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The murine Hox-2 genes display dynamic dorsoventral patterns of expression during central nervous system development

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Summary

This report demonstrates that the genes in the murine Hox-2 cluster display spatially and temporally dynamic patterns of expression in the transverse plane of the developing CNS. All of the Hox-2 genes exhibit changing patterns of expression that reflect events during the ontogeny of the CNS. The observed expression correlates with the timing and location of the birth of major classes of neurons in the spinal cord. Therefore, it is suggested that the Hox-2 genes act to confer rostrocaudal positional information on each successive class of newly born neurons. This analysis has also revealed a striking dorsal restriction in the patterns of Hox-2 expression in the spinal cord between 12.5 and 14.5 days of gestation, which does not appear to correlate with any morphological structure. The cellular retinol binding protein (CRBP) shows a complementary ventral staining pattern, suggesting that a number of genes are dorsoventrally restricted during the development of the CNS. The expression of Hox-2 genes has also been compared with the Hox-3.1 gene, which exhibits a markedly different dorsoventral pattern of expression. This suggests that, while genes in the different murine Hox clusters may have similar A–P domains of expression, they are responding to different dorsoventral patterning signals in the developing spinal cord.

Key words: homeobox genes, CNS, neural development, Hox-2.

Introduction

The homeobox is an ancient and widespread motif highly conserved in evolution, having been described in the genomes of many higher metazoan organisms (Holland and Hogan, 1986). The murine genome contains at least 35 '*Antp*'-like homeobox genes that are organised into four clusters termed Hox-1, Hox-2, Hox-3 and Hox-4. The clustering of this class of homeobox genes is a general phenomenon in most species and relates to their expression along the anteroposterior (A–P) axis. In *Drosophila*, there is a correlation between the physical order of the ANT-C and BX-C genes and their expression along the A–P axis (Harding *et al.* 1985; Akam, 1987). More recently, it has been shown that the murine Hox clusters display a similar correlation between the position of a gene in the cluster and the relative expression along the A–P axis in the ectoderm and mesoderm, such that each adjacent 3′ gene shows a successively more rostral limit of expression (Duboule and Dolle, 1989; Graham *et al.* 1989). This relationship between the murine and *Drosophila* clusters also extends to the level of sequence and organisation, suggesting that the murine and *Drosophila* homeobox clusters have evolved from a common ancestral cluster that existed prior to the split between the deuterostome and protostome lineages (Akam, 1989; Duboule and Dolle, 1989; Graham *et al.* 1989). These results support the idea that the similarities between the mouse and *Drosophila* homeobox clusters reflect a common evolutionary role in positional signalling along the A–P axis.

Many of the *Drosophila* segmentation and homeotic genes are expressed in the embryonic nervous system The segmentation genes *eve, en* and *ftz*, are expressed in a specific subset of neurons in every segment of the developing central nervous system and seem to be involved in neuronal determination (Doe *et al.* 1988*a*,*b*). The homeotic genes are expressed at their highest levels in the embryonic nervous system (Doe and Scott, 1988). However, unlike the segmentation genes, they are not expressed in every segment of the CNS and are therefore not believed to be involved in the specification of segmentally reiterated features of the CNS. The homeotic genes tend to show peaks of expression in given parasegments and lower levels of expression in more posterior regions. Mutations in these genes affect those regions of the CNS that exhibit

the highest levels of expression for each gene, suggesting that the homeotic genes are involved in regulating correct anteroposterior differentiation (Doe and Scott, 1988).

The A–P expression patterns of the murine homeobox genes in the CNS are remarkably similar to the general observations in *Drosophila*. The Hox-2 genes are expressed at high levels near their anterior boundaries, and expression is reduced in more posterior regions. This is clearly seen at earlier stages of development where the boundaries of expression of several Hox-2 genes correlate with segmental units in the hindbrain, termed rhombomeres (Lumsden and Keynes, 1989; Wilkinson *et al.* 1989). The Hox-2.7, -2.8 and -2.9 genes all display high levels of expression in one or several rhombomeres, and low levels in caudal regions of the neural tube These results suggest that the Hox genes may play a role in specifying the rostrocaudal identity of the rhombomeres; although, unlike *Drosophila*, the role of the murine homeobox genes in the ontogeny of the central nervous system is not clear.

