Spatially restricted patterns of expression of the homeobox-containing gene *Hox 2.1.* during mouse embryogenesis

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Summary

The mouse *Hox 2.1* gene contains a homeobox sequence and is therefore a candidate for a vertebrate gene involved in the control of embryonic patterning or positional specification. To investigate this possibility, we have used *in situ* hybridization to determine the pattern of *Hox 2.1* expression during mouse embryogenesis. At 8·5 days *post coitum*, Hox 2.1 is expressed at a low level in the posterior neuroectoderm and mesoderm, and in the neuroectoderm of the presumptive hindbrain. At 12·5 days *p.c.*, Hox 2.1 is expressed in an anteroposterior restricted domain extending from the hindbrain throughout the length of the spinal cord, predominantly in the dorsal region. Between 12·5 and 13·5 days *p.c.* the domain becomes localized to the occipital and cervical regions. We also detect *Hox 2.1* RNA in the embryonic lung, stomach,

mesonephros and metanephros, as well as in myenteric plexus, dorsal root ganglia and the nodose ganglion, and in mature granulocytes. The embryonic expression of *Hox 2.1* in neural tissue is compared with that of *Hox 3.1*, which also shows anteroposterior restricted domains of gene expression. These patterns of expression are not clearly consistent with *Hox 2.1* or *Hox 3.1* having roles in segmental patterning. However, the data are consistent with these genes having regulatory roles in anteroposterior positional specification in the neuroectoderm and mesoderm, and suggest that *Hox 2.1* may also have functions during organogenesis.

Key words: homeobox, *Hox 2.1*, mouse embryo, *in situ* hybridization, gene expression, spinal cord, mesoderm.

Introduction

The homeobox is a conserved DNA sequence of approximately 183 bp, present within the coding region of several genes in the fruitfly, *Drosophila*. At least 18 homeobox-containing genes have been identified in *Drosophila*, most of which correspond to previously characterized developmental genes (for reviews see Gehring, 1985, 1987). These include genes implicated in the control of body pattern (e.g. pair-rule segmentation genes) and positional specification (e.g. homeotic genes).

Homeobox-containing genes are present in a wide range of metazoa, including vertebrates (McGinnis, 1985; Holland & Hogan, 1986; Colberg-Poley *et al.* 1987), but as yet their functions are unclear. One approach to investigating the possible functions of a

gene is to analyse its spatial pattern of expression. Such an approach in the *Drosophila* embryo has revealed that the domains of expression closely correlate with the spatial regions within which different homeobox genes exert their developmental roles (Harding *et al.* 1985; Gehring, 1987).

We have therefore used *in situ* hybridization to investigate in detail the patterns of homeobox gene expression in the developing mouse embryo. We have concentrated on the mouse *Hox 2.1* gene (Hart *et al.* 1985; Hauser *et al.* 1985; Jackson *et al.* 1985; Krumlauf *et al.* 1987), which has been shown to be expressed in a tissue-specific manner during embryonic development (Jackson *et al.* 1985; Krumlauf *et al.* 1987; Utset *et al.* 1987). Aspects of this pattern are compared with the expression of the mouse *Hox 3.1* gene (Awgulewitsch *et al.* 1986; Breier *et al.* 1986; Utset *et al.* 1987).

Materials and methods

In situ *hybridization*

In situ hybridization was performed as previously described (Holland *et al.* 1987), except that the exposure times were 6 to 12 days and probes $(2\times10^9\,\text{disints min}^{-1}\,\mu\text{g}^{-1})$ were used at $1-2\times10^5\,\text{disints min}^{-1}\,\mu\text{l}^{-1}$. The Hox 2.1 probes used were the 750 bp probe 1 and the 430 bp probe 2 of Krumlauf *et al.* (1987). No difference in hybridization pattern was observed between these probes and, hence, they are not distinguished in the text. Probe 1 was used for the examples shown in Figs 2, 3, 6, 7C–F, 8, 9; probe 2 was used for Figs 1, 4, 5, 7A–B. Specificity was monitored by performing control hybridizations with the sense strands of probes 1 and 2. As previously reported (Krumlauf *et al.* 1987), these did not give differential hybridization, except for a weak signal over red blood cells. The Hox 3.1 probe used was the 330 bp pm31.le probe of Breier *et al.* (1986).

Removal of autoradiographic grains, as required in Fig. 9, was performed as suggested by Rogers (1979). Sections were then stained with Mayer's haematoxylin.

Computer-aided three-dimensional reconstruction

In situ hybridization was performed to serial sagittal sections taken at 50–100 μm intervals from a 12·5-day *p.c.* embryo. Camera-lucida tracings were then made of the brain, spinal cord, major ganglia and sites of expression of *Hox 2.1* from each section. These data were used to reconstruct the spatial pattern of *Hox 2.1* expression using the SSRCON serial section reconstruction program described by Shepherd *et al.* (1984), on a DEC PDP11/34 computer.

Results

(A) *Expression of* Hox 2.1 *at the early somite stage*

The anteroposterior axis of the mouse embryo becomes visible at gastrulation (6·5–7·0 days *p.c.*), and the subsequent two days see the commencement of anteroposterior specialization and the first establishment of a metameric pattern (Snow, 1977; Hogan *et al.* 1986).

In situ hybridization of sense and antisense probes to sections of embryos at 6·5, 7·0 and 7·5 days *p.c.* did not reveal any clear differential hybridization (data not shown). It is important to stress that this result need not imply that *Hox 2.1* is not expressed at 6·5 to 7·5 days *p.c.*, since the technique employed may not be capable of detecting very low levels of RNA.

In situ hybridization of sense and antisense probes to sections of 8·5 days *p.c.* (3- to 4-somite-stage) embryos indicated a low level of *Hox 2.1* expression. Hybridization of antisense probe to sagittal sections resulted in a higher grain density over the neuroectoderm and mesoderm of the posterior regions than over head folds and heart mesoderm (Fig. 1A,B). Although the difference in grain density between posterior and anterior is small, it is clearly greater

than the difference observed with the sense strand probe on adjacent sections (Fig. 1C).

The posterior limit of detectable specific hybridization in the embryo is sharp and is at the base of the allantois. The anterior limit between labelled and unlabelled regions is not sharply defined, but seems to lie within the somitic region of the mesoderm and the presumptive hindbrain region of the neuroectoderm.

Hybridization of antisense probe to transverse sections at 8·5 days *p.c.* confirms that grain density is higher in the posterior region of the embryo than in the anterior head fold, visceral yolk sac and parietal yolk sac (Fig. 1D,E). Weak hybridization is seen over the posterior region of the head fold, in the region of the presumptive hindbrain.

(B) *Expression of* Hox 2.1 *in the embryonic central nervous system (CNS)*

The body plan and organs of the embryo are progressively formed throughout midgestation. We have therefore investigated in detail the pattern of *Hox 2.1* expression at 12·5–15·5 days *p.c.*

