975). Consistent with this hypothesis is the dem-

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onstration, by in situ RNA hybridization (Fjose et al. 1985; Ingham et al. 1985; Kornberg et al. 1985; Weir and Kornberg 1985) and antibody localization of the *en* protein product (DiNardo et al. 1985), that *en* is expressed only in the cells of the posterior compartments during the early stages of embryonic development. Molecular studies have led to the identification of a gene adjacent to *en*, now designated *invected* (*inv*), which has extensive sequence homology with it (Poole et al. 1985). Sequence comparisons of *en* and *inv* cDNA clones (Coleman et al. this issue) have revealed two regions of homology between the genes. One region includes a stretch that encodes 17 contiguous amino acids that are identical in the two genes. This region is separated, by sequences encoding 5 (*en*) or 26 (*inv*) nonconserved amino acids, from a second downstream region, which contains a homeo box that is very similar in *en* and *inv*, but quite divergent from the homeo boxes found in other *Drosophila* genes. Additional homologous sequences encoding 31 amino acids are found immediately downstream of the homeo box. In situ RNA hybridization analysis of *inv* expression in developing *Drosophila* embryos has shown that, like *en*, it is expressed selectively in the cells of the posterior compartments (Coleman et al., this issue). These results suggest that the *en* and *inv* genes have related functions during embryogenesis. As yet, however, no mutations of *inv* are known that would suggest its role in development.

In a previous study (Joyner et al. 1985), we attempted to determine whether the *Drosophila engrailed* gene had been conserved during the evolution of higher vertebrates. Southern blot hybridization analysis revealed two regions of the mouse genome with sequence homology to an *en* cDNA clone. These regions are designated *En-1* and *En-2* (previously they were termed *Mo-en.1* and *Mo-en.2*). One of these genomic regions, *En-1*, was cloned and mapped to mouse chromosome 1. Nucleotide sequence analysis of *En-1* genomic DNA revealed that it contains at least part of the conserved coding sequences common to the *Drosophila en* and *inv* genes. These include the homeo box and 21 amino acids immediately downstream from it. The studies described here were undertaken in part to determine whether the second region of homology between the *Drosophila en* and *inv* genes, 5' to the homeo box, is also conserved in the *En-1* gene, and to determine to what extent the mouse genomic region designated *En-2* conserves the sequences common to the two *Drosophila* genes. To achieve this, we isolated and sequenced *En-2* genomic clones and cDNA clones representing embryonic mRNAs encoded by both the *En-1* and *En-2* genes. Moreover, we have extended our previous studies, showing that *En-1* is expressed in cultures of teratocarcinoma cells and in mouse embryos from 9.5 to 17.5 days of gestation, to define the stage-specificity of *En-2* RNA expression during embryogenesis. We have also carried out experiments aimed at defining the tissue specificity of *En-1* and *En-2* in the embryo and at obtaining estimates of the positions of these genes in the mouse linkage map.

Results

Conservation in *En-1* and *En-2* of the sequences common to *en* and *inv*

Two overlapping *En-2* genomic clones, λ En-2.1 and λ En-2.2, were isolated by screening a mouse genomic DNA library with an *En-1* homeo box region probe (see Materials and methods). A partial restriction map of the genomic region represented by these clones is shown in Figure 1a. The same probe was also used to screen a 12.5-day mouse embryo cDNA library (see Materials and methods) for *En-1* and *En-2* cDNA clones. Eight unique phage were isolated. Restriction enzyme analysis of their inserts demonstrated that three represented one mRNA and five another. Each of the shorter cDNAs appeared to be identical to one end of the longest cDNA in its respective group, and the largest cDNA of each group, 1.2 and 3 kb, respectively, was chosen for further analysis. Hybridization of these cDNAs to Southern blots of cloned *En-1* and *En-2* genomic DNA indicated that they represent transcripts of the *En-1* and *En-2* genes, respectively (Fig. 1a and data not shown). The 1.2-kb cDNA clone was therefore designated *En-1c* and the 3.0-kb cDNA clone *En-2c* (Fig. 1b).