The mammalian CNS, which lies dorsally, is considerably more complex than the ventral CNS of *Drosophila* The mammalian CNS develops from a pseudostratified neuroepithelium, in an ordered manner and, over a period of time, generates a diverse range of different cell types. The transverse organisation of the mammalian CNS, particularly of the spinal cord and hindbrain, is relatively similar throughout its A–P length, except for differences in the timing of maturation of given classes of neurons. The different neuronal cell types are highly organised and show a controlled pattern of spatial and temporal development in the dorsal–ventral plane (Altman and Bayer, 1984). The development of the CNS occurs with rough ventrodorsal and rostrocaudal temporal gradients The large motor neurons of the ventral horn are the first major group of neurons that are born and among the earliest cells of the spinal cord to mature (Altman and Bayer, 1984; Wentworth, 1984*a*). This is followed by the generation of the laterally located relay neurons and finally by the sensory interneurons of the dorsal horn, which are among the latest neurons born. There is a progression in the development of the CNS from rostral to caudal so the process of neuron maturation does not occur simultaneously along the rostrocaudal axis. Hence, while the motor neurons are the first major class to be born, their development occurs in a progressive wave along the rostrocaudal axis and is more advanced in rostral as compared to caudal regions.

To approach the role of murine homeobox genes in the ontogeny of the CNS, it is important to analyse their expression patterns, temporally and spatially, in the transverse plane. A number of groups have analysed the expression of some Hox genes at isolated stages in the development of the CNS and the results reveal several quite divergent patterns ranging from uniform to dorsal or ventral patterns of expression (Brier *et al.* 1988; Dony and Gruss, 1987; Toth *et al.* 1987; Graham *et al.* 1988; Duboule and Dolle, 1989; Krumlauf *et al.* 1987, Bogarad *et al.* 1989). The fact that one observes changing patterns and no clear correlation between individual genes underlines the importance of analysing the expression of an entire cluster of Hox genes at several time points during the ontogeny of the CNS.

In this study, the expression patterns of seven members of the Hox-2 cluster have been analysed during CNS development. Expression of all of these genes have been analysed on adjacent sections at multiple levels along the A–P axis between 10.5 and 14.5 days *post coitum* (*p.c.*). The Hox-2 genes all show a consistent dynamic pattern of expression, which suggests that they are not involved in differential dorsoventral patterning. However, the Hox-2 genes show patterns of expression that mirror cell maturation in the dorsoventral axis, suggesting that they are expressed transiently to confer anteroposterior position on newly born neuronal cells.

Materials and methods

Hybridisation probes

All Hox-2 probes that were used in this analysis have been previously described in Graham *et al.* (1989). The Hox-3.1 probe was a gift from Dr Stephen Gaunt and is described in Gaunt (1988).

In situ *hybridisation*

The protocol used was essentially that of Wilkinson *et al* (1987) with some modifications, and is essentially as follows. Mouse embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C, and then embedded in paraffin wax. Sections (6 μm) were cut and dried onto TESPA-coated slides. The sections were dewaxed in xylene, subjected to proteinase K treatment and treated with acetic anhydride After dehydration, probes were added at a final activity of $1–2\times10^5$ disints min^{-1} kb^{-1} μl^{-1} in hybridisation buffer (50% formamide, 0.3 M NaCl, 20 mM Tris, 5 mM EDTA, 10 mM sodium phosphate, 10% dextran, 1×Denhardts solution, 0.5 $\mu g\,\mu l^{-1}$ tRNA). Hybridisation was overnight at 55°C. Sections were washed at 65°C in 50% formamide, 2×SSC, 100 mM DTT for 40 min followed by incubation with RNAase A at 20 $\mu g\,ml^{-1}$ in 0.5 M NaCl, 10 mM Tris, 5 mM EDTA, for 30 min followed by two 15 min washes in 2×SSC and 0.1×SSC Sections were dehydrated through alcohol solutions containing 0.3 M ammonium acetate Slides were dipped in a mix of Ilford K5 nuclear emulsion and glycerol water (6 ml emulsion in 8.82 ml H_2O and 0 18 ml glycerol) and kept at 4°C until developed. Sections were stained in 0.02% toludine blue for 1 min and then mounted in permount.