It has previously been shown, by analysis of isolated RNA (Jackson *et al.* 1985; Krumlauf *et al.* 1987) and *in situ* hybridization (Krumlauf *et al.* 1987; Utset *et al.* 1987), that the embryonic spinal cord is enriched for Hox 2.1 RNA. In this study, we have used *in situ* hybridization to serial sections of 12·5 days *p.c.* mouse embryos, coupled with computer-aided three-dimensional reconstruction, to investigate more precisely the spatially restricted pattern of this expression. Strong, specific, hybridization to Hox 2.1 RNA was observed in the spinal cord and posterior (caudal) hindbrain of the embryo (Fig. 2; see also Krumlauf *et al.* 1987). The anterior (rostral) limit of *Hox 2.1* expression lies within the myelencephalon (medulla oblongata), although this limit is not uniform along the dorsoventral axis. Three-dimensional reconstruction from serial sagittal sections hybridized with Hox 2.1 antisense probe (Fig. 3) reveals that the domain of expression extends anterior and dorsally into the roof plate of the fourth ventricle, and anterior and ventrolaterally towards the presumptive pons. These regions receive a substantial cellular contribution from the dorsal region of the myelencephalon (Hamilton & Mossman, 1972). Hence, due to the complication of cell migration, it is not possible to precisely determine the original anteroposterior position of the expressing cells. Immediately posterior to, and continuous with, these extensions is the region of strongest hybridization to Hox 2.1 RNA, within the posterior region of the myelencephalon.
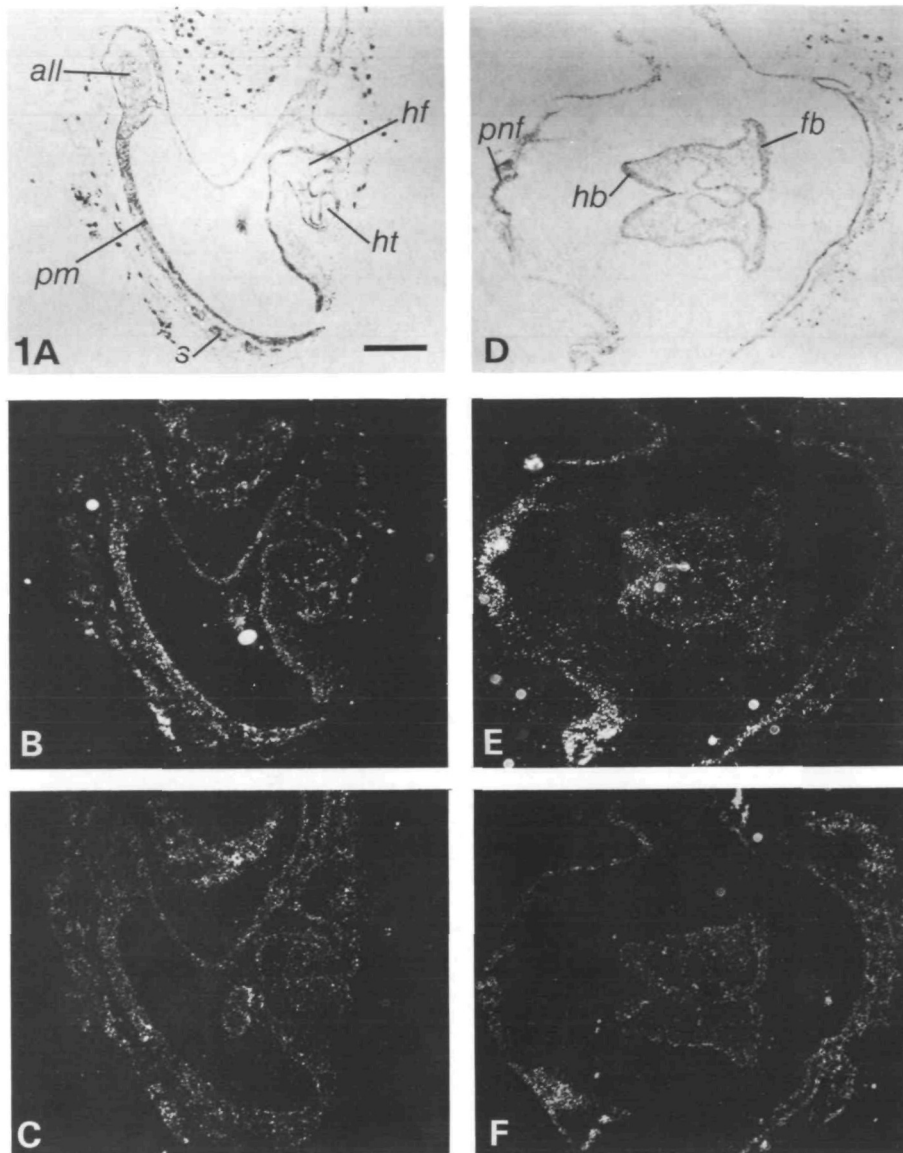
Fig. 1. Expression of *Hox 2.1* at the early somite stage. Antisense and sense strand Hox 2.1 probes were hybridized *in situ* to sections of 8·5-day *p.c.* embryos, and exposed for 12 days. (A) Sagittal section of an embryo with three to four somites, hybridized with Hox 2.1 antisense probe, and photographed under bright-field illumination. (B) Same field as A, photographed under dark-ground illumination such that autoradiographic grains are white. Highest grain density is over the posterior region. (C) Adjacent section to A,B, hybridized with Hox 2.1 sense strand (control) probe and photographed under dark-ground illumination. (D) Transverse section through head folds and posterior neural fold, hybridized with Hox 2.1 antisense probe. Bright-field illumination. (E) Same field as D, photographed under dark-ground illumination. Highest grain density is over the posterior neural fold, and weak hybridization is seen over the posterior region of the head folds. (F) Adjacent section to D,E, hybridized with Hox 2.1 sense strand probe and photographed under dark-ground illumination. A nonspecific signal is seen over blood cells. *all*, allantois; *fb*, presumptive forebrain; *hb*, presumptive hindbrain; *hf*, head folds; *ht*, heart; *pm*, presomitic mesoderm; *pnf*, posterior neural folds; *s*, somite. Bar, 250 μm.

The anterior limit of this expression is located posterior to the otic vesicle. We estimate that the anterior limit of *Hox 2.1* gene expression in the 12·5 days *p.c.* CNS, in relation to the original somite pattern laid down during gastrulation, lies within the region corresponding to occipital somites 1–3 (Fig. 10).

The hybridization seen in the myelencephalon is continuous with a domain of expression in the spinal cord, extending to the most posterior regions identifiable in our sections (around the level of the 26th dorsal root ganglion or 30th somite). In individual sections, intensity of hybridization was not uniform along the anteroposterior axis posterior to the
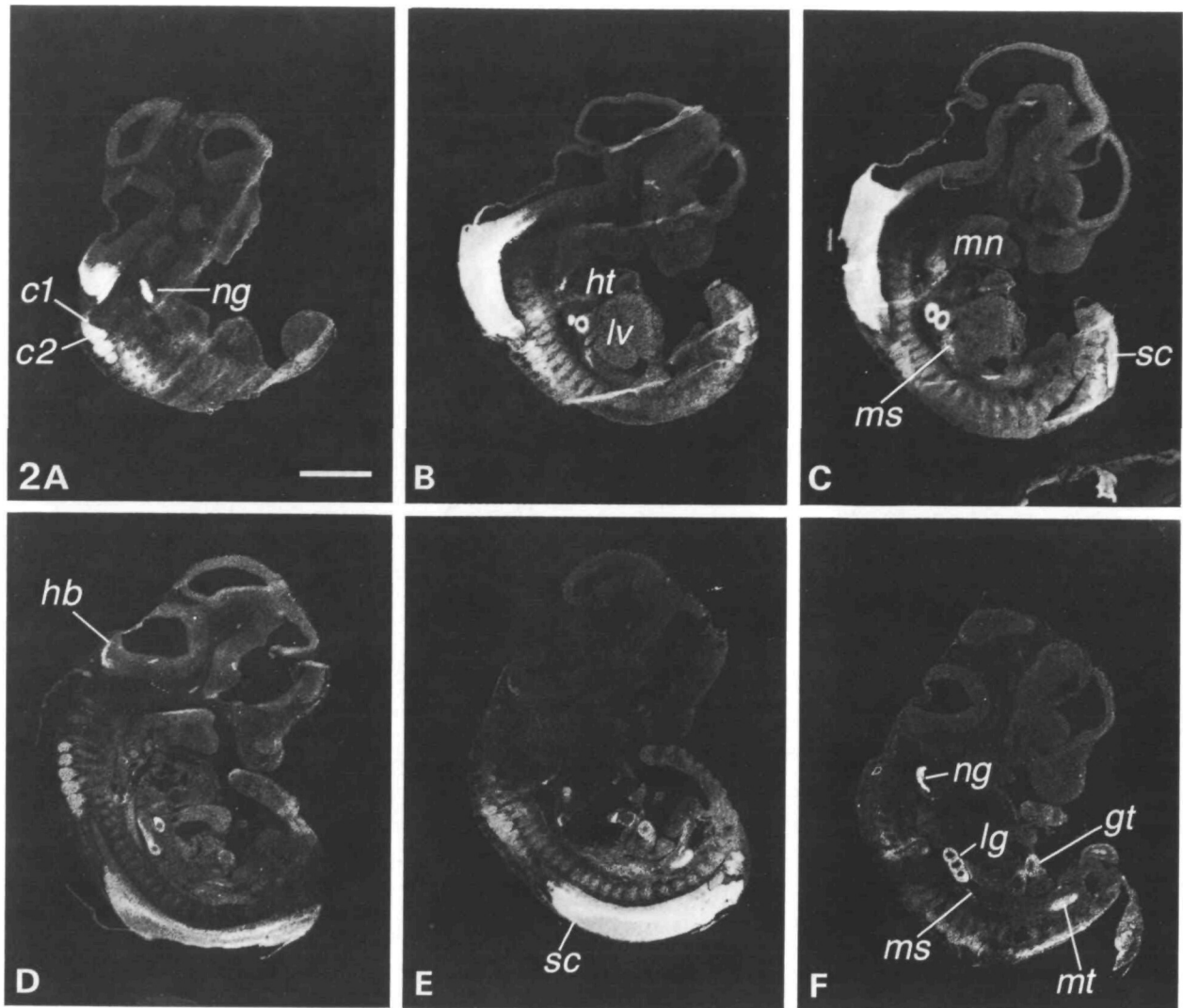
Fig. 2. Representative sagittal sections of a series through a 12·5-day *p.c.* mouse embryo, hybridized with Hox 2.1 antisense probe. Slides were exposed for 12 days and photographed under dark-ground illumination, such that autoradiographic grains are white. Strong hybridization is evident in the spinal cord, hindbrain, dorsal root ganglia, nodose ganglia, mesonphros, metanephros and gut. The stomach, which also expresses Hox 2.1 RNA, is not present in these examples. Slight section folding, leading to artefactual probe trapping, is evident in sections, A,B,C. *c1*, first cervical dorsal root ganglion; *c2*, second cervical dorsal root ganglion; *gt*, gut; *hb*, hindbrain; *ht*, heart; *lg*, lung (see also sections B–D); *lv*, liver; *mn*, mandible; *ms*, mesonephric tubules; *mt*, metanephros (see also section E); *ng*, nodose ganglion; *sc*, spinal cord. Bar, 1 mm.

medulla and was generally highest in the most anterior spinal cord. However, there is no clear evidence for either a uniform gradient or a periodic pattern in this region.

Although not evident in all regions of each section, there is a tendency for stronger hybridization to the dorsal region of the spinal cord. This pattern was analysed further by hybridizing Hox 2.1 antisense probes to serial transverse sections through the spinal cord at 12·5 days *p.c.* (Fig. 4). Hox 2.1 RNA is detected predominantly in the dorsolateral regions of the spinal cord. This region of the cord contains developing sensory nerve tracts.

In summary, Hox 2.1 RNA shows spatial restriction within the central nervous system at 12·5 days *p.c.* There is a clear anterior boundary around the level of the first to third somite, but no identifiable posterior boundary. Along the anteroposterior axis the highest expression is detected in the myelencephalon and anterior spinal cord; dorsoventrally, expression is highest in the dorsolateral region and its derivatives.

