

## Mouse homeo-genes within a subfamily, Hox-1.4, -2.6 and -5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation

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### Summary

By use of *in situ* hybridization experiments on mouse embryo sections, we compare the transcript patterns of three homeo-genes from the Hox-1.4 subfamily (Hox-1.4, -2.6 and -5.1). Genes within a subfamily are true homologues, present in the genome as a result of duplication of an ancestral homeo-gene cluster.

We show that Hox-1.4, -2.6 and -5.1 are similar, although apparently not identical, in the limits of their transcript domains along the anteroposterior axis. Within the prevertebral column of the 12½ day embryo, for example, the anterior boundary of transcripts for each of the three genes was most obvious at the junction of the first and second prevertebrae. Similarly, all three genes showed an anterior boundary of transcripts within the central nervous system that was located in the mid-myelencephalon of the hindbrain. Both in the prevertebral column and hindbrain, however, Hox-2.6 and Hox-5.1 transcripts extended slightly anterior to the anteriormost limits detected for Hox-1.4.

In spite of close similarities in the positions of their transcript domains, Hox-1.4, -2.6 and -5.1 displayed

striking stage- and tissue-dependent differences in the relative abundance of their transcripts. For example, Hox-5.1 transcripts were abundant within mesoderm and ectoderm of early stages (8½ and 9½ days), yet were detected only weakly in mesodermal components of the lung and stomach at 10½ days, and were apparently absent from these tissues at 12½ days. In contrast, Hox-1.4 and Hox-2.6 transcripts were relatively weakly detected at 8½ and 9½ days, but were abundant within the lung and stomach at 12½ days. Our findings suggest, but do not prove, that genes within the Hox-1.4 subfamily might be coordinately regulated in their expression.

We discuss the patterns of mouse homeo-gene expression now observed in terms of models originally devised for *Drosophila*. We also propose how our new findings may help to explain any selective advantage to the vertebrates of homeo-gene duplication to form subfamilies.

Key words: mouse embryo, homeo-gene subfamily, *in situ* hybridization.

### Introduction

*In situ* hybridization studies have provided much circumstantial evidence to support the hypothesis that the *Antennapedia*- (*Antp*-) like homeobox-containing genes of the mouse, like those of *Drosophila* (e.g. Gehring, 1987), may play a role in pattern formation during embryogenesis. Thus, mouse homeo-genes are expressed in spatially restricted domains within the developing embryo. Different homeo-genes are usually found to be expressed in different domains, and these lie within a series of partially overlapping transcript domains along the body axis (e.g. Gaunt, 1988; Gaunt *et al.* 1988; Holland and Hogan, 1988a). The domains are first established before organogenesis within the ectoderm and mesoderm germ layers at 7½ days gestation (Gaunt *et al.* 1986; Gaunt, 1987, 1988; Mahon *et*

*al.* 1988), and subsequently they persist within the developing nervous system, the prevertebral column, and at least some of the organs at 12½ days.

Four separate clusters of *Antp*-like homeo-genes have been identified in the genome of the mouse. These are the Hox-1, Hox-2, Hox-3 and Hox-5 loci (e.g. Duboule and Dollé, 1989; Graham *et al.* 1989). The four clusters display similarities in their intergene spacing and nucleotide sequence, suggesting that they arose by duplication of a common ancestral cluster (Hart *et al.* 1987; Odenwald *et al.* 1987; Graham *et al.* 1988; Duboule *et al.* 1989). This ancestral cluster has ancient origins in animal evolution, and it is a related cluster of homeo-genes in *Drosophila* (Duboule and Dollé, 1989; Graham *et al.* 1989) that regulates pattern formation in this species. In both *Drosophila* and mouse, there is apparently a strict correspondence between the relative

position of homeo-genes within their clusters and that of their transcript domains within the developing embryo (for *Drosophila*: Harding *et al.* 1985; for mouse Hox-1: Gaunt *et al.* 1988; Duboule and Dollé, 1989; for Hox-2: Graham *et al.* 1989; for Hox-5: Duboule and Dollé, 1989).

It is not yet known why the mouse genome contains four related clusters of *Antp*-like homeo-genes. Each gene within a cluster has homologues in other clusters, and the term 'subfamily' has been used as the collective name for such a grouping of homologous genes (Hart *et al.* 1987; Duboule *et al.* 1989). Initial studies by Gaunt *et al.* (1988) indicated that homeo-genes within the same subfamily (Hox-1.4 and Hox-5.1; Hox-1.2 and Hox-6.1) display similar or identical positions of their transcript domains in the developing central nervous system and prevertebral column. However, recent studies have identified two genes within another subfamily that do not show similar domains of transcription: Hox-2.5 (Graham *et al.* 1989) and Hox-5.2 (Duboule and Dollé, 1989).

In this paper, we compare the transcript patterns of three genes from the Hox-1.4 subfamily (Hox-1.4, Hox-2.6 and Hox-5.1). Some aspects of our findings have been reported in earlier *in situ* hybridization studies (for Hox-1.4: Toth *et al.* 1987; Gaunt *et al.* 1988; for Hox-2.6: Graham *et al.* 1988, 1989; for Hox-5.1: Featherstone *et al.* 1988) but we have attempted in this work to provide an accurate comparison of Hox-1.4, -2.6 and -5.1 transcript patterns as detected on adjacent or nearby sections of the same embryo. We show that all three genes are closely similar in the limits of their transcript domains along the anteroposterior axis. Between genes, however, we note some striking stage- and tissue-dependent differences in the relative abundance of their transcripts.

## Materials and methods

### Preparation of embryo sections

Embryos were obtained from natural matings between F<sub>1</sub> (CBA×C57BL/6) mice. For aging of embryos, midday on the day of the vaginal plug was designated day ½ of pregnancy. Embryos of 8½ and 9½ days (but not 10½ and 12½ days) were fixed within their deciduae. Methods used for fixation, embedding and sectioning (7 µm thick sections) of embryos were all as previously described (Gaunt *et al.* 1986).

### Preparation and use of <sup>35</sup>S-labelled RNA probes

Labelled RNA probes (ribo-probes) were prepared to 3' regions of coding sequence after subcloning genomic DNA (Hox-1.4 and Hox-5.1) and cDNA (Hox-2.6) fragments into transcription vectors (pGEM-1, Promega; or Bluescript pKSM13+, Stratagene). For Hox-1.4 and Hox-5.1, ribo-probes were prepared from a sequence (about 700 bp) which extends 3' of the homeobox *Bg*III site (*Bg*III–*Hind*III fragments). For restriction maps of these genes, see Duboule *et al.* (1986, for Hox-1.4) and Featherstone *et al.* (1988, for Hox-5.1). Hox-2.6 ribo-probes were prepared from a sequence (about 700 bp) which extends 3' of the *Bam*HI site (at nucleotide 1147 in Fig. 2 of Graham *et al.* 1988). Each of the three ribo-probes used in *in situ* hybridization gave its own

characteristic pattern of labelling (as described in detail in this paper), showing that there was no significant cross-reactivity under the experimental conditions employed. Identical patterns of labelling to those now reported were also obtained by using a shorter Hox-1.4 probe that did not include any of the homeobox sequence (a probe of about 200 bp, representing the 3' end of the 700 bp probe: *Ava*I–*Hind*III fragment), and by using a longer (1.4 kb) Hox-2.6 probe that included the 700 bp sequence described above but which also extended 5' up to the homeobox *Bg*III site. Although not shown in this paper, specificity of the probes for their respective genes has also been established by us in Northern blotting experiments.