A comparison of the sequences in the region surrounding the homeo box (Fig. 2a) revealed a remarkable degree of homology between *En-1* and *En-2*. For the genomic sequences, this region of homology includes the nucleotides at positions 59–375 in Figure 2a. Each of these sequences constitutes an open reading frame that contains the homeo box (positions 100–279) and ends with a signal for the termination of protein synthesis. However, 5' of the homeo box, at a position 41 bp from its start (position 59 in Fig. 2a), the *En-1* genomic sequence diverged significantly from that of *En-2*, and both were found to contain a consensus splice acceptor site (Mount 1982) adjacent to and including the nucleotides at position 59 (sequences not shown). The cDNA sequences of *En-1* and *En-2* were determined for the region extending 5' from the *Sst*I site in the homeo box through to the nucleotides at position 1 in Figure 2a. For each gene, the cDNA sequence was found to be identical to that of its corresponding genomic clone up to the consensus splice site and then to diverge. The *En-1* and *En-2* cDNA sequences 5' of position 59 were found to be highly homologous to each other and extended the open reading frame containing the homeo box. In the composite cDNA and genomic sequences shown in Figure 2a, 295 of 375 nucleotides (79%) are identical. The results of preliminary sequencing studies and analysis of cross-hybridization between the cDNA and genomic clones suggest that the sequences following the stop codon in the *En-2* transcript constitute an exceptionally long 3' untranslated region of ≈ 1.5 kb, whereas the corresponding region of the *En-1* transcript contains only ≈ 500 bp (see Fig. 1b).

The 124 amino acids that can be encoded by the *En-1* and *En-2* homeo box regions are shown in comparison with the corresponding sequences of the *Drosophila en* and *inv* genes in Figure 2b. Although these four se-

Figure 1. Restriction maps of *En-1* and *En-2* genomic and cDNA clones. (a) A partial restriction map of the *En-1* genomic region, as previously determined by Joyner et al. (1985), is shown in comparison with a partial restriction map of the *En-2* genomic region. The open boxes mark the ends of the cloned regions. For *En-2*, the upper horizontal line represents the total combined length of mouse genomic DNA contained in the λ En-2.1 and λ En-2.2 clones. The open and filled triangles indicate the lengths of the λ En-2.1 and λ En-2.2 inserts, respectively. The lower horizontal line is an enlargement of the *Hind*III–*Bgl*III restriction fragment containing the homeo box, which is shown as a filled box. The direction of transcription of *En-1* was previously determined using a single-stranded DNA probe to detect the *En-1* transcripts in embryo RNA samples (Joyner et al. 1985). The direction of transcription of *En-2* was inferred from the sequence of the open reading frame including the homeo box; this is supported by sequencing data showing that the predicted 3' end of the *En-2c* cDNA clone contains a poly(A)⁺ tail. The open boxes marked mp and mp2 represent the subcloned fragments used as probes for the mapping studies. For *En-1*, the wavy lines above the genomic DNA indicate the restriction fragments that hybridize with the 1.2-kb *En-1c* cDNA clone shown in Fig. 1b; similarly, for *En-2* the wavy line indicates those fragments that hybridize with the 3-kb *En-2c* cDNA clone. (b) The horizontal lines indicate the DNA inserts in the λ En-1c and λ En-2c cDNA clones, with the small open boxes indicating the ends of the inserts, which contain synthetic *Eco*RI sites. The filled boxes represent the homeo box in each clone and the large open boxes surrounding them delineate the extent of the region of homology between *En-1* and *En-2*, as it is presently known from comparison of the DNA sequences that were determined. The space interrupting each horizontal line denotes the position of the splice site 5' of the homeo box. In each cDNA, the vertical arrow indicates the end of the open reading frame that includes the homeo box. The cDNA regions that were sequenced are shown as horizontal arrows pointing in the direction of sequencing. (E) *Eco*RI; (S) *Sst*I; (Bg) *Bgl*III.