In order to investigate temporal changes in this spatial pattern, *in situ* hybridization was performed to sagittal sections of mouse embryos at 13·5–15·5 days
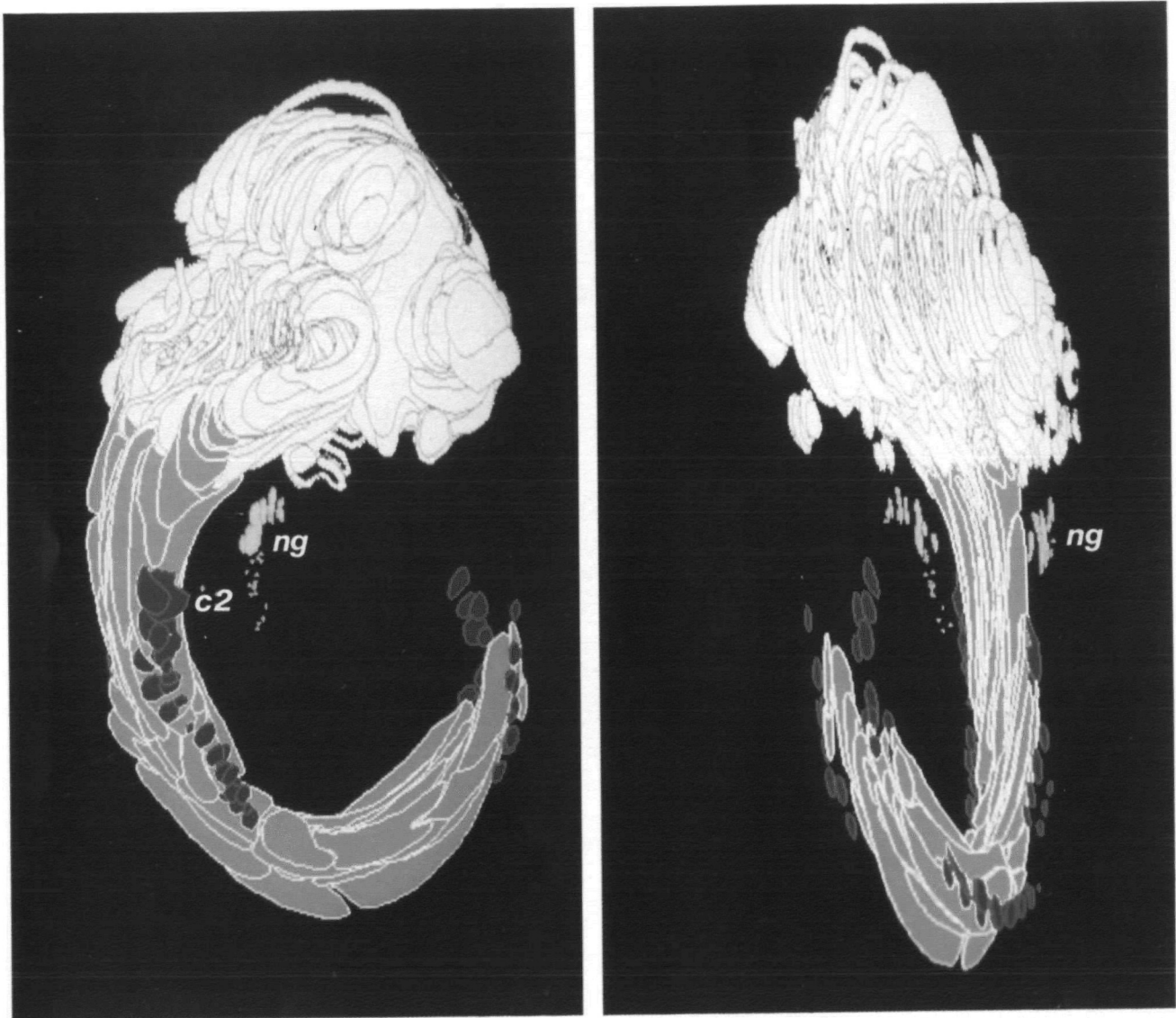
Fig. 3. Three-dimensional reconstructions showing the distribution of Hox 2·1 RNA in the CNS, spinal ganglia and nodose ganglion at 12.5 days *p.c.* Serial sagittal sections were hybridized with Hox 2.1 antisense probe (examples shown in Fig. 2), and used to reconstruct three-dimensional images of the regions of *Hox 2.1* expression. The reconstructions shown include only the brain, spinal cord, dorsal root ganglia and nodose ganglion, displayed using hidden line elimination. Due to section damage, not all dorsal root ganglia are shown. For clarity, the body outline and visceral organs are not shown. The regions of the spinal cord and hindbrain displayed in red express *Hox 2.1*; no expression was detected in the brain regions displayed in white. Dorsal root ganglia displayed in blue also express *Hox 2.1*, as does the nodose ganglion (yellow). *c2*, second cervical dorsal root ganglia; *ng*, nodose ganglion.
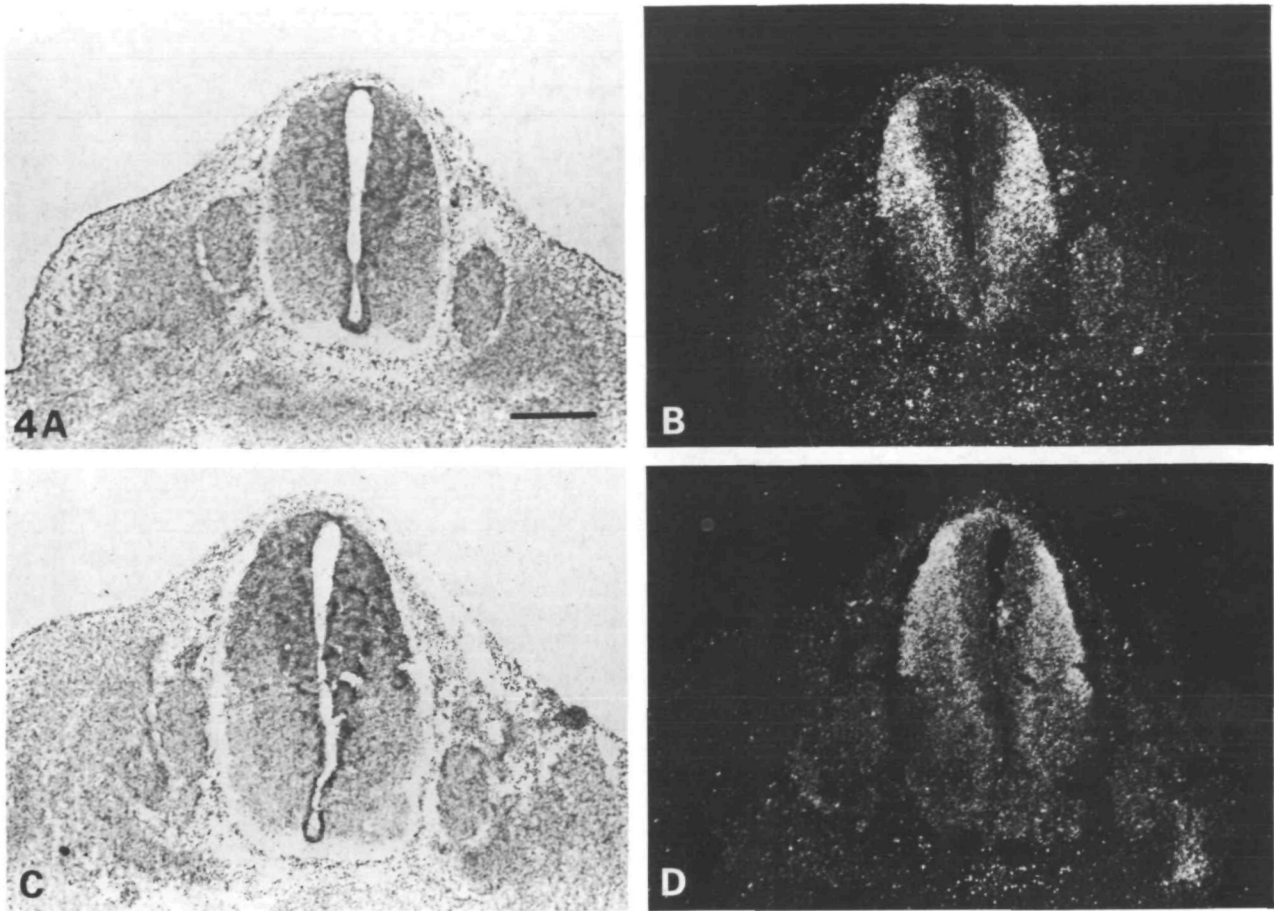
Fig. 4. Dorsoventral and mediolateral distribution of Hox 2.1 RNA in the spinal cord at 12·5 days *p.c.* Serial transverse sections were hybridized with Hox 2.1 antisense probe and exposed for 9 days. (A,B) Transverse section through the spinal cord in the cervical region, photographed under bright-field illumination (A) and dark-ground illumination (B). The strongest hybridization is in the dorsal and lateral regions. Under the conditions used in this experiment, only a weak positive signal is seen over dorsal root ganglia. (C,D) Transverse section through the spinal cord in the thoracic region, photographed under bright-field illumination (C) and dark-ground illumination (D). Hybridization is again strongest in the dorsal and lateral regions. Bar, 300 μm.

p.c. These experiments revealed that *Hox 2.1* continues to be expressed in a spatially restricted manner in the embryonic central nervous system, but the domain of major expression is constricted, so that by 13·5 days *p.c.* strong hybridization extends only to the level of the second cervical somite. Posterior to this point, the hybridization is weaker. No change in the anterior limit of high expression is observed and the boundary remains within the myelencephalon (Fig. 5A). Since the overall intensity of hybridization is reduced, it is unclear to what extent the apparent shortening of the spatial domain reflects a localized *versus* a general reduction in RNA levels. The constricted domain of *Hox 2.1* expression is maintained at 14·5 (not shown) and 15·5 days (Fig. 5C). Weak hybridization to this region is also seen in sections of newborn mice (not shown).