Immunohistochemistry

The CRBP staining was done on adjacent sections used for the Hox-2 5 probe. The P2 antibody was a rabbit polyclonal antipeptide antibody directed against the rat CRBP protein and was generated by Dr U. Eriksson, Ludwig Institute, Stockholm, Sweden The staining was carried out as previously described by Maden *et al.* 1989*a*.

Results

The isolation and use of gene-specific probes for seven

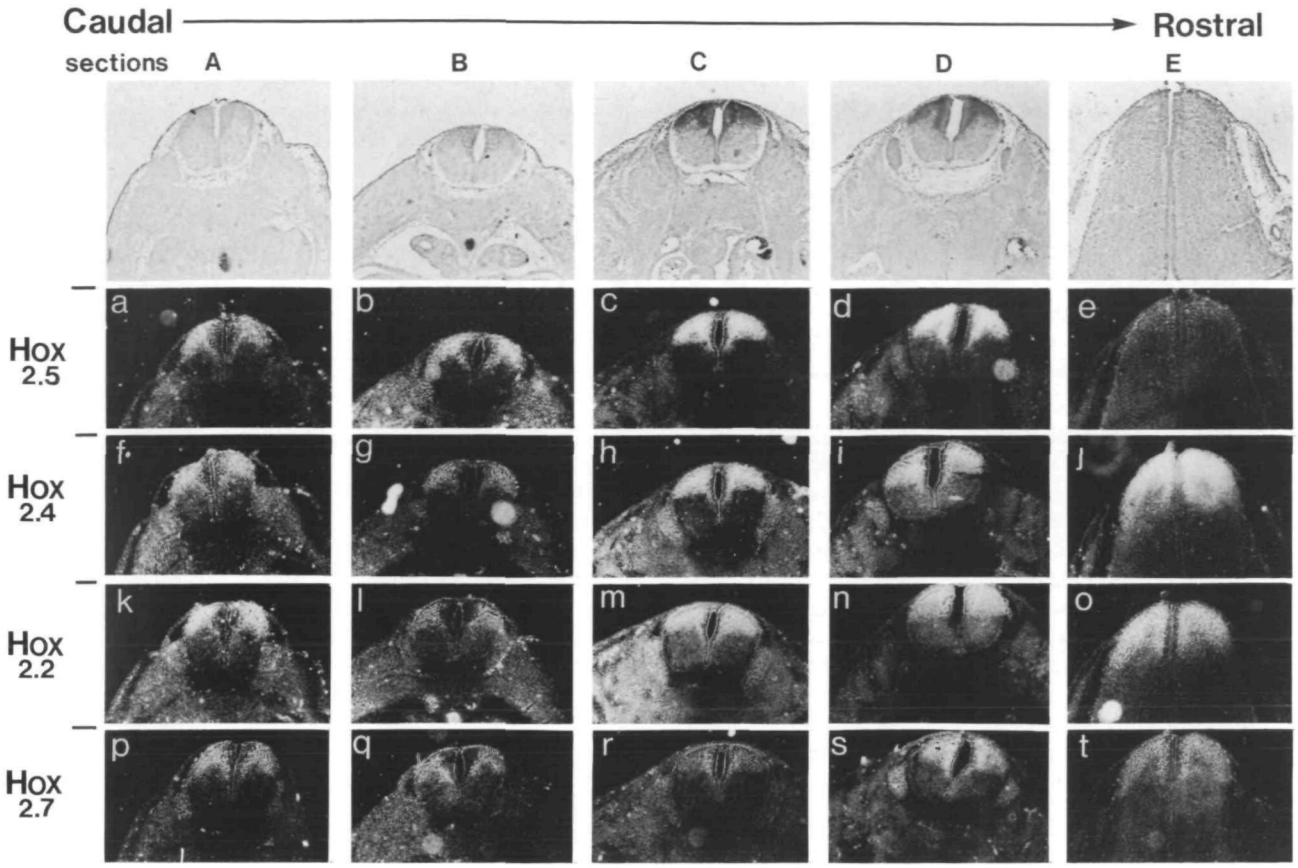

Fig. 1. Expression patterns of four members of Hox-2 at different regions points along the rostrocaudal axis of a 12.5 day *p.c.* mouse embryo Sections were taken at five different points labelled A–E. The rostral-to-caudal order of the sections is indicated at the top of the figure. Representative bright-field photographs are shown for each plane of section The dark-ground photographs display the patterns of expression of each gene at each of the five different points. The probe used for each row of sections is marked on the left, the position of the section is marked at the top of each column.

members of the Hox-2 complex (Hox-2.1 to 2.7) has been previously described (Graham *et al.* 1989). In this study, identical probes were used in *in situ* hybridisation experiments to compare the patterns of expression of these genes in serial transverse sections of the nervous system. Embryos from different stages were analysed at several points along the anteroposterior axis.

Hox-2 genes are dorsally restricted at 12.5 days p.c.

The stage at which the Hox-2 genes show their highest levels of expression is 12.5 days *p.c.* and this time point has been used as a convenient stage for the initial analysis of the patterns of expression. Fig. 1 shows the patterns of expression of four Hox-2 members on adjacent transverse sections at five different points along the rostrocaudal axis (labelled A–E). One can easily see that Hox-2.5, -2.4, -2.2 and -2.7 all display similar patterns of expression in each of the five sets of sections at this stage. In Fig. 1, the sections from position A (a,f,k,p) are caudal and cut through the metanephric kidney. All of the genes exhibit an 'M'-shaped pattern with strong lateral/dorsal labelling. As one moves rostrally along the axis of the