Figure 2. Sequence analysis of the region conserved in *En-1* and *En-2* and the *Drosophila en* and *inv* genes. (a) Composite genomic and cDNA sequences of the regions surrounding the homeo boxes in *En-1* and *En-2*. The direction of transcription is from left to right. For both genes the arrow indicates the boundaries of an intron, and the box delineates the homeo box. The *SstI* site in the homeo box is indicated. Asterisks denote nucleotides that are identical in *En-1* and *En-2*. The last three nucleotides encode a termination signal for translation of the open reading frame. The sequences of 400 bp of *En-1* and *En-2* genomic DNA surrounding the *SstI* site in the homeo box were determined; most of the *En-1* genomic DNA sequence shown was previously published (Joyner et al. 1985). The cDNA clones were sequenced from the nucleotides at positions 1–211. For both *En-1* and *En-2*, respectively, the genomic and cDNA sequences were identical from the nucleotides at positions 59 through 211. (b) Translation of the *En-1* and *En-2* DNA sequences shown in a, in comparison with the corresponding amino acid sequences of the *Drosophila en* and *inv* genes. The sequences were aligned to achieve the best match. To accomplish this a space, denoted by a dash (-), was left between amino acids that are actually contiguous in *En-1* and *En-2*. In addition, 22 amino acids in the *inv* sequence, denoted by brackets ([]), are not shown. The dots represent six amino acids in the *inv* sequence immediately upstream from the stop codon. The homeo box is delineated by a box. An asterisk denotes amino acids in the *En-2*, *en*, and *inv* sequences that are identical to the amino acid in the corresponding position in the *En-1* sequence. The *en* and *inv* sequences were determined by Poole et al. (1985) and Coleman et al. (this issue). (*en*) *engrailed*; (*inv*) *invected*.

quences are not perfectly colinear, they can be aligned by making certain assumptions: *en* contains one additional amino acid, and *inv* contains 22 amino acids between positions 32 and 33 in the *En-1* and *En-2* sequences; in addition, *inv* contains two amino acids inserted between positions 20 and 21 in the mouse sequences. It is obvious from this comparison that there is a region that is remarkably conserved between the two mouse and two *Drosophila* genes that extends over most of the sequences shown. The conserved region

begins with a stretch of 17 contiguous amino acids that are identical in all four genes (positions numbered 4–20). Each of the four genes contains an intron that interrupts the nucleotide sequence coding for the last of these 17 amino acids (position 20 in Fig. 2b). The first nine of the next 13 amino acids in the sequence (positions numbered 21–29) represent a conserved domain in which seven are identical in *En-1* and *En-2*, and of these seven, four are also conserved in *en* and six are conserved in *inv*. The remaining four amino acids in this

stretch (positions 30–33) represent a region in which the most variation occurs in the four genes; the two *Drosophila* genes each contain additional amino acids in this region. The next 60 amino acids in the sequence, delineated by a box in Figure 2b, constitute the homeo box in which 56 of 60 amino acids (93%) are identical in the two mouse genes, and 42 of 60 amino acids (70%) are identical in all four genes. In the two *Drosophila* genes, but not the mouse genes, the homeo box is interrupted by an intervening sequence. Immediately downstream of the homeo box there is a region (amino acids numbered 94–114) in which 18 of 21 amino acids (86%) are identical in the two mouse genes and 15 of 21 amino acids (71%) are identical in all four genes. Although the predicted amino acid sequences of *en* and *inv* are homologous for an additional 10 amino acids, these amino acids are not conserved in either mouse gene.

Stage and tissue specificity of En-1 and En-2 during embryogenesis

In our previous study (Joyner et al. 1985) we examined the expression of the *En-1* gene by Northern blot hybridization analysis of poly(A)⁺ RNA extracted from PSA-1 teratocarcinoma cells, which serve as an in vitro model system for the mouse embryo at the peri-implantation stages of development (3.5–6.5 days of gestation; Martin 1980), and from whole mouse embryos at the mid- and late-gestation stages of development. Using a 150-base single-stranded *En-1* homeo box region probe, we detected a relatively low-abundance *En-1* transcript in

RNA from undifferentiated teratocarcinoma stem cells. Following differentiation of the PSA-1 cells, an additional, larger *En-1* transcript became detectable. Both transcripts were observed in RNA from embryos at 9.5 through 17.5 days of gestation. However, whereas the less abundant, smaller transcript appeared to be expressed at relatively constant levels in all samples tested, the levels of the more abundant, larger transcript decreased late in development. These results were confirmed in the present study using the *En-1* cDNA probe (Fig. 3a, and data not shown).