(C) *Expression of* Hox 2.1 *in the embryonic peripheral nervous system*

In situ hybridization to serial transverse and sagittal sections at 12·5 days *p.c.* revealed that Hox 2.1 RNA is present in specific ganglia of both the sensory and autonomic nervous systems. The dorsal root (spinal) ganglia are paired sensory ganglia, arranged in a repeating pattern either side of the spinal cord, reflecting the segmented organization of the adjacent somitic mesoderm (Keynes & Stern, 1985). *In situ* hybridization clearly indicates that Hox 2.1 RNA is present in the dorsal root ganglia (Figs 2, 3), although the intensity of hybridization is lower than in the spinal cord. All dorsal root ganglia identified at 12·5 days *p.c.* are positive, including the most rostral well-defined ganglion, which we believe to be the second cervical ganglion (C2). The first cervical ganglion, C1, degenerates around 11 days *p.c.* (Dawes, 1930;

Rugh, 1968) and cannot be positively identified. However, a small ganglion is visible anterior to C2 and may represent C1. Hox 2.1 RNA is also present in this ganglion (Figs 2, 3). We cannot, however, identify occipital ganglia, which are reported to be vestigial (Froriep, 1882; Hunter, 1935).

In the cranial region, the ganglia that are most comparable to the dorsal root ganglia are those associated with the major mixed and sensory cranial nerves (V, VII, VIII, IX, X). In our sections, the major ganglia of the Vth, VIIth and VIIIth cranial nerves were clearly visible, but did not hybridize to Hox 2.1 antisense probes. However, intense hybridization was seen to one large postotic ganglion situated dorsal to the anterior oesophagus (Figs 2, 3, 6). Comparison between hybridized sections and adjacent serial sections stained with anti-neurofilament antibodies (not shown) suggests that this ganglion is almost certainly the nodose ganglion of the Xth cranial nerve. The nodose ganglion has a mixed
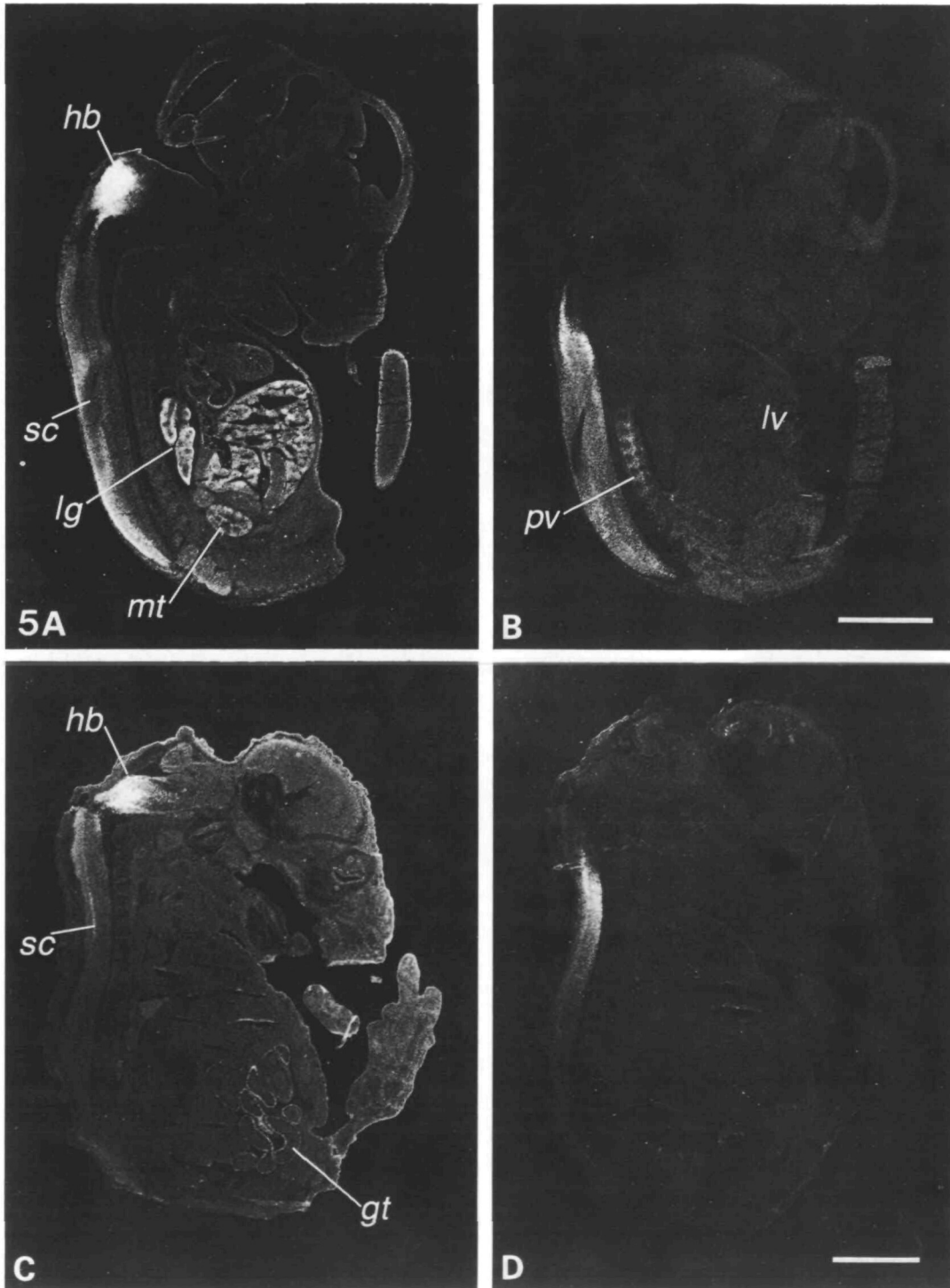
origin, with the neurones being predominantly of placodal origin and the neuroglia of neural crest origin (Le Douarin, 1982). Although we cannot state which cell types are expressing *Hox 2.1*, it is of interest that expression is nonuniform, with a punctate appearance (Fig. 6), often limited to the posterior region of the ganglion.

Ganglia associated with the autonomic nervous system are typically much smaller than the dorsal root ganglia and were not clearly identifiable in our sections. However, *in situ* hybridization to sections passing through the embryonic gut revealed a pattern of hybridization consistent with *Hox 2.1* expression in the neural-crest-derived parasympathetic ganglia of the enteric plexus. An irregular and punctate pattern of hybridization was seen in sections through most loops of the gut (Fig. 2), localized within the developing longitudinal and circular muscle layers (Fig. 6). This expression is still apparent at 13·5–15·5 days *p.c.* (Fig. 5), remaining punctate and localized even though the enveloping longitudinal muscle layer has proliferated extensively. This pattern suggests that the hybridization signals correspond to ganglia of the myenteric (Auerbach's) plexus, which are reported to have a very similar distribution (Cochard & Paulin, 1984; Payette *et al.* 1984; Jessen *et al.* 1987).

(D) *Expression of* Hox 2.1 *in mesodermal structures*
As described earlier, *Hox 2.1* is expressed at a low level in the posterior (presomitic) regions of the ingressing mesoderm at 8·5 days *p.c.*

At later stages of embryogenesis (12·5–15·5 days *p.c.*) little or no hybridization was observed to the

Fig. 5. Distribution of Hox 2.1 and Hox 3.1 RNA at 13·5 and 15·5 days *p.c.*, revealed by *in situ* hybridization. Slides were exposed for 6 days and photographed under dark-ground illumination. (A) Midsagittal section of a 13·5 day *p.c.* embryo, hybridized with Hox 2.1 antisense probe. Strongest hybridization is in the posterior (caudal) hindbrain and anterior (rostral) spinal cord. Expression is also evident in the lung, metanephric kidney and gut. The signal over the liver is only seen when section folding occurs, and thus may be artefactual. (B) Almost adjacent section to A, hybridized with Hox 3.1 antisense probe. Hybridization in the spinal cord extends as far anterior as the midcervical region. Expression is also evident in the prevertebrae as far anterior as the midthoracic region. (C) Midsagittal section of a 15·5-day *p.c.* embryo, hybridized with Hox 2.1 antisense probe. Hybridization is evident in the posterior hindbrain, anterior spinal cord and around the loops of the gut. Weak hybridization is seen to the lung and kidney. (D) Adjacent section to C, hybridized with Hox 3.1 antisense probe. Expression is detected in the spinal cord posterior to the midcervical region. *gt*, gut; *hb*, hindbrain; *lg*, lung; *lv*, liver; *mt*, metanephric kidney; *pv*, prevertebrae; *sc*, spinal cord. Bar, 1·5 mm.

major derivatives of the somites, such as the myotome and the sclerotome-derived prevertebrae (Figs 2, 5).

The axial mesoderm differentiates into the notochord. This structure was clearly visible in transverse sections of embryos at 12·5 days *p.c.*, but did not hybridize with probes to *Hox 2.1*.

In contrast, strong hybridization was observed to certain visceral organs derived predominantly from the lateral plate mesoderm (lung and stomach) and from the paraxial mesoderm immediately lateral to the somites (mesonephros and metanephros). *In situ* hybridization to 12·5-day *p.c.* embryos indicates that *Hox 2.1* is expressed in the embryonic lung, specifically in the mesodermally derived mesenchymal cells (Fig. 2; see also Krumlauf *et al.* 1987). However, hybridization to serial sagittal sections (not shown), and hybridization to transverse sections (Fig. 7), revealed that expression is not uniform throughout the mesodermal component. Around the tips of the growing and branching epithelial outgrowths, expression is detected in all mesodermal cells. In contrast, expression in more medial regions is confined to the most peripheral mesodermal cells, away from the epithelial cell layer. This mesodermal expression persists to 13·5 days *p.c.* by which time extensive branching has occurred (Fig. 5).