For use in *in situ* hybridization, <sup>35</sup>S-labelled antisense RNA probes were synthesised in a direction opposite to that of normal transcription, as described by Gaunt *et al.* (1986) and Gaunt (1987). Control (sense) probes, synthesised in the direction of normal transcription, gave no specific labelling in *in situ* hybridization. The three gene probes were used in *in situ* hybridization experiments at similar specific activities and concentrations. Methods used for alkaline hydrolysis of labelled probes, *in situ* hybridization and autoradiography were all as previously described (Gaunt *et al.* 1986).

## Results

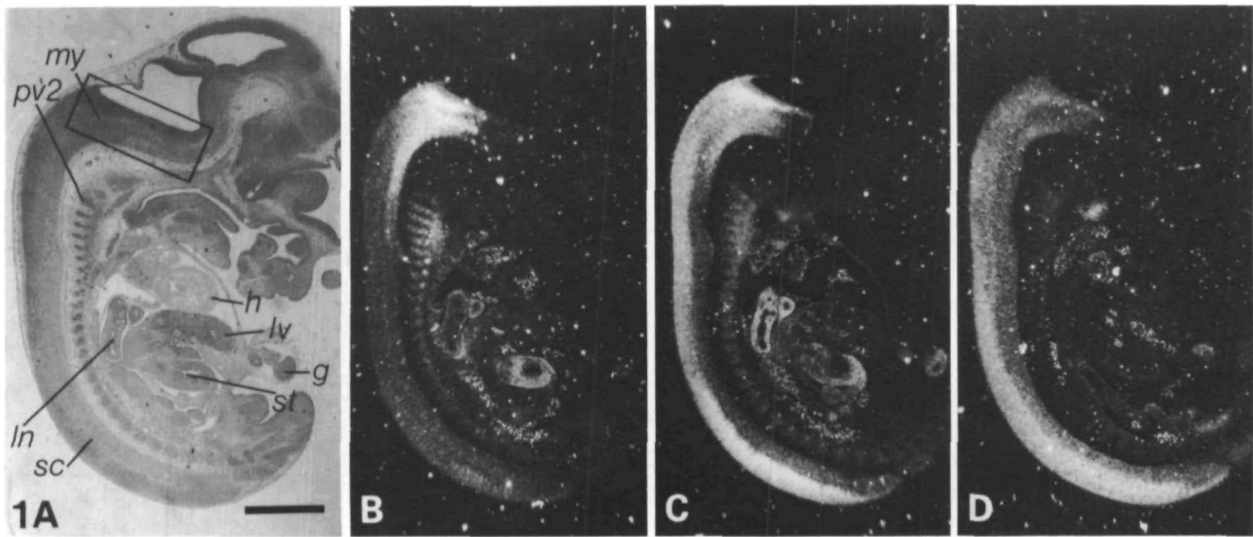
### The central nervous system at 12½ days

Hox-1.4, -2.6 and -5.1 transcript domains in the central nervous system were examined and compared in near-sagittal sections (Figs 1, 2).

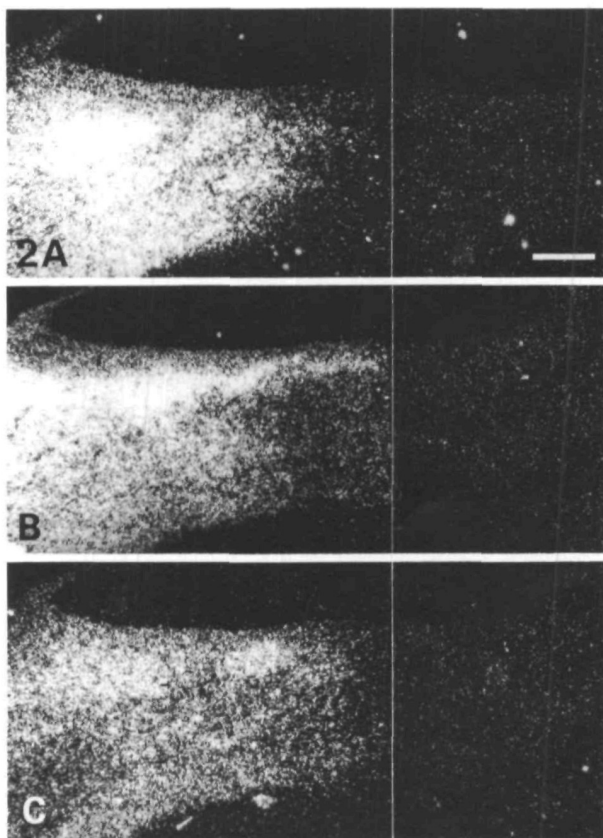
For each of the three genes, the anterior boundary of transcripts was found in the mid-myelencephalon. Between genes, small differences were found in the exact position and shape of the boundaries. Thus, the Hox-1.4 boundary (Fig. 2A) was located slightly posterior to the Hox-2.6 (Fig. 2B) and Hox-5.1 (Fig. 2C) boundaries, and a characteristic feature of the Hox-2.6 boundary was a dorsal tongue of strong labelling which usually extended anterior to the most anterior position of the Hox-5.1 boundary. From comparison with adjacent sections hybridized to a Hox-1.5 probe (not shown), the tongue of Hox-2.6 labelling did not extend as far forwards as the position of the Hox-1.5 boundary (e.g. Gaunt *et al.* 1988).

Posterior to the hindbrain boundaries, Hox-2.6 and Hox-5.1 probes usually gave an approximately constant intensity of labelling along the entire length of the central nervous system, but transcripts of both genes were often seen to be most concentrated in dorsal parts of the spinal cord (Figs 1C,D). In contrast, Hox-1.4 transcripts showed a marked anterior-to-posterior fall in abundance over the anterior region of the spinal cord (Fig. 1B). This fall in transcript abundance was anterior to prevertebra 1 (pv1) in dorsal parts of the spinal cord, but posterior to pv5 in ventral parts. Posterior to the level of pv6, the abundance of Hox-1.4 transcripts was low in comparison to those of Hox-2.6 and Hox-5.1.

Since the abundance of homeo-gene transcripts may vary across the lateral and dorsoventral axes of the spinal cord (e.g. Utset *et al.* 1987; Toth *et al.* 1987; Holland & Hogan, 1988b), a complete analysis of Hox-1.4, -2.6 and -5.1 transcripts in the central nervous system would require examination of nervous tissue in



**Fig. 1.** Hox-1.4 (B), Hox-2.6 (C) and Hox-5.1 (D) transcripts detected by *in situ* hybridization within complete parasagittal sections of the 12½ day mouse embryo. (A) Bright-field, (B–D) dark-field illumination. The figure shows nearby sections cut from the same embryo. my, myelencephalon; pv2, prevertebra 2, ln, lung; sc, spinal cord; st, stomach; g, gut; lv, liver; h, heart. Box, area examined in Fig. 2. Bar, 1.0 mm.