In contrast to the pattern described for *En-1*, a single 3.7-kb *En-2* RNA was readily detected at approximately equivalent levels in all teratocarcinoma samples tested. In samples of poly(A)⁺ RNA from embryos, the pattern

Figure 3. Expression of *En-1* and *En-2* RNA during mouse embryogenesis. (a) Expression of *En-2* in teratocarcinoma cells and mouse embryos at various stages of gestation. A Northern blot of poly(A)⁺ RNA (5 μ g) isolated from PSA-1 teratocarcinoma stem cells maintained in the undifferentiated state (un) and at three stages of differentiation (diff 1, 2, 3; see Joyner et al. 1985), and from whole embryos at the days of gestation indicated, was analyzed by hybridization under conditions of high stringency using the *En-2c* cDNA insert probe. For comparison, the filter was stripped and rehybridized under the same conditions with the *En-1c* cDNA insert probe; the lane containing the 12.5-day embryo sample is shown on the right. The sizes of the two *En-1* transcripts were previously estimated to be 2.0 and 3.1 kb (Joyner et al. 1985), but are now calculated to be 1.8 and 2.8 kb, respectively. (b) Tissue specificity of *En-1* and *En-2* expression at midgestation. The tissues indicated were dissected from mouse fetuses at 12.5 days of gestation. The brain was removed from the head and divided into the anterior portion, containing the two frontal lobes, and the posterior portion consisting of midbrain, cerebellum, pons, and medulla; the remainder of the head constituted the "face" sample. The spinal cord sample was free of the spinal column but included the spinal ganglia. The "viscera" sample contained a pool of internal organs, and the "carcass" sample contained what remained of the fetus after the other tissues were removed. In addition, yolk sac and placenta samples were dissected from the conceptuses. A Northern blot of total RNA (15 μ g) from each tissue was analyzed by reiterative hybridization and stripping using the *En-1c* and *En-2c* cDNA insert probes, and a probe for β -actin.

of *En-2* expression was found to be more complex: The 3.7-kb *En-2* RNA was detected in all embryo samples tested, but two smaller transcripts, of approximately 2.5 and 1.8 kb in length, became detectable by 15.5 days and were found at relatively high levels in the samples from embryos at 16.5 and 17.5 days of gestation. The increase in abundance of these two smaller transcripts coincided with a decrease in the abundance of the 3.7-kb transcript (Fig. 3a). In most samples, other very weakly hybridizing transcripts were also detected. The results of rehybridizing the blot shown in Figure 3a with a probe for β -actin indicated that the apparent peak in abundance of the 3.7-kb *En-2* RNA at 9.5 days of gestation was probably an artifact due to differences in the amount of RNA present in each lane (data not shown).

To examine the tissue-specificity of *En-1* and *En-2* transcripts, conceptuses at 12.5 days of gestation were dissected into seven fetal and two extraembryonic parts and total RNA was extracted from each sample. Northern blot hybridization analysis showed that the 2.8-kb *En-1* RNA was most abundant in the sample containing the posterior portion of the brain. This sample consisted primarily of midbrain, but also included cerebellum, pons, and medulla. As shown in Figure 3b, the 2.8-kb *En-1* RNA was approximately three- to fivefold more abundant in the posterior brain sample than in those from the anterior portion of the brain, the spinal cord, face, limbs, or carcass; samples derived from the viscera appeared to be completely negative. Although there were several faint bands of hybridization in the lanes containing RNA from yolk sac and from placenta, neither of these extraembryonic tissue samples contained detectable amounts of the 2.8-kb *En-1* RNA. The 1.8-kb *En-1* RNA observed in all poly(A)⁺ RNA samples from teratocarcinoma cells and embryos was not observed in any of the total RNA samples from fetal tissues, possibly because it is expressed in most tissues at levels too low to detect under the conditions we employed.