In situ hybridization to serial sections at 12·5 days *p.c.* also revealed that the embryonic stomach expresses high levels of Hox 2.1 RNA. Again, strong hybridization was observed to the mesodermally derived mesenchymal cells, while the endodermally derived epithelial cell layer was negative (Fig. 6). Hybridizations to serial sections revealed regions where the entire mesodermal component expresses *Hox 2.1* (Fig. 7) and regions where expression is restricted to dorsal mesoderm (Fig. 7). Expression does not extend into the mesodermal component of the hindgut.

At 12·5 days *p.c.* strong expression was also detected in all mesonephric tubules identified (Figs 2, 8). These develop from the nephrotome region of the paraxial mesoderm and constitute examples of mesodermally derived epithelial structures. The most-posterior nephrogenic tissue condenses to form the metanephric mass, which shows little morphological differentiation at 12·5 days *p.c.* *In situ* hybridization revealed that *Hox 2.1* is expressed in the metanephric mass; but with no apparent spatial restriction (Fig. 2). By 16 days *p.c.* the metanephros has differentiated, *via* reciprocal inductive interactions with the invading nephrogenic ureteric bud, into a complex organ consisting predominantly of mesenchymal cells surrounding epithelial renal tubules (Saxen *et al.* 1986). Hybridization of *Hox 2.1* antisense probes to sections of the meta-

nephric kidney at 16 days *p.c.* indicated that, during this differentiation process, *Hox 2.1* expression becomes spatially localized, predominantly to the epithelial cells of the developing tubules. Hybridization

is seen in the epithelial cells of all metanephric tubules and hence it is likely that *Hox 2.1* is expressed in tubules derived from both the metanephric blastema and the ureteric bud (Fig. 8).
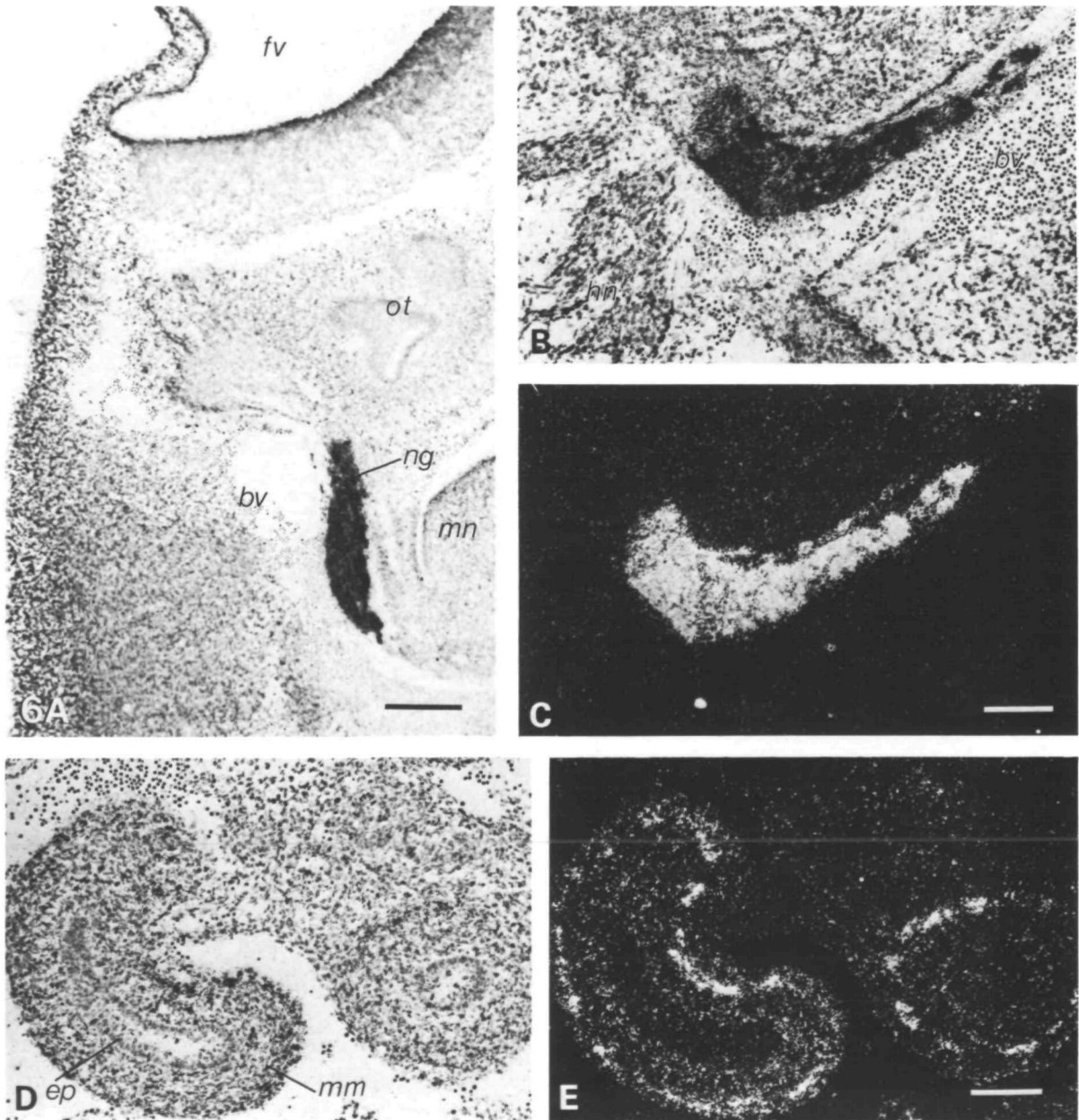

Fig. 6. Expression of Hox 2.1 RNA in ganglia of the peripheral nervous system, revealed by *in situ* hybridization. Sections were hybridized with Hox 2.1 antisense probe and exposed for 12 days. (A) Parasagittal section through the cervical region at 12·5 days *p.c.*, photographed under bright-field illumination, such that autoradiographic grains are black. Intense hybridization is seen to a large postotic ganglion, which we believe to be the nodose. Bar, 100 μm. (B,C) Higher magnification of the nodose ganglion, showing adjacent blood vessels and cranial nerve tracts. The position of the latter suggests they are roots of the hypoglossal nerve. (B) bright field; (C) dark-ground illumination. The intense hybridization to the ganglion is clearly punctate. No hybridization is seen to adjacent nerve tracts. Bar, 100 μm. (D,E) Section through the gut at 12·5 days *p.c.*, photographed under bright-field illumination (D) and dark-ground illumination (E). Comparison of D and E clearly shows that *Hox 2.1* expression in the gut is punctate and irregular within the developing muscle layers. This distribution is consistent with expression in parasympathetic ganglia of the myenteric plexus (see text). Bar, 100 μm. *bv*, blood vessel; *ep*, epithelium of gut; *fv*, fourth ventricle; *hn*, hypoglossal nerve; *mm*, mesodermal muscle layer; *mn*, mandible; *ng*, nodose ganglion; *ot*, otic vesicle.

Two major organs which were consistently found to be negative for *Hox 2.1* expression were the liver (from 12·5 to 15·5 days *p.c.*) and the heart (from 8·5 to 15·5 days *p.c.*). The liver develops *via* an interaction between endoderm and mesoderm but, at the stages studied, the major component is of endodermal origin. Hence, we cannot state whether liver mesoderm expresses Hox 2.1 RNA. The heart forms from mesoderm in the cranial region and was found not to express Hox 2.1 RNA. This emphasizes that

not all visceral organs derived from mesoderm express high levels of *Hox 2.1*.