**Fig. 2.** Anterior boundaries of homeo-gene transcript domains within the 12½ day central nervous system. (A) Hox-1.4; (B) Hox-2.6; (C) Hox-5.1. The hybridizations were made to nearby parasagittal sections, and the fields shown here under dark-field illumination are outlined on the bright-field view of the whole embryo (Fig. 1A). Bar, 0.2 mm.

all planes of section. We have not yet undertaken such a detailed comparison.

*The prevertebral column at 12½ days*

For each of the three genes, transcripts were most abundant within the cervical and anterior thoracic prevertebrae (Figs 1, 3). As judged from the density of silver grains, the intensities of prevertebral labelling given by the Hox-1.4 and Hox-2.6 probes were clearly greater than that given by the Hox-5.1 probe (Table 1).

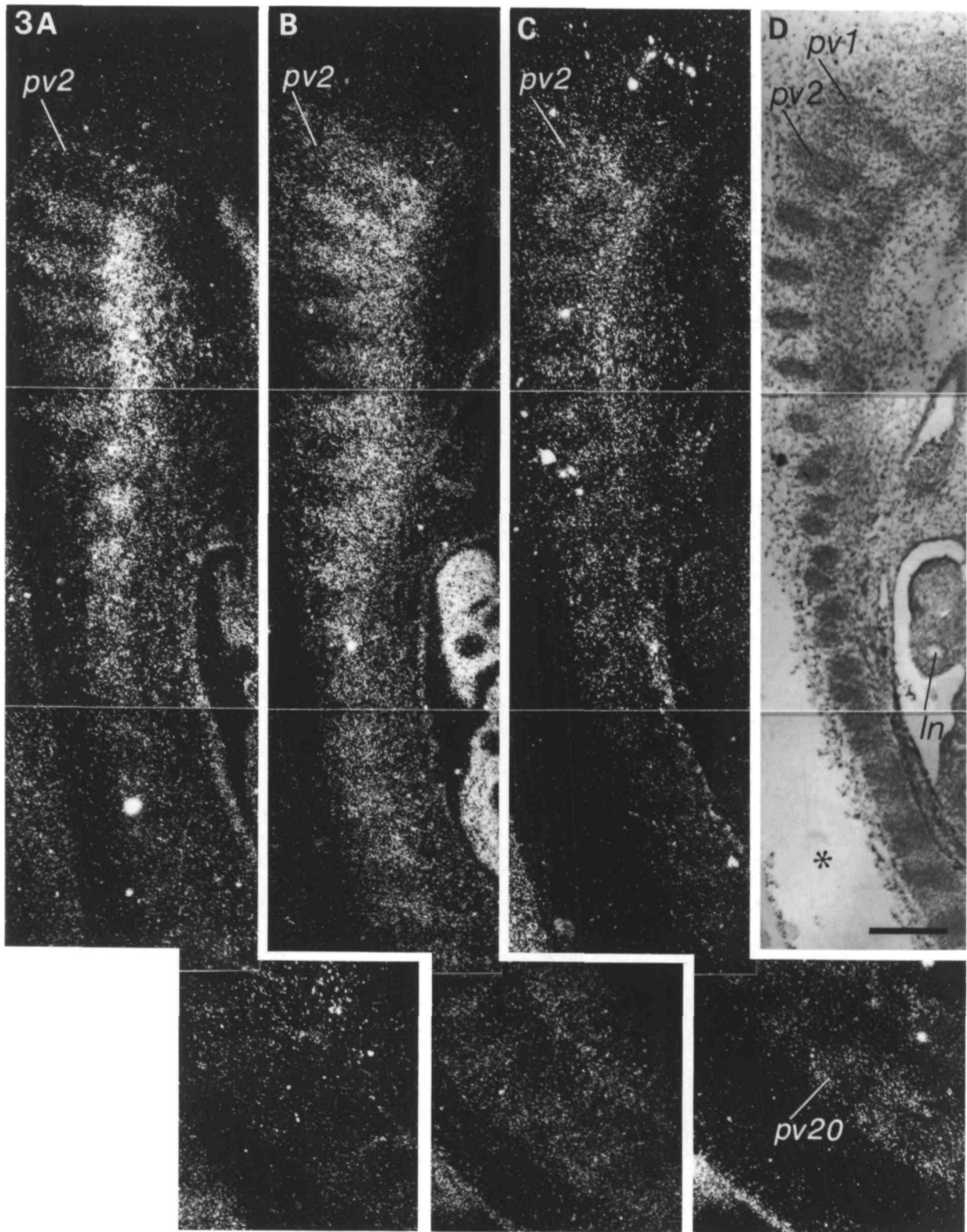
Hox-1.4 transcripts (Fig. 3A) were absent in pv1, weak in pv2, and then strong in pv3. Hox-2.6 (Fig. 3B) and Hox-5.1 (Fig. 3C) transcripts were weakly detected in pv1, especially in ventral parts, and were then strong in pv2. Thus, the anterior boundary of the Hox-1.4 transcript domain was apparently located slightly posterior to that of Hox-2.6 and Hox-5.1. Pv1 showed, when cut in most planes of section, a lower cell density than other cervical prevertebrae (Figs 3D, 4C). Lower cell density did not account for the low labelling intensities detected for Hox-1.4, -2.6 and -5.1 since pv1 displayed strong labelling in parallel control experiments using the Hox-1.5 probe (Fig. 4B; see also Gaunt, 1988).

For Hox-1.4, a gradual and progressive decline in the abundance of transcripts was noted posterior to pv7, but the level of labelling remained above background even within the lumbar region (Figs 1B, 3A). For Hox-2.6, the anterior-to-posterior decline was more gradual (Figs 1C, 3B). For Hox-5.1, labelling intensity had fallen to background level by pv14 but a second, more posterior domain of prevertebral labelling was detected in the lumbar region (Figs 1D, 3C). In this posterior Hox-5.1 domain, labelling intensity increased from pv20 to pv25, declined from pv27 to pv30, and was

then weak in more posterior parts. For Hox-2.6, but not Hox-1.4, we also noted a very slight increase in the abundance of transcripts in the lumbar region.