The data in Figure 3b show that the 3.7-kb *En-2* transcript is also abundant in the RNA sample from the posterior portion of the brain and no *En-2* RNA is detectable in the other samples. However, upon longer exposure of such autoradiographs, the 3.7-kb *En-2* transcript was detectable in all samples except that from the anterior brain (not shown). Abundant expression of both *En-1* and *En-2* in the posterior portion of the brain continues after development, since the 2.8-kb *En-1* and 3.7-kb *En-2* transcripts were also readily detectable in total RNA samples from this portion of the brain of 17.5-day fetuses and of newborn mice (data not shown); in the other tissues tested (see Materials and Methods), the 2.8-kb *En-1* RNA was detectable only in spinal cord, at relatively low abundance, whereas *En-2* RNA was not detectable. Moreover, the 2.8-kb *En-1* and 3.7-kb *En-2* transcripts were also detected in poly(A)⁺ RNA from adult brain (data not shown). The two smaller *En-2* transcripts were not detected in any of the tissue samples analyzed from 17.5-day fetuses, or from newborn or adult mice.

Genetic mapping of the *En-1* and *En-2* loci

Previous studies employing a panel of hamster–mouse somatic cell hybrids showed that *En-1* maps to mouse chromosome 1 (Joyner et al. 1985). To determine its map position, we carried out a recombinant inbred (RI) strain analysis (Taylor 1981), using a set of 26 RI strains derived from C57BL/6 (B) and DBA (D) mice (B $\times$ D strains). Such analysis is feasible because there is a restriction fragment length polymorphism (RFLP) between *En-1* sequences in the B and D strains (see Materials and methods). The strain distribution pattern (SDP) of the *En-1* alleles was determined by hybridizing the *En-1* mapping probe (mp; see Fig. 1a) to Southern blots of *Taq*I digests of genomic DNA from each of 26 B $\times$ D RI strains. Table 1 shows this SDP in comparison with SDPs for the *Pep-3* and *Sas-1* loci on mouse chromosome 1: 2 of 26 B $\times$ D RI strains are recombinant between *En-1* and *Pep-3*, and 3 of 26 strains are recombinant between *En-1* and *Sas-1*. Using the method of Silver (1985) for estimating confidence intervals for linkage estimates based on RI strain analysis, these results place *En-1* 3.5 cM proximal to *Pep-3* (95% confidence intervals of 1.2–18.3 cM). These results are consistent with the preliminary results of a recombinational analysis which show that *En-1* is approximately 7.5 cM proximal to *Ren-1,2* (G.R. Martin and A.L. Joyner, unpubl.) and the finding of Buchberg et al. (1986), which showed that *En-1* is 8 cM proximal to *Pep-3*.

A similar type of RI strain analysis was carried out to determine the map position of the *En-2* locus. By hybridizing the *En-2* mapping probe (mp2; see Fig. 1a) to Southern blots of *Msp*I digests of genomic DNA, the SDP of *En-2* alleles was determined in 12 RI strains derived from C57BL/6 and C3H (H) mice (B $\times$ H strains) and 7 RI strains derived from BALB/c (C) and C57BL/6 mice (C $\times$ B strains). The data indicate that *En-2* is closely linked to the *Emv-1* locus on chromosome 5. Only 1 of 19 RI strains tested is recombinant between *En-2* and *Emv-1* (Table 1). These results indicate that the *En-2* locus lies within 1.4 cM of *Emv-1* (95% confidence interval of 0.03–10.7 cM). Preliminary results of a recombinational analysis confirm that *En-2* maps to mouse chromosome 5 (G.R. Martin and A.L. Joyner, unpubl.). The genetic maps shown in Figure 4 depict the regions surrounding *En-1* on chromosome 1 and *En-2* on chromosome 5.