(E) *Expression of* Hox 2.1 *in adult connective tissue*

In situ hybridization to sections of adult mouse organs (kidney, lung, submaxillary gland) revealed one striking case of high levels of Hox 2.1 RNA in a specific subset of cells. In the adult submaxillary gland, scattered cells situated within the connective tissue matrix were found to hybridize intensely to Hox 2.1 antisense probes (Fig. 9A). These cells did not form
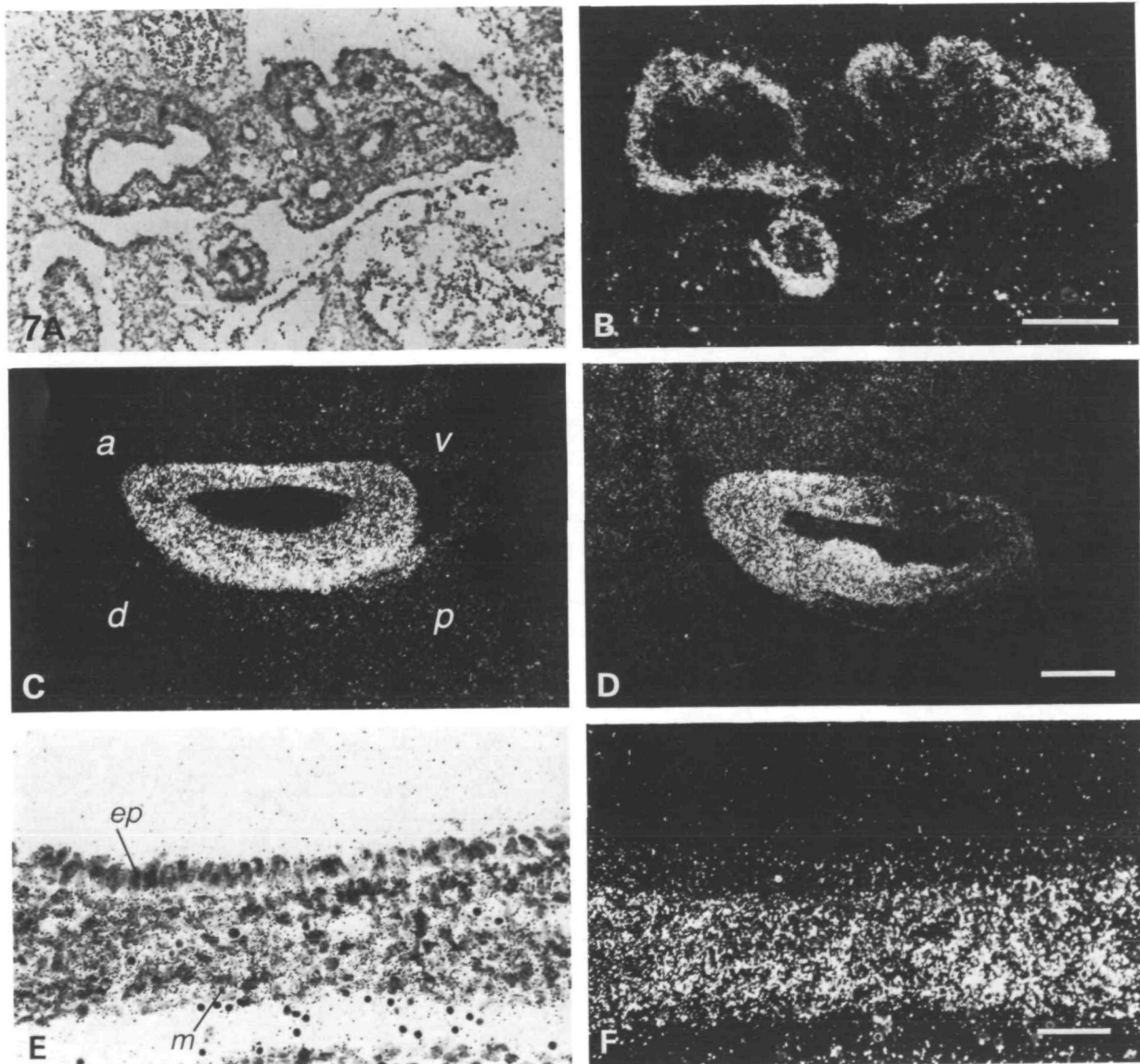

Fig. 7. Expression of *Hox 2.1* in mesodermal regions of the lung and stomach. (A,B) Transverse sections through the lung at 12·5 days *p.c.*, hybridized with Hox 2.1 antisense probe and exposed for 9 days. Bright-field illumination (A) and dark-ground illumination (B). Hybridization is limited to the mesodermal layer of the lung and is strongest around the tips of the growing buds. Bar, 200 μm. (C,D) Transverse sections through the stomach at 12·5 days *p.c.*, hybridized with Hox 2.1 antisense probe and exposed for 12 days. Expression is uniform in C, but highest dorsally in D. Bar, 200 μm. (E, F) Higher magnification of a stomach section hybridized with Hox 2.1 antisense probe, showing that high expression is localized to the mesodermal cells. (E) Bright-field; (F) dark-ground illumination. Bar, 40 μm. *a*, anterior; *d*, dorsal; *p*, posterior; *v*, ventral; *ep*, epithelium; *m*, mesoderm.
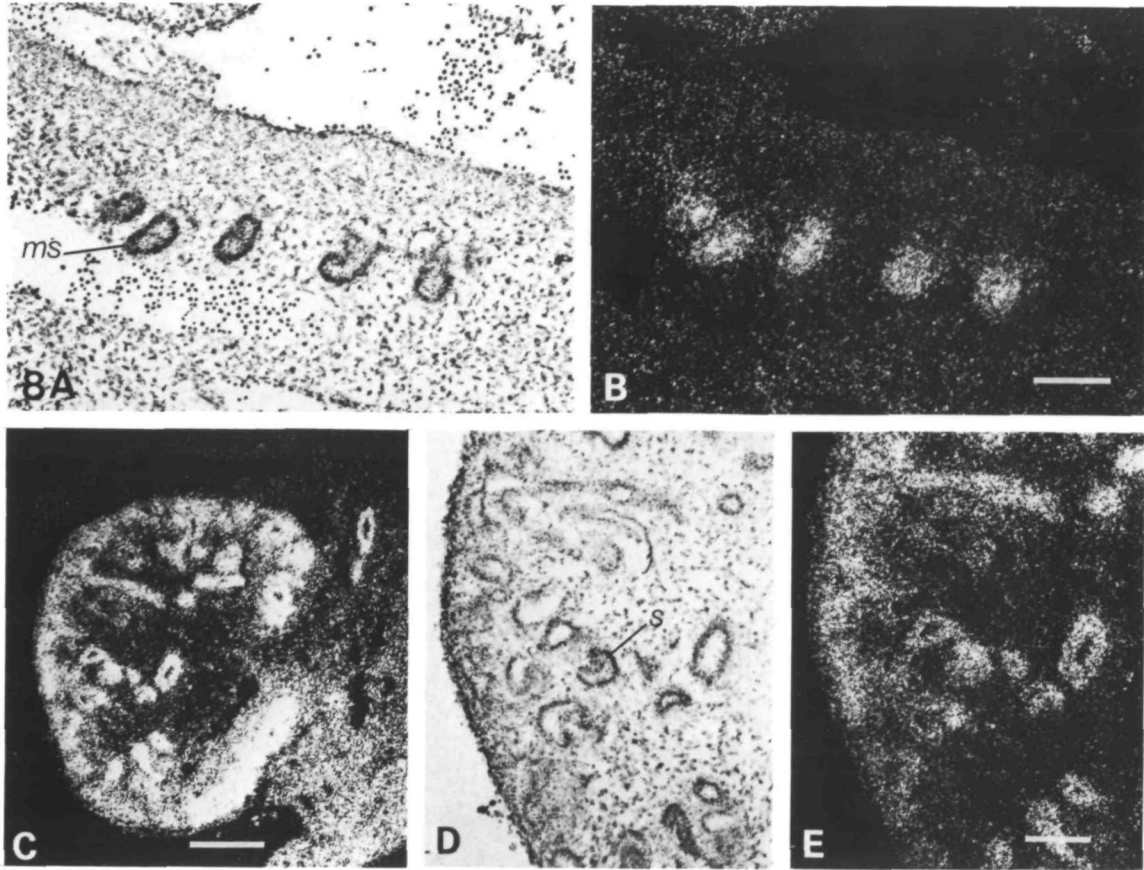
Fig. 8. *Hox 2.1* expression in the mesonephros and metanephros. (A,B) Sagittal section through the thoracic region of an embryo at 12·5 days *p.c.*, hybridized with Hox 2.1 antisense probe and exposed for 7 days. Clear hybridization is evident to the mesonephric tubules. Bright-field (A) and dark-ground (B) illumination. Bar, 100 μm. (C) Horizontal section through the metanephric kidney from a 16-day *p.c.* embryo, hybridized with Hox 2.1 antisense probe and exposed for 10 days. Dark-ground illumination. Bar, 200 μm. (D,E) Higher magnification of the section shown in C, photographed under bright-field (D) and dark-ground (E) illumination. *Hox 2.1* expression is detected in all metanephric tubules visible. These include developing nephrons (S-shaped bodies) around the periphery. Bar, 100 μm. *ms*, mesonephric tubules; *s*, S-shaped bodies.
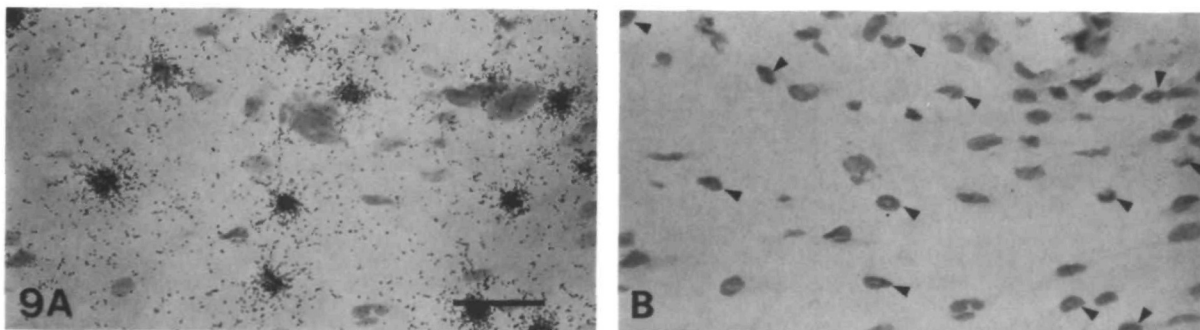

Fig. 9. Expression of *Hox 2.1* in adult connective tissue. Sections of adult submaxillary gland were hybridized with Hox 2.1 antisense probe and exposed for 10 days. (A) Region of connective tissue showing several dispersed cells which hybridize strongly. Bright-field illumination. (B) Same field as A after removal of autoradiographic grains and staining with haematoxylin. Cells which hybridized are indicated by arrowheads. Most of these have characteristic horseshoe-shaped nuclei. Bar, 40 μm.

part of any multicellular structure and were estimated to constitute only approximately 5 % of connective tissue cells.

In order to allow histological examination of the individual hybridizing cells, it proved necessary to remove the overlaying silver grains and to restain the

cells (Fig. 9B). Comparison of the cells before and after grain removal indicated that most, although not all, of the hybridizing cells had characteristic horse-shoe-shaped nuclei. Furthermore, in no case was a cell with such a nuclear morphology not associated with hybridization grains. This evidence, coupled with the location of the cells, suggests that the majority of the connective tissue cells which express Hox 2.1 RNA are mature granulocytes. These cells originate from the bone marrow and not the submaxillary gland itself.