*Pharyngeal, thoracic and abdominal organs at 12½ days*

In the pharyngeal region, in planes just lateral to



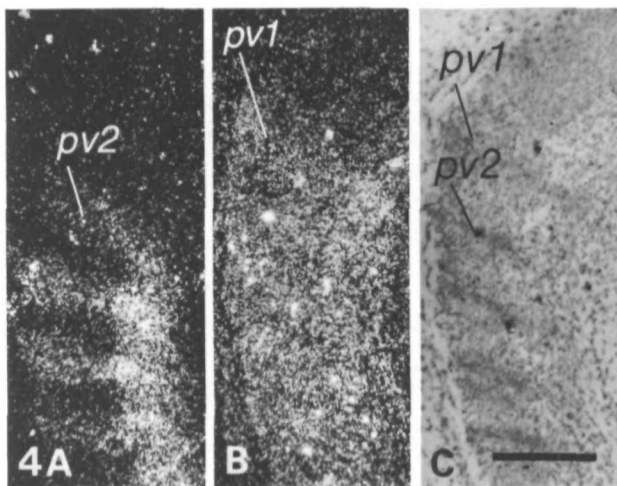
**Fig. 3.** Homeo-gene transcript domains within the 12½ day prevertebral column and lung. (A) Hox-1.4; (B) Hox-2.6; (C) Hox-5.1. (A-C) Dark-field, (D) bright-field illumination. The hybridizations were made to nearby parasagittal sections. pv1, pv2, pv20, prevertebrae 1, 2 and 20; In, lung; \*, artefactual space generated by tearing of mesenchymal tissue between the prevertebral column and the spinal cord. Bar, 0.2 mm.

**Table 1.** Tissue- and stage-dependent differences in the abundance of *Hox-1.4*, *-2.6* and *-5.1* transcripts

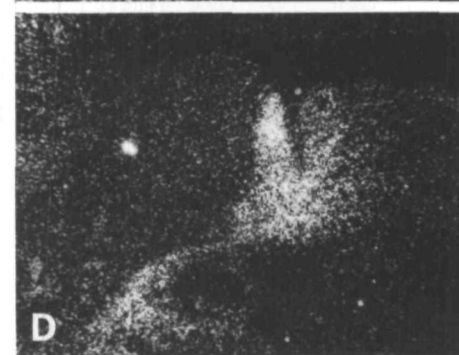
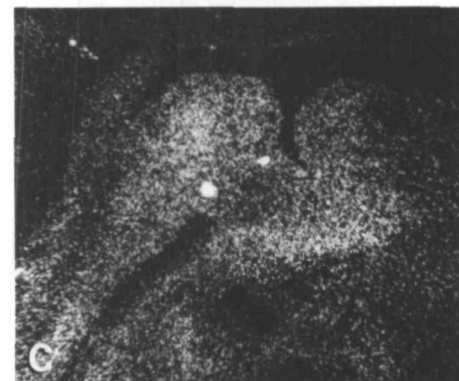
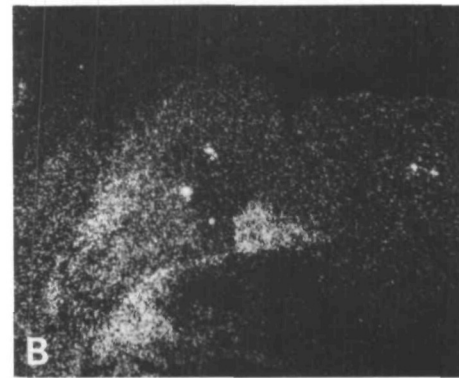
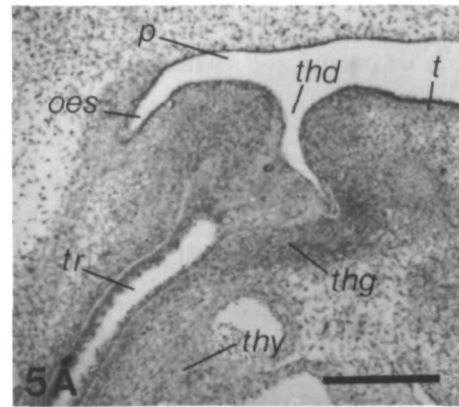
Tissue	Grain densities		
	<i>Hox-1.4</i>	<i>Hox-2.6</i>	<i>Hox-5.1</i>
12½ d prevertebra*	+++	+++	++
12½ d lung	++	+++	-
12½ d stomach	+++	+++	-†
12½ d mesonephros	+	++	+++
12½ d metanephros	+	++	+++
12½ d testis	-	-	++
8½ d neurectoderm	+	++	+++
9½ d mesoderm	+	++	+++
10½ d lung	nt	+++	+
10½ d stomach	nt	+++	+

Grain densities in autoradiograms were measured by counting silver grains over unit areas of photographs. Most of the photographs used are presented in this paper. Results consistent with those shown were obtained in different experiments. Grain densities were scored as follows: -, ≤ background count over the forebrain, tongue, liver or decidual tissue; +, 2-4× background; ++, 4-8× background; +++, >8× background. For the lung, stomach and testis, counts were made over the outer mesodermal components. At 8½ and 9½ days, counts were made over posterior regions of neurectoderm and mesoderm tissue. \* grains were counted over the ventral part of prevertebra 3. † grains within discrete patches (assumed to be overlying autonomic ganglia) were not counted. nt, not tested.

sagittal, each of the three genes displayed a characteristic and reproducible pattern of transcripts. *Hox-1.4*, as described earlier (Gaunt *et al.* 1988), was detected in the thyroid gland, the mesodermal derivatives (but not the lining endothelium) of the trachea, and in a structure that we tentatively identify as the thymus (Fig. 5B). In a nearby section, *Hox-2.6* transcripts (Fig. 5C) were detected in all of these *Hox-1.4*-labelled structures and, in addition, were found in the pharyngeal floor posterior and anterior to the thyroid duct. *Hox-2.6* transcripts did not, however, extend over the



**Fig. 4.** Anterior boundaries of *Hox-1.4* (A) and *Hox-1.5* (B) transcripts within the 12½ day prevertebral column. (A, B) Dark-field, (C) bright-field illumination. The hybridizations were made to nearby parasagittal sections. pv1, pv2, prevertebrae 1 and 2. Bar, 0.2 mm.



**Fig. 5.** *Hox-1.4* (B), *Hox-2.6* (C) and *Hox-5.1* (D) transcript patterns within the pharyngeal region of the 12½ day embryo. (A) Bright-field, (B-D) dark-field illumination. The hybridizations were made to nearby parasagittal sections. p, pharynx; oes, oesophagus; tr, trachea; thy, structure that we tentatively identify as the thymus; thg, thyroid gland; t, tongue; thd, thyroid duct. Bar, 0.2 mm.