Discussion

The mouse has two genes, *En-1* and *En-2*, that have conserved the majority of the sequences that are homologous in the two *Drosophila* genes, *engrailed* and *invected*. The region of homology, containing a centrally located homeo box, can code for 107 amino acids (positions 4–29 and 34–114; Fig. 2b), of which 78 (73%) are identical in all four genes. No other regions of significant homology have been found between the two *Drosophila* genes (Coleman et al., this issue), and relevant data for the two mouse genes are not yet available. The

Table 1. Segregation of *En-1* with linked markers in *B* × *D* RI strains and segregation of *En-2* with linked markers in *B* × *H* and *C* × *B* RI strains

	B × D RI strain ^a																															
Locus	1	2	5	6	8	9	11	12	13	14	15	16	18	19	20	21	22	23	24	25	27	28	29	30	31	32						
<i>En-1</i>	B	B	D	D	B	B	D	B	D	B	D	D	D	B	D	B	B	D	D	B	D	D	D	D	B	D						
<i>Pep-3</i>	B	B	D	D	B	B	D	B	D	B	D	B	B	B	D	B	B	D	D	B	D	D	D	D	B	D						
<i>Sas-1</i>	B	B	D	D	B	B	D	B	D	B	B	B	B	B	D	B	B	D	D	B	D	D	D	D	B	D						
	B × H RI strain ^b												C × B RI strain ^b																			
Locus	2	3	4	6	7	8	9	10	11	12	14	19	Locus	D	E	G	H	I	J	K												
<i>En-2</i>	H	H	B	H	B	H	H	B	B	H	B	H	<i>En-2</i>	B	C	B	C	C	C	C												
<i>Emv-1</i>	H	H	B	H	B	H	H	B	B	H	B	H	<i>Emv-1</i>	B	C	B	C	C	B	C												

^a The letters B and D represent the alleles inherited from progenitor strains C57BL/6J and DBA/2J, respectively. The X indicates crossover regions. *En-1* alleles were typed with the mp probe as described in the text. The SDP for *Pep-3* was determined by Wilson et al. (1978) and for *Sas-1* by Rosenstreich et al. (1978).

^b The letters B, H, and C represent the alleles inherited from progenitor strains C57BL/6J, C3H/HeJ, and BALB/c, respectively. The X indicates crossover regions. *En-2* alleles were typed with the mp-2 probe as described in the text. The SDP for *Emv-1* in the B × H RI strains was determined by Jenkins et al. (1982) and in the C × B RI strains by N. Jenkins and N. Copeland (pers. comm.).

conserved sequences represent a carboxy-terminal protein domain with a molecular mass of approximately 12,000 daltons. The existence of such extensive homology suggests that the conserved domain in the products of all four genes performs a similar biochemical function. Although this function may be related to that of other homeo box-containing genes, it is likely to have some unique features specified by the conserved sequences outside of the homeo box. Such conserved sequences have thus far not been found in any other homeo box-containing genes in *Drosophila* or vertebrate species.

Although the two mouse genes might be the homologs of the *Drosophila engrailed* and *invected* genes with respect to their specific biochemical functions, it remains to be determined whether *En-1* and *En-2* play some role in the control of embryogenesis in mammals that is in any way analogous to the developmental functions of their counterparts in *Drosophila*. Our studies establish that both genes are expressed in PSA-1 teratocarcinoma cells maintained in the undifferentiated state and at three different stages of differentiation. Such undifferentiated cells are thought to be equivalent in many respects to the inner cell mass (ICM) cells of the preimplantation mouse blastocyst (3.5–4.5 days of gestation), and the differentiating cultures at stages 1, 2, and 3 appear to represent or have features in common with mouse embryos at stages prior to (4.5–6.5 days of gestation), during (6.5–8.5 days), and after (8.5–10.5 days) gastrulation, respectively (Martin and Evans 1975a,b; Martin et al. 1977). Thus, the data imply that both genes are expressed early in embryogenesis, during periods when fundamental cell lineage decisions are being made and when the basic embryo body plan is being established. The finding that later in development both *En-1* and *En-2* are expressed most abundantly in the posterior

portion of the brain suggests that both genes have some function in the development of the central nervous system. Expression of both *engrailed* and *invected* has also been detected in the central nervous system of developing *Drosophila* embryos (Ingham et al. 1985; Weir and Kornberg 1985; Coleman et al., this issue). Although it is an attractive idea that there are similarities in the

Figure 4. Chromosome maps of the *En-1* and *En-2* regions. The long vertical lines marked 1 and 5 represent mouse chromosomes 1 and 5, respectively. The open circles indicate the centromeres. The shorter vertical lines represent the region of each chromosome in which *En-1* or *En-2* is located, and the linkage positions of the mouse genes or markers that have been mapped to these regions are indicated (from Mouse News Letter 1985).

developmental functions of the mouse and *Drosophila* genes, at present this is highly speculative. Some insight into this question may be gained from in situ RNA hybridization and protein localization studies of *En-1* and *En-2* products on sections of mouse embryos, which will serve to define the spatial distribution and cell type specificity of the expression of *En-1* and *En-2* during mouse embryogenesis.