(F) *A comparison of the spatial patterns of* Hox 3.1 *and* Hox 2.1 *expression*

Since *Hox 2.1* is expressed in a complex spatial and temporal pattern, it was of interest to compare this with the pattern of expression of other homeobox-containing genes. The *Hox 3.1* gene was used for this comparison, since it has been shown to be expressed predominantly in the cervical region of the spinal cord of embryonic and newborn mice (Awgulewitsch *et al.* 1986; Utset *et al.* 1987). *In situ* hybridization with a Hox 3.1 antisense probe to sections of 12·5–15·5 days *p.c.* embryos confirmed that *Hox 3.1* has a spatially restricted pattern of expression in the embryo (Fig. 5). Strong hybridization to Hox 3.1 RNA was observed in the spinal cord, although this expression did not extend as far anterior as *Hox 2.1*. The anterior limit of expression in the spinal cord was around the level of the fourth to fifth cervical vertebrae, but was again nonuniform along the dorso-ventral axis. As previously reported (Utset *et al.* 1987), Hox 3.1 hybridization was also apparent in several of the sclerotome-derived prevertebrae, out of register with expression in the spinal cord (Fig. 5). Thus, *Hox 3.1* is expressed in specific anteroposterior domains of both the spinal cord and the sclerotomes during embryogenesis.

Discussion

In this study, we have used *in situ* hybridization to analyse the embryonic expression of a mouse homeobox-containing gene, *Hox 2.1*. The earliest that Hox 2.1 RNA could be detected *in situ* was 8·5 days *p.c.*, when it was predominantly localized to the posterior mesoderm and neuroectoderm. The posterior limit of expression is at the base of the allantois. In the mesoderm, the anterior limit is diffuse, while in the neuroectoderm, expression extends into the region of the presumptive hindbrain.

Localization to the posterior region of the early mouse embryo has also been reported for another mouse homeobox-containing gene, *Hox 1.5* (Mo10; Gaunt *et al.* 1986). It is intriguing that Hox 2.1 RNA is spatially localized along the anteroposterior axis at

the early somite stage, although the weak and diffuse nature of the signal makes elucidation of the spatial limits of expression difficult. This information is essential for assessing the possible roles of *Hox 2.1* during embryogenesis. *In situ* hybridization was therefore used to investigate in detail the expression pattern of *Hox 2.1* from 12·5 to 15·5 days *p.c.* These experiments clearly demonstrate that expression of the gene is much more spatially complex than had been previously reported (Jackson *et al.* 1985; Krumlauf *et al.* 1987; Utset *et al.* 1987) and is detected in specific regions of the developing central nervous system, peripheral nervous system and visceral mesoderm.

Within the central nervous system (CNS), expression is detected in the posterior myelencephalon and the spinal cord. The anteroposterior limits of this expression do not seem to reflect any visible change in histological cell type, and can therefore be viewed as reflecting an anteroposterior spatial domain. Expression within a restricted region of the CNS has also been reported for several *Drosophila* homeotic genes thought to be involved in anteroposterior specification (Akam, 1983; Hafen *et al.* 1983; Levine *et al.* 1983; White & Wilcox, 1984; Akam & Martinez-Arias, 1985; Beachy *et al.* 1985; Harding *et al.* 1985; Kuroiwa *et al.* 1985; Regulski *et al.* 1985; White & Wilcox, 1985; Martinez-Arias, 1986; Chadwick & McGinnis, 1987). Genetic evidence suggests that *Drosophila* homeotic genes are directly involved in the control of positional specification in the CNS (Teugels & Ghysen, 1983; Ghysen *et al.* 1985; Ghysen & Lewis, 1986), and hence it is possible that *Hox 2.1* has an analogous function in the vertebrate CNS.

The domain of *Hox 2.1* expression in the CNS is dynamic, since between 12·5 and 13·5 days *p.c.* the posterior limit of high expression changes from the extreme posterior end of the spinal cord to the anterior cervical region. This temporal change in the major expression domain, which may reflect either a localized or a general reduction in RNA levels, could explain why *Hox 2.1* expression in the posterior spinal cord was not detected in a previous study (Utset *et al.* 1987), in which 13·5 days *p.c.* was the earliest stage analysed.

Dynamic changes in the spatial domain of gene expression have also been reported for a *Xenopus* homeobox-containing gene, *Xeb1* (Carrasco & Malacinski, 1987). It is therefore of interest that the expression domains of several *Drosophila* homeotic genes are also dynamic and, in particular, become progressively localized during development (Levine *et al.* 1983; Akam & Martinez-Arias, 1985; Harding *et al.* 1985; Martinez-Arias, 1986). Furthermore, there is a close correlation between the localized expression domain of several *Drosophila* homeotic genes and the
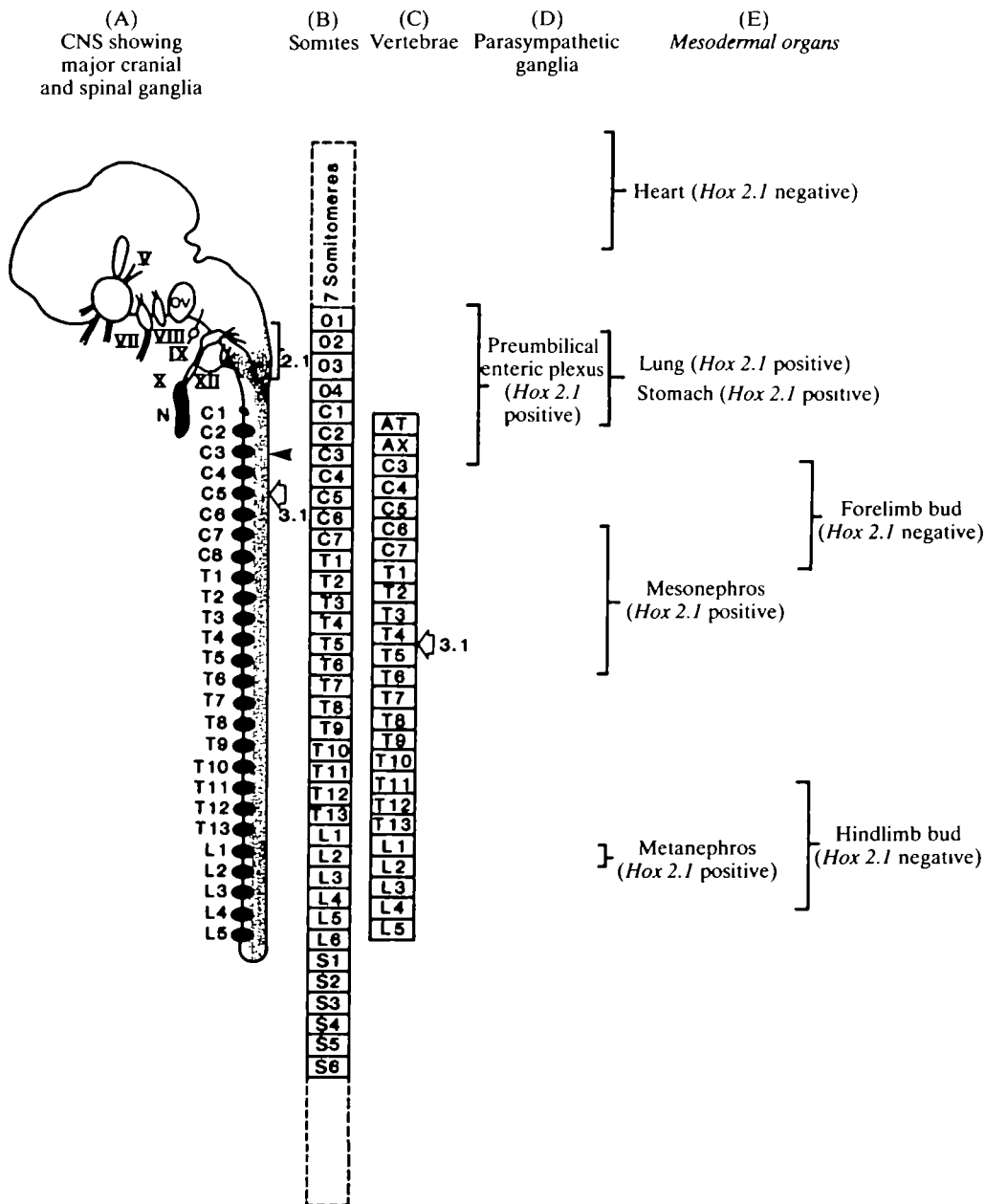
regions in which they exert their major functions. By analogy, this suggests that certain functions of *Hox 2.1* may be restricted to the occipital and anterior cervical regions.

Comparison of the anteroposterior domains of *Hox 2.1* and *Hox 3.1* expression in the spinal cord reveals that the major domains are overlapping at 12·5 days *p.c.*, but not at 13·5 days *p.c.* This feature is again suggestive of a functional similarity to *Drosophila* homeotic genes, which may act combinatorially to effect positional specification (Struhl, 1982; Morata *et al.* 1983).

Hox 2.1 expression in the spinal cord was found to have a dorsoventral restriction at 12·5 days *p.c.*, with the highest levels in the dorsolateral region. The implication of this restriction is unclear, although it is

of interest that expression persists in cells that secondarily migrate ventrally, towards the presumptive pons. In contrast to this observation, two recent reports describe localization of vertebrate homeobox gene expression in the ventral region of the embryonic spinal cord (mouse *Hox 3.1*, Utset *et al.* 1987; *Xenopus Xeb1*, Carrasco & Malacinski, 1987).