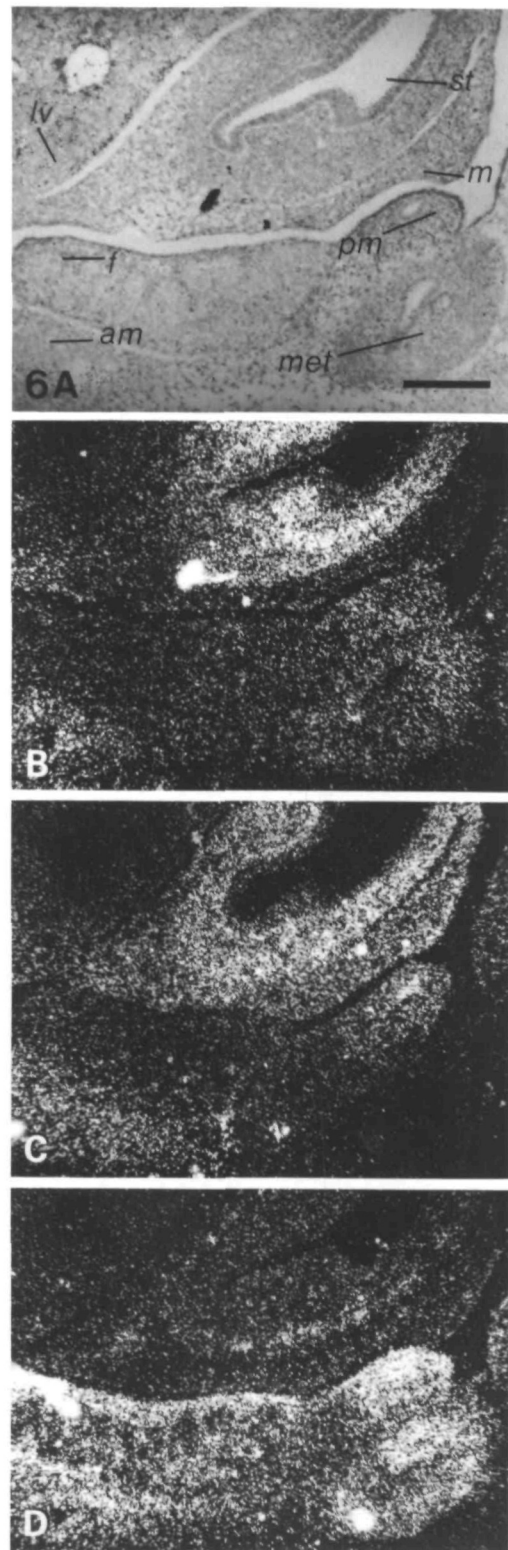
tongue. This pattern for Hox-2.6 is similar or identical to the pattern detected earlier for Hox-1.5 (Gaunt, 1988). Hox-5.1 transcripts (Fig. 5D) were detected in the thyroid and 'thymus' glands and in the floor of the pharynx posterior and anterior to the thyroid duct. In contrast to the results for Hox-1.4 and Hox-2.6, Hox-5.1 transcripts were not detected in the trachea. Hox-5.1 transcripts were also absent in posterior parts of the pharyngeal floor. We consider, however, that the Hox-2.6 labelling found in this region (and also found for Hox-1.4, but not in the plane of section shown in Fig. 5B; see Gaunt *et al.* 1988) could simply be due to transcripts present in anterior parts of the tracheal wall.

Hox-1.4 and Hox-2.6 transcripts were detected in the mesodermal derivatives, but not the lining endothelium, of the lung (Fig. 3A,B). These findings are consistent with observations published earlier for Hox-1.4 (Gaunt *et al.* 1988) and for Hox-2.6 (Graham *et al.* 1988), and they are typical of the transcript distribution found within the lung for several homeo-genes (e.g. Gaunt *et al.* 1988). Transcripts within the lung were more abundant for Hox-2.6 than for Hox-1.4 (Table 1). The Hox-5.1 probe gave only background levels of labelling over the 12½ day lung (Fig. 3C; Table 1).

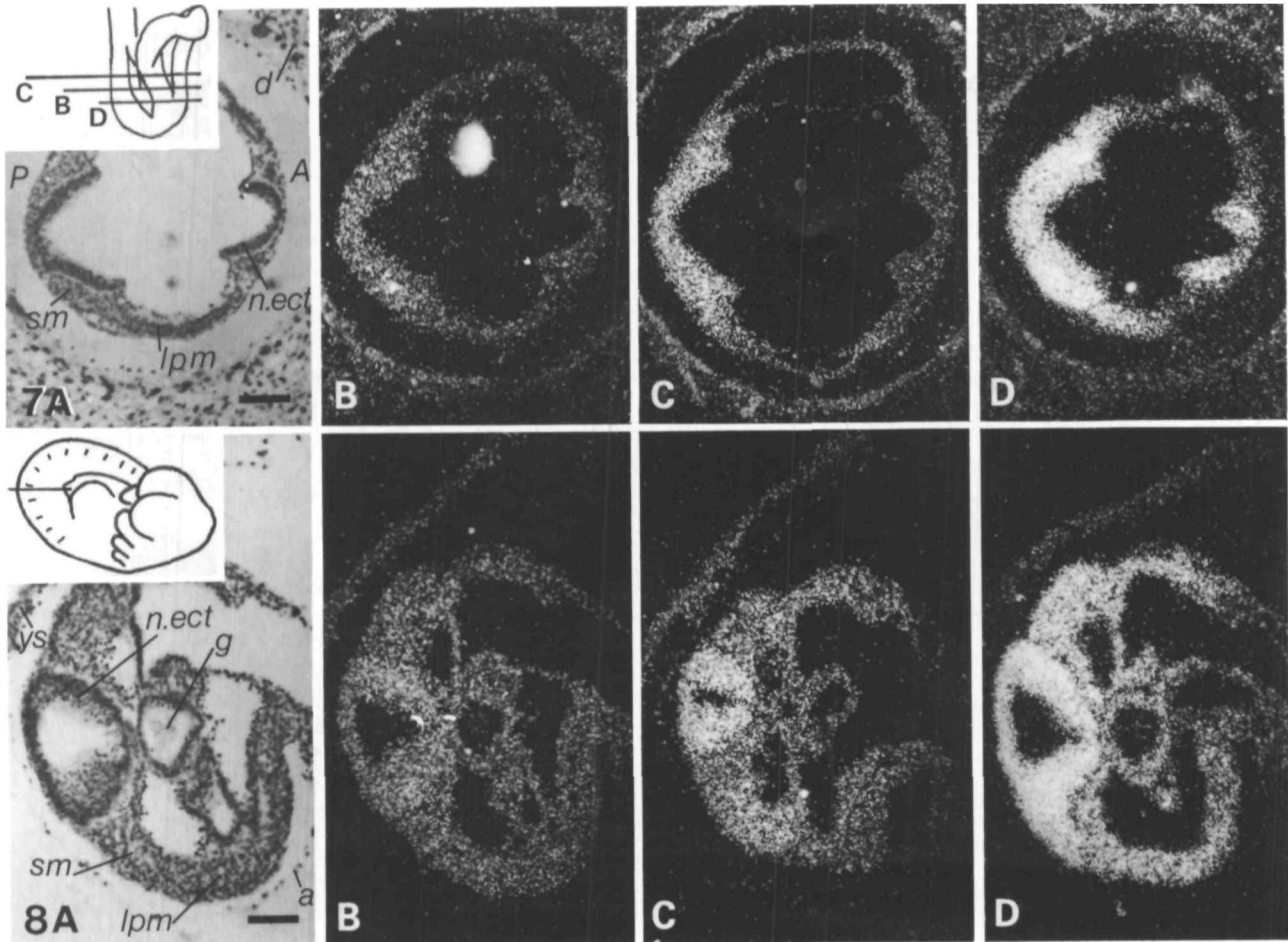
Hox-1.4 and Hox-2.6 transcripts were abundant in the mesodermal derivatives, but not the lining endothelium, of the stomach (Fig. 6B,C; Table 1). This finding is in agreement with earlier reports (for Hox-1.4; Gaunt *et al.* 1988; for Hox-2.6: Graham *et al.* 1988) and is typical of the transcript distribution found in the stomach wall for a variety of other mouse homeo-genes (e.g. Gaunt *et al.* 1988). For Hox-2.6, but not Hox-1.4, weaker labelling was also detected in the mesentery underlying the stomach (Fig. 6C). In contrast to the results for Hox-1.4 and Hox-2.6, Hox-5.1 transcripts in the stomach were restricted to patches located in a peripheral zone of the wall (Fig. 6D). An identical pattern of punctate labelling has been noted before for Hox-2.1 transcripts in the intestine (Holland and Hogan, 1988b). This pattern probably indicates that Hox-5.1 transcripts in the 12½ day stomach are restricted to neural-crest-derived parasympathetic ganglia of the enteric plexus (Holland and Hogan, 1988b), and are absent from mesodermal components (Table 1). The descriptions now given for the distributions of Hox-1.4, -2.6 and -5.1 transcripts within the stomach are also applicable to the patterns of transcripts observed in more posterior regions of the intestine (not shown). The liver was not labelled above background by any of the three gene probes (Fig. 6).