The data reported here show that *En-1* and *En-2* are unlinked, whereas the *engrailed* and *invected* genes are adjacent to each other in the *Drosophila* genome (Coleman et al., this issue). Thus, any functional significance in the close linkage of the *Drosophila* genes has not been conserved in the evolution of the mammalian *engrailed*-like genes. Both *En-1* and *En-2* map in the vicinity of mutations that are known to cause developmental abnormalities (Green 1981). *En-1* on chromosome 1 is in the vicinity of *Dominant hemimelia* and *En-2* on chromosome 5 is in the vicinity of a pair of genes, *Hemimelic extra toes* (*Hx*) and *Hammertoe* (*Hm*), which may be allelic since they have never been separated by recombination. It is interesting to note, but perhaps coincidental, that the *Dh*, *Hx*, and *Hm* genes all cause similar types of abnormalities in skeletal development. *En-2* also maps in the vicinity of *reeler* (*rl*), a recessive mutation that causes a reduction in the size of the cerebellum with consequent effects on motor activity. Recombination studies to determine whether any of these genes might represent mutant alleles of either *En-1* or *En-2* are in progress. The identification of an extant mutant allele(s) of one or both of these mouse *engrailed*-like genes would greatly facilitate the analysis of their function in mammalian development.

Materials and methods

All experimental procedures were carried out according to the methods described by Maniatis et al. (1982), unless otherwise specified.

DNA probes

All probes except the *En-1* homeo box region probe were radio-labeled to a specific activity of at least 2×10^8 cpm/ μ g, using a nick-translation kit (Bethesda Research Laboratories). The *En-1* homeo box region probe that was used for screening genomic and cDNA libraries was a single-stranded DNA probe derived from an M13 clone, as described previously (a-s probe; Joyner et al. 1985). It consisted of the approximately 150 nucleotides immediately 3' to the *Sst*I site in the *En-1* homeo box.

The cDNA probes used in the Northern blot hybridization analysis were as follows: the 1-kb *Eco*RI fragment of the λ En-1c clone that contains the homeo box, and the entire 3-kb *Eco*RI insert of the λ En-2c clone (see Fig. 1b).

The probes used for the linkage analysis were the *En-1* mapping probe (mp), a 3.6-kb *Eco*RI–*Sst*I subclone of the Mo-en.1 genomic clone (Joyner et al. 1985), and the *En-2* mapping probe (mp2), a 2-kb *Sst*I fragment of λ En-2.2 (see Fig. 1a). The mp2 fragment was subcloned into the *Sst*I site of the plasmid SP65. The mp probe hybridizes to fragments of 3.5 and 3.3 kb in *Taq*I-digested C57BL/6 genomic DNA and fragments of 4.4 and 3.3 kb in *Taq*I digests of DBA/2J DNA. The mp2 probe hybridizes to fragments of 1.5 and 1.3 kb in *Msp*I digests of genomic DNA from C57BL/6 (B) mice and to a fragment of 2.8 kb

in *Msp*I digests of genomic DNA from C3H (H) and BALB/C (C) mice.

The chicken β -actin probe was kindly provided by Dr. D. Cleveland (Cleveland et al. 1980).

Isolation of genomic and cDNA clones

A recombinant λ EMBL3A library containing 12- to 20-kb fragments of genomic DNA, derived from C3H strain mice, which had been partially digested with *Sau*3A, was kindly provided by Drs. J. Vogel and R. Goodenow (Department of Genetics, University of California, Berkeley). Approximately 600,000 recombinant phage were screened with the *En-1* homeo box region probe under conditions of moderate stringency (see below), and one of the phage obtained was found by restriction analysis to contain the *En-1* genomic fragments that were previously isolated. The other two clones represent the *En-2* genomic region, as described in the Results.