The neural crest, like the dorsolateral region of the spinal cord, is derived from the more lateral part of the neural plate. It is therefore of interest that Hox 2.1 RNA was also detected in specific ganglia of the embryonic peripheral nervous system, which receive neural crest contributions. Hox 2.1 RNA was detected in all dorsal root ganglia visible at 12·5 days *p.c.* These sensory ganglia are formed by neural crest cells derived from the same anteroposterior domain

(A) CNS showing major cranial and spinal ganglia (B) Somites (C) Vertebrae (D) Parasympathetic ganglia (E) *Mesodermal organs*

Heart (*Hox 2.1* negative)

Preumbilical enteric plexus (*Hox 2.1* positive)

Lung (*Hox 2.1* positive)
Stomach (*Hox 2.1* positive)

Forelimb bud (*Hox 2.1* negative)

Mesonephros (*Hox 2.1* positive)

Metanephros (*Hox 2.1* positive)

Hindlimb bud (*Hox 2.1* negative)

as seen for *Hox 2.1* expression in the central nervous system. In addition, we detected Hox 2.1 RNA in the parasympathetic ganglia of the myenteric plexus, which are also formed from neural crest (Le Douarin, 1982). In the chick, most of these ganglia, including all in the preumbilical region, derive from neural

Fig. 10. Schematic representation of the domains of homeobox gene expression in the 12·5-day *p.c.* mouse embryo. (A) Diagram of the brain and spinal cord, showing the approximate positions of the major mixed and sensory cranial nerves and ganglia (V, VII, VIII, IX, X), and the hypoglossal nerve (XII). *N*, nodose ganglion of the Xth cranial nerve (vagus). O.V., otic vesicle. Posterior to the XIIth cranial nerve, the dorsal root ganglia are indicated. C1-8, T1-13, L1-5, spinal ganglia of the cervical, thoracic and lumbar regions, respectively. Ganglia shaded in black (N, and spinal ganglia) express Hox 2.1 RNA, as do the regions of the spinal cord and medulla indicated by stippling. The approximate anterior limit of *Hox 2.1* expression is indicated by the bracket, but this has not been determined exactly (see text). The arrowhead denotes the posterior limit of the major domain of *Hox 2.1* expression in 13·5- to 15·5-day *p.c.* embryos. The anterior limit of *Hox 3.1* expression in the spinal cord is indicated by the white arrow. (B) Schematic representation of the somitic organization of the embryo. The number of somites anterior to the cervical region is variable between species and their fate is unclear. Here we assume there to be four true occipital somites and that the 'rudimentary' and transient somite anterior to these (Butcher, 1929; Dawes, 1930) is part of the cranial somitomeric region (Meier & Tam, 1982). We number the cervical somites such that C1 is associated with the transient first dorsal root ganglion (Dawes, 1930; Rugh, 1968). The vestigial Froriep's ganglion (not shown) is then the dorsal root ganglion from the last occipital somite (Froriep, 1882; Butcher, 1929; Dawes, 1930; Hunter, 1935). Subsequent numbering is based on the presumptive fate of the sclerotomal derivatives (Theiler, 1972). O1-4, C1-7, T1-13, L1-6, S1-6, occipital, cervical, thoracic, lumbar and sacral somites, respectively. The numerical relationship between somites and dorsal root ganglia is based on Hebel & Stromberg (1986). (C) The positional relationship between somites and presumptive vertebrae is indicated. It is unclear whether each vertebra derives from one or two somites (Dawes, 1930; Snow & Gregg, 1986); hence the spatial relationship between somites and vertebrae may disagree with the embryonic origin by up to half a somite. *AT*, atlas; *AX*, axis. The white arrow indicates the anterior limit of *Hox 3.1* expression in the presumptive vertebrae. (D) The approximate anteroposterior origin of the neural crest cells which give rise to the parasympathetic ganglia of the enteric plexus in the chick (Le Douarin, 1982). (E) The approximate anteroposterior origin of the mesodermal component of the major visceral organs and limb buds, indicating *Hox 2.1* expression (Torrey, 1943; Sorokin, 1965; Le Douarin, 1984, 1982; Theiler, 1972; Snow, 1981; Meier & Tam, 1982).

crest in the region corresponding to the first seven somites (Le Douarin, 1982); that is, within the major region of *Hox 2.1* expression in the central nervous system.

A structure we believe to be the nodose ganglion of the Xth cranial nerve also expresses high levels of *Hox 2.1* RNA, in contrast to preotic cranial ganglia, which are negative. Glial cells within the nodose ganglion are derived from vagal neural crest (Narayanan & Narayanan, 1980) and hence this expression could represent another example of *Hox 2.1* expression in neural-crest-derived cells. In view of the possibility that vertebrate homeobox-containing genes may have roles in anteroposterior positional specification, expression in the neural crest is of particular interest. That is, experimental evidence suggests that the fate of some neural crest cells may be intrinsically specified and not primarily determined by their site of arrest (Noden, 1983). Furthermore, it is intriguing to note that a mutation in the cat has been identified which affects this intrinsic specification of the cranial neural crest (perhaps acting *via* positional programming from the central nervous system). This mutation can result in a homeotic-like transformation of cranial neural crest derivatives (Noden & Evans, 1986).

In situ hybridization also demonstrated that *Hox 2.1* is expressed in a subset of mesodermal derivatives. Expression was detected in the mesonephros, metanephros and the mesodermal components of the lung and stomach. Expression is not limited to a specific morphological cell type common to these organs, since high levels were detected in mesenchyme within lung and stomach, and in epithelia within the metanephros and mesonephros. However, there is spatial restriction of the expression detected in lung, metanephros and stomach, although it is unclear what this internal pattern reflects. One possibility is that *Hox 2.1* regulates sets of genes with specific functions in organogenesis. For example, within the lung and metanephros, *Hox 2.1* expression is highest in the mesodermal cells which are responsible for inducing morphological remodelling of an epithelial tubular bud (Spooner & Wessells, 1970; Wessels, 1970; Saxen *et al.* 1986). The possibility that homeobox-containing genes have functions in processes not directly concerned with positional specification is further suggested by the detection of Hox 2.1 RNA in mature granulocytes. However, since these cells may have abnormally low levels of protein synthesis (Zucker-Franklin, 1980), and may accumulate RNA species without significantly translating them (e.g. *c-fos*, Kriepe *et al.* 1986), it is unclear whether this expression is of functional relevance.

Within the embryo, it is intriguing that the mesodermal organs which express *Hox 2.1* appear to be

confined to a particular spatial domain. First, *Hox 2.1* seems to be expressed in a specific lateral domain. Expression is not detected in derivatives of axial mesoderm (notochord and somites), but is seen more laterally, in derivatives of the paraxial mesoderm immediately lateral to the somites (mesonephros and metanephros). In addition, *Hox 2.1* expression is detected in lung and stomach, organs which in the chick have been shown to contain mesoderm derived from the most medial region of the lateral plate mesoderm (Le Douarin, 1964). Second, it appears that only those visceral mesodermal organs that have an embryonic origin in the postcranial region express *Hox 2.1*. Thus, the lung bud emerges approximately at the level of the fourth to fifth somite (Sorokin, 1965; M. H. L. Snow, personal communication), the mesonephric tubules originate from somite levels 11 to 17 (Torrey, 1943), while the metanephros apparently arises at somite level 26 (Torrey, 1943). The origin of the lung and stomach mesenchyme has been mapped in the chick to the region of the second to seventh somites (Le Douarin, 1964, 1982). In contrast, the heart mesoderm, which does not express *Hox 2.1*, forms in the cranial somitomeric region (Le Douarin, 1964; Snow, 1981; Meier & Tam, 1982). Thus, anteroposterior domains of *Hox 2.1* expression may exist for mesodermal as well as for ectodermal derivatives.

It is intriguing that, although *Hox 3.1* is also expressed in embryonic mesoderm, it shows a very different spatial restriction to *Hox 2.1*. Within the prevertebrae there is again an anteroposterior restriction, but this domain is not coincident with that of *Hox 3.1* in the spinal cord nor with *Hox 2.1*. The patterns of *Hox 2.1* and *Hox 3.1* expression are shown diagrammatically in Fig. 10.

In the long term, the investigation of vertebrate homeobox-containing genes may give insights into the molecular mechanisms controlling pattern formation and regional specification in vertebrate embryos. Both experimental embryology and genetics have suggested that positional specification does occur in the somitic mesoderm (Kieny *et al.* 1972), the spinal cord (Narayanan & Hamburger, 1971; Lewis & Wolpert, 1976) and the neural crest (Noden, 1983; Noden & Evans, 1986). Although there is some evidence to suggest that mesoderm may induce some regional specification in the neuroectoderm (Snow, 1981; Cooke, 1985; Gurdon, 1987), the mechanisms by which this information is generated, stored and communicated between cells are unclear. It is therefore of interest that homeobox-containing genes are expressed in a wide variety of embryonic tissues, deriving from somitic mesoderm, lateral mesoderm, neural crest and neural tube. In this study, we have demonstrated that, within each of these embryonic

regions, homeobox-containing genes are expressed in spatially restricted anteroposterior domains. This may indicate that, in all these regions, homeobox-containing genes have functions in the control of anteroposterior positional specification, rather than in metameric patterning. If this hypothesis is correct, it is possible that each of the major embryonic regions may utilize a different set of homeobox-containing genes to code for a given position, since we find that at a given position the combination of homeobox genes expressed differs between the somitic mesoderm and the lateral mesoderm, neural crest and central nervous system. A similar conclusion has recently been reached for the epidermis, nervous system and musculature of *Drosophila* (Lawrence & Johnston, 1984; Akam & Martinez-Arias, 1985; Ghysen *et al.* 1985; Martinez-Arias, 1986; Hooper, 1986).

However, this hypothesis seems unable to account for all aspects of the expression patterns observed, suggesting that mammalian homeobox-containing genes may have additional functions, for example in the control of organogenesis.

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