Both mesonephric and metanephric kidneys were labelled by all three gene probes (Fig. 6B,C,D). The metanephric kidney is not included within the section shown for Hox-2.6 (Fig. 6C) but was seen to be labelled in neighbouring sections. The most intense labelling within kidney tissue was given by the Hox-5.1 probe; the least intense labelling was given by the Hox-1.4 probe (Table 1).

At 12½ days, the gonad lies on the inner surface of the mesonephros (Fig. 6A). In the embryo under illustration, the presence of developing testis cords (which



**Fig. 6.** Hox-1.4 (B), Hox-2.6 (C) and Hox-5.1 (D) transcript patterns in the stomach, testis, mesonephric and metanephric kidneys of the 12½ day embryo. (A) Bright-field, (B–D) dark-field illumination. The hybridizations were made to nearby parasagittal sections. lv, liver; st, stomach; m, mesentery; pm, anterior and posterior parts of mesonephric kidney; met, metanephric kidney; f, testis; am, anterior part of mesonephric kidney. Bar, 0.2 mm.



**Figs 7, 8.** Homeo-gene transcripts within lateral plate mesoderm, somitic mesoderm and neurectoderm of 8½ day (Fig. 7) and 9½ day (Fig. 8) embryos. (B) Hox-1.4; (C) Hox-2.6; (D) Hox-5.1. (A) Bright-field, (B–D) dark-field illumination. The hybridizations were made to nearby transverse sections, and the planes of section are shown diagrammatically in 7A and 8A. Diagrams are based on figures provided by Rugh (1968). A, anterior; P, posterior; sm, somitic mesoderm; lpm, lateral plate mesoderm; n.ect., neurectoderm; d, decidual tissue; ys, yolk sac; a, amnion; g, gut. Bars, 0.1 mm.

contain primordial germ cells and Sertoli cells; Fig. 6A) indicate that the gonad is a testis. No specific labelling of the 12½ day testis was given by the Hox-1.4 or Hox-2.6 probes (Figs 6B,C). However, strong labelling of the testis was given by the Hox-5.1 probe. Hox-5.1 transcripts were located mainly within the peripheral mesenchymal tissue of the testis, and were less intense or absent within the testis cords (Fig. 6D). This distribution of transcripts within the developing testis is similar to that found earlier for Hox-5.2 (Dollé and Duboule, 1989) and Hox-6.1 (Sharpe *et al.* 1988).

#### *Somitic and lateral plate mesoderm at 8½ and 9½ days*

The mesodermal component of the 12½ day trachea, lung and gut is derived from the lateral plate mesoderm of earlier stages (e.g. Hogan *et al.* 1985). It has been assumed (e.g. Gaunt, 1988) that cells that express a homeo-gene at 12½ days are derived from cells that express the gene at much earlier stages of development. Following this assumption, we anticipated that Hox-1.4 and Hox-2.6 transcripts, abundant in the 12½ day trachea, lung and gut mesoderm, would be readily

detectable in the lateral plate mesoderm at 8½ to 9½ days. We further reasoned that the absence of Hox-5.1 transcripts in lateral plate mesoderm derivatives at 12½ days (e.g. mesodermal components of the trachea, lung and gut) might follow an earlier absence of Hox-5.1 expression in the lateral plate mesoderm at 8½ to 9½ days.

To test the above hypotheses, we examined Hox-1.4, -2.6 and -5.1 transcript distributions in serial transverse sections of 8½ and 9½ day embryos. Unexpectedly, we found that all three genes were expressed in both somitic and lateral plate mesoderm (Figs 7, 8). In all cases, transcripts were confined to more posterior parts. Hox-5.1 transcripts (absent from mesodermal derivatives of the gut wall at 12½ days) were clearly detected in lateral plate mesoderm as it contacted and surrounded gut endoderm at 9½ days (Fig. 8D). Examination of additional serial transverse sections taken at all positions through 8½ and 9½ day embryos (not shown) did not reveal any clear differences between the three genes in the spatial limits of their transcripts. Hox-5.1 transcripts at these stages were present in lateral plate

mesoderm, somitic mesoderm and neurectoderm at greater abundance than Hox-2.6 or Hox-1.4 transcripts (Table 1).

#### *Mesodermal components of the lung and stomach at 10½ days*

From the above findings, we considered it likely that the founder cells of lung and stomach mesoderm, present within the lateral plate mesoderm at 8½ to 9½ days, may be strongly positive for Hox-5.1 transcripts, but that with continued development to 12½ days there is selective loss of Hox-5.1 transcripts within these cell lineages. In contrast, we considered it likely that Hox-1.4 and Hox-2.6 transcripts may increase in abundance within these cell lineages. To test these hypotheses, we examined the 10½ day lung and stomach for Hox-5.1 and Hox-2.6 transcripts.

In contrast to results obtained for the 12½ day lung (Fig. 3C), the mesodermal component of the lung at 10½ days was found to be labelled by the Hox-5.1 probe (Fig. 9E; Table 1). The labelling was weak, but was clearly greater than the background labelling seen over the adjacent lining endothelium. Hox-2.6 transcripts were abundant within mesodermal derivatives of the 10½ day lung (Fig. 9C; Table 1).

At 10½ days, labelling given by the Hox-5.1 probe was weakly but uniformly distributed over the mesodermal components of the stomach wall (Fig. 9F; Table 1). The pattern of labelling observed was clearly different to the punctate pattern confined to peripheral parts of the stomach wall at 12½ days (Fig. 6D). The lining endothelium of the 10½ day stomach was not labelled by the Hox-5.1 probe. Hox-2.6 transcripts were abundant within mesodermal derivatives of the 10½ day stomach (Fig. 9D; Table 1).