A λ gt10 cDNA library was constructed according to the procedure of Huynh et al. (1985), and was kindly provided by Dr. Craig Hauser (Department of Biochemistry, University of California, Berkeley). It contains *Eco*RI cDNA inserts derived from poly(A)⁺ RNA extracted from C3H mouse embryos at 12.5 days of gestation. Four phage that carry DNA inserts homologous to the *En-1* homeo box region probe were obtained by screening an aliquot of the library that contained 500,000 unique phage. Four additional unique phage were obtained by screening a similar number of phage from another aliquot of the same library following its amplification.

The replica nitrocellulose filters were hybridized for 16 hr at 64°C in $5 \times$ SSC, $10 \times$ Denhart's solution, 0.1% SDS, 0.1% sodium pyrophosphate, and 15% deionized formamide. The filters were washed once for 30 min in $2 \times$ SSC, 0.1% SDS and twice for 20 min each in $0.2 \times$ SSC, 0.1% SDS at 50°C.

Southern blot hybridization analysis

Recombinant λ phage DNAs (1 μ g) or total genomic DNAs (10 μ g) isolated from mouse tissues were cleaved with restriction endonucleases, electrophoretically fractionated in 1% agarose, and transferred to GeneScreen (New England Nuclear) in $10 \times$ SSC. Subsequent to transfer, the nylon filters were exposed to UV light according to the methods of Church and Gilbert (1984). The filters were hybridized at 65°C using the following "moderate hybridization conditions": 1% bovine serum albumin, 0.5 M NaPO₄ (pH 7.2), 1 mM EDTA, 7% SDS, and 15% formamide. The filters were washed twice for 20 min in $2 \times$ SSC, 1% SDS and once for 30 min in $0.2 \times$ SSC, 0.1% SDS at 50°C. Genomic DNA isolated from RI strain mice was purchased from the Jackson Laboratory (Bar Harbor, Maine).

Nucleotide sequence analysis

All DNA sequences were obtained by the dideoxynucleotide chain termination technique (Sanger et al. 1977) using the universal 15-mer primer (Pharmacia) or using appropriate synthetic oligonucleotide primers. The λ En-2.2 genomic fragments or En-1c and En-2c cDNA fragments for sequencing were cloned into M13 vectors (Messing 1983; Pharmacia) and the recombinant phages to be sequenced were identified by hybridizing replica filters of bacterial host cells (JM101) that had been transfected with the appropriate ligation mixtures to a radioactively labeled *En-1* homeo box region probe. DNA for sequencing was then isolated from cross-hybridizing phages (Sanger et al. 1980). All sequences shown were determined by independently sequencing both strands. For each M13 clone, only the length of DNA that was sequenced is indicated in Figure 1.

Northern blot hybridization analysis

All procedures were similar to those previously described by Joyner et al. (1985). Mouse embryos at various stages of gestation were obtained by mating random-bred ICR animals (Simenson Laboratories, Gilroy, California). The day on which the vaginal plug was detected was considered 0.5 day of gestation. Embryos at the designated stages were dissected from the implantation site and separated from the extraembryonic membranes. Total cellular RNA was isolated from the following samples using the guanidinium thiocyanate/CsCl₂ method of Chirgwin et al. (1979) with some minor modifications: PSA-1 cells cultured under the conditions described by Martin et al. (1977); pools of whole mouse embryos at various times of gestation (see text); tissues dissected from conceptuses at 12.5 days of gestation (see text); anterior and posterior portions of the brain, the spinal cord, heart and lungs, liver, and other viscera from fetuses at 17.5 days of gestation; anterior and posterior portions of the brain, the spinal cord, and the liver from newborn mice; the brain from adult mice. Poly(A)⁺ RNA was selected from total RNA isolated from whole embryos, adult brain or PSA-1 cells by oligo(dT)-cellulose column chromatography. RNA was fractionated by gel electrophoresis, transferred to GeneScreen nylon membranes and hybridized under conditions of "high stringency" as described by Joyner et al. (1985).

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Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under the accession numbers Y00201 for *En-1* and Y00203 for *En-2*.

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