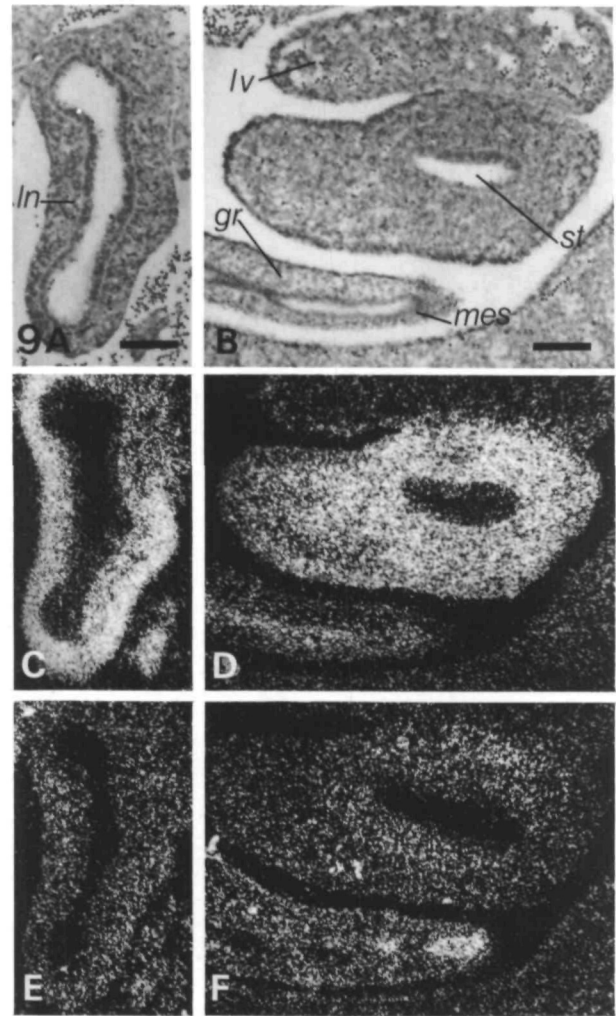
## Discussion

### *Similarities in the positions of the Hox-1.4, -2.6 and -5.1 transcript domains*

Within the prevertebral column of the 12½ day mouse embryo, the anterior boundary of the transcript domain for each of the three genes examined was most obvious at the junction of pv1 and pv2. The exact position of the boundary was not, however, identical for all three genes, and was slightly more posterior for Hox-1.4 (which showed weak labelling of pv2 relative to pv3) than for Hox-2.6 and Hox-5.1 (which also showed some weak labelling in pv1). All three genes showed greatest abundance of transcripts over cervical prevertebrae 3–7, and an anterior-to-posterior decline of transcripts over the thoracic prevertebrae.

Within the central nervous system, the anterior boundaries of the Hox-1.4, -2.6 and -5.1 transcript domains were located in the mid-myelencephalon. As in the prevertebral column, the boundary for Hox-1.4 was apparently located at a position slightly posterior to that of Hox-2.6 and Hox-5.1.

These observations are consistent with results published earlier for Hox-1.4 and Hox-5.1 transcript



**Fig. 9.** Hox-2.6 (C,D) and Hox-5.1 (E,F) transcript patterns in the lung and stomach of the 10½ day embryo. (A,B) Bright-field, (C–F) dark-field illumination. The hybridizations were made to nearby parasagittal sections. ln, lung; lv, liver; gr, genital ridge; mes, mesonephros; st, stomach. Bar, 0.1 mm.

domains within the prevertebral column and central nervous system (for Hox-1.4: Gaunt *et al.* 1988; for Hox-5.1: Featherstone *et al.* 1988), and for Hox-2.6 within the central nervous system (Graham *et al.* 1988, 1989) but they are an advance on earlier work since they permit accurate comparison of the domains on nearby sections of the same embryo.

The findings are in accordance with an earlier suggestion (Gaunt *et al.* 1988) that mouse homeo-genes within a subfamily display similar transcript domains in the embryo. Some additional support for this is also given by the apparently similar transcript boundaries displayed in the myelencephalon by Hox-1.5 (e.g. Gaunt *et al.* 1988) and Hox-2.7 (Graham *et al.* 1989). However, two members of another subfamily, Hox-2.5 (Graham *et al.* 1989) and Hox-5.2 (Duboule and Dollé, 1989) have clearly shown marked differences in the anterior boundaries of their transcripts within the central nervous system. Taken together, the findings suggest that



subfamily similarities in transcript patterns (as have been found for members of the Hox-1.2, -1.4 and -1.5 subfamilies) may be a feature only of those genes that are expressed in anterior parts of the embryo, and are not a feature of genes expressed in more posterior parts (such as Hox-2.5 and Hox-5.2).

*Tissue- and stage-dependent differences in the relative abundance of Hox-1.4, -2.6 and -5.1 transcripts*

In addition to the similarities noted above, there were also some striking differences between the transcript patterns of Hox-1.4, -2.6 and -5.1. At 12½ days, these were observed mainly within some pharyngeal, thoracic and abdominal organs. These differences in labelling patterns have a practical value in demonstrating that there is no significant cross-reactivity between the three gene probes used in *in situ* hybridization.

Within pharyngeal tissue, Hox-2.6 and Hox-5.1 transcripts, unlike Hox-1.4 transcripts, extended anterior to the thyroid duct. This may be a consequence of the slightly more anterior position observed for Hox-2.6 and Hox-5.1 transcripts in the myelencephalon, since it is neural crest cells from this region that give rise to the mesenchymal tissues of the pharyngeal floor (Le Douarin, 1982).

Within mesodermal derivatives of the 12½ day trachea, lung and gut, Hox-1.4 and Hox-2.6 transcripts, but not Hox-5.1 transcripts, were readily detected. Conversely, Hox-5.1 transcripts, but not Hox-1.4 or Hox-2.6 transcripts, were abundant in the mesenchymal component of the 12½ day gonad. In addition to these qualitative differences in the patterns of Hox-1.4, -2.6 and -5.1 expression, quantitative differences in transcript abundance were also noted. As shown, for example, in Table 1, the 12½ day prevertebral column, mesonephric and metanephric kidneys each displayed clear differences in the relative abundance of Hox-1.4, -2.6 and -5.1 transcripts.

In addition to these tissue-dependent differences noted at 12½ days in the relative abundance of Hox-1.4, -2.6 and -5.1 transcripts, stage-dependent differences were also found (Table 1). Thus, although Hox-5.1 transcripts were apparently absent from the mesodermal component of the lung and stomach at 12½ days, Hox-5.1 transcripts were clearly detectable in these tissues at 10½ days. Lung and stomach mesoderm are derivatives of the lateral plate mesoderm present at earlier stages (e.g. Hogan *et al.* 1985). Lateral plate mesoderm of 8½ and 9½ day embryos was labelled strongly by the Hox-5.1 probe. We conclude, therefore, that Hox-5.1 transcripts displayed a fall in abundance from 8½ to 12½ days in tissues destined to form the mesodermal components of the lung and stomach. In contrast, Hox-1.4 and Hox-2.6 transcripts within these tissues displayed a rise in abundance from 8½ to 12½ days. Similarly, Hox-5.1 transcripts displayed fall in abundance within tissues that give rise to the prevertebral column (since the Hox-5.1 probe gave strong labelling of somitic mesoderm at 8½ days, but weak labelling of prevertebrae at 12½ days), while Hox-1.4 and Hox-2.6 transcripts displayed a rise in abundance

within these tissues. These observations suggest, but do not prove, that genes within the Hox-1.4 subfamily might be coordinately regulated in their patterns of expression.

Two quite separate mechanisms could account for these tissue- and stage-dependent differences in expression now observed between genes within the Hox-1.4 subfamily. As one possibility, homeo-gene expression might be modulated within a cell lineage in a tissue- and stage-dependent way. This assumes, for example, that transcripts from each of the three genes are present at 8½ to 9½ days within all cells of the posterior lateral plate mesoderm. Then, with further development and divisions of one cell to form, say, mesodermal components of the stomach, transcript abundance falls for Hox-5.1 but rises for Hox-1.4. As a second possibility, Hox-1.4, -2.6 and -5.1 might be expressed in separate cell populations within the lateral plate mesoderm. Mesodermal components of the stomach would then develop from Hox-1.4 and Hox-2.6, but not Hox-5.1, -positive subpopulations. The *in situ* hybridization technique does not permit distinction between these two possibilities since it does not permit us to localize homeo-gene expression within individual cells.

We do not favour the second of the mechanisms discussed above. Most of the cells within the lateral plate mesoderm would, in this model, presumably be of the Hox-5.1-positive subpopulation. Yet Hox-5.1-positive cells apparently contribute little or nothing to derivatives of lateral plate mesoderm at 12½ days. Results obtained in *Drosophila* show that expression of homeo-genes may be modulated in a tissue- and stage-dependent way. This is consistent with the first of our above proposals for the mouse. As one example of modulation of homeo-gene expression in *Drosophila*, some epidermal cells of parasegment 5 (ps5) initially express both *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) transcripts, but at later stages groups of the cells express either *Antp* alone or *Ubx* alone (Carroll *et al.* 1988). As a second example of modulation, the epidermal cells of ps3 apparently switch from expressing *Antp* at early stages to *Sex combs reduced* (*Scr*) at later stages (Martinez-Arias *et al.* 1987; Carroll *et al.* 1988). In the following section, we assume that the changing patterns of homeo-gene transcripts now observed in tissues of the developing mouse are due to modulations in gene expression.

*Mouse homeo-gene transcript patterns considered in terms of Drosophila models*

It is of interest to consider two models that have been proposed to describe homeotic gene activity in *Drosophila*. Both models may be applicable to observations made on the expression of mouse homeo-genes. The two models are related, and the second is able to accommodate observations made on modulation of homeo-gene expression.

The Lewis model (Lewis, 1978; Lawrence and Morata, 1983), originally suggested for genes of the Bithorax complex, proposes that each homeotic gene is

switched on according to position along the anteroposterior axis of the embryo. The genes are postulated to be expressed in a series of partially overlapping domains. This gives each body segment a different combination of active homeotic genes, and it is this combination that defines segment identity. As earlier discussed (Gaunt, 1988; Gaunt *et al.* 1988), the patterns of embryonic expression observed for several different mouse homeo-genes conform closely to these predictions of Lewis's model. Indeed, the patterns for mouse genes fit better than do those of some *Drosophila* genes, especially those expressed in more anterior parts of the fly (e.g. *Labial*, *Proboscipedia* and *Scr*: Mahafey *et al.* 1989).

The 'open for business' model (Akam *et al.* 1988; Peifer *et al.* 1988) conforms to the formal structure of Lewis's model, but differs from it in proposing that each homeotic gene is made accessible for transcription, or 'open for business', according to position along the anteroposterior axis of the embryo (rather than being simply switched on as in Lewis's model). The molecular mechanism by which this is achieved is unknown (but see Akam *et al.* 1988). Modulations of homeo-gene expression in *Drosophila* are not easily reconciled with Lewis's model, but they can be accommodated by the new model (see Akam *et al.* 1988). This model may similarly be of value in describing some of the new features of mouse homeo-gene expression now reported in this paper. Thus, Hox-1.4, -2.6 and -5.1 might best be considered as 'open for business' within the same spatial domain along the anteroposterior axis of the mouse embryo. The stage- and tissue-dependent modulations in expression that we have seen in structures within the domain, such as mesodermal components of the stomach and lung, might then be a consequence of other factors (so far unknown) which determine whether or not, or to what extent, a gene 'open for business' is actually switched on.

#### *The selective advantage of homeo-gene duplication to form subfamilies*

We, and others, have suggested that mouse homeo-gene expression might be required for specification of tissue identities according to position along the anteroposterior axis (e.g. Gaunt *et al.* 1988). We have now provided evidence that Hox-1.4, -2.6 and -5.1, derivatives of a common ancestral gene, have evolved to display both similarities and differences in their patterns of expression. Although we do not discount the possibility that the similarities found might indicate some overlap and redundancy of function between these genes, we suggest below two possible ways in which the differences observed might usefully increase the variety of instructions available in the developing embryo.

First, although all three subfamily members show basically similar domains of transcription, some small differences in their anterior boundaries were noted. These might specify fine differences in position along the anteroposterior axis. Second, at any given position along the anteroposterior axis of the embryo, a multi-

plicity of different structures pursue different pathways of development. We consider, therefore, that some mechanism must exist to provide different developmental instructions to different tissues located at the same anteroposterior position. Theoretically, a single homeo-gene could provide a variety of different instructions by changes in the nature or abundance of its transcripts. But we suggest that stage- and tissue-dependent differences in expression within a group of homeo-genes possessing basically similar domains of transcription (as we have now found for genes of the Hox-1.4 subfamily) could potentially provide a much wider variety of developmental instructions effective at the same anteroposterior position.

We propose that one or both of the above mechanisms could provide a selective advantage to species evolving a more complex body structure, and could explain, at least in part, the advantage for the vertebrates to have duplicated the ancestral homeo-gene cluster (Duboule and Dollé, 1989; Graham *et al.* 1989; Akam, 1989). In terms of this hypothesis, less complex species, including *Drosophila*, would not require more than a single cluster.

Following from this, we can suggest a possible explanation as to why subfamily similarities in expression domains are not also observed for homeo-genes expressed in more posterior parts of the mouse embryo (Hox-2.5 and Hox-5.2; Graham *et al.* 1989; Duboule and Dollé, 1989). Posterior parts, being less complex than anterior parts, may not require such a wealth of developmental instructions effective at similar positions along the anteroposterior axis. Following duplication of the ancestral homeo-gene cluster, there would therefore have been less selective pressure upon 'posterior' genes to conserve the position of their transcript domains. Boundaries for posterior genes could then shift to acquire new positions along the anteroposterior axis. We consider, therefore, that posterior genes of the Hox clusters might be viewed as forming collectively a unique, functional homeo-gene complex.

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