embryo, the 'M' shape is still evident up to the level of the lungs (region B, sections b,g,l,q). The pattern then changes in more rostral sections (regions C and D), such that all genes exhibit a strong dorsal domain of expression with a sharp restriction. This boundary in the expression pattern is particularly interesting as it does not seem to correlate with any known morphological structure. In all cases, there is generally a lower or undetectable level of expression in the ventricular layer, which lies immediately adjacent to the lumen.

In the hind brain (region E, sections e,j,o,t), there is no expression of Hox-2.5 but the other three genes (Hox-2.2, -2 4 and -2.7) are still expressed at high levels. The observed difference between the expression of Hox-2.5 and that of the other three genes in the hindbrain results from the fact that there is a correlation between the physical order of Hox genes and their expression along the rostrocaudal axis (Gaunt *et al.* 1988; Duboule and Dolle, 1989; Graham *et al.* 1989; Wilkinson *et al.* 1989). Thus Hox-2.5 is not expressed in the hindbrain (Fig. 1e) since its rostral extent of expression maps in the spinal cord. It is important to note that the only major difference observed between Hox-2 genes in a transverse analysis of CNS expression is that imposed by their rostrocaudal boundaries along the axis. Otherwise, at any given A–P position the Hox-

2 genes all have similar patterns of expression. The pattern that one observes at 12.5 days *p c.* changes along the A–P axis from an 'M' shape to a sharp dorsal restriction, which most likely reflects differences resulting from the anteroposterior gradient of CNS development.

We were interested in how these changing patterns were generated and how they develop. To address these points we have also analysed Hox-2 expression at both earlier and later stages during the development of central nervous system.

Hox-2 genes are expressed uniformly across the nerve cord at 10.5 days p.c.

There is clearly a temporal aspect to the patterns of expression for the Hox-2 genes in the developing CNS, since the dorsal restriction in the expression of these genes at earlier stages is not evident. Expression of seven Hox-2 genes at 10.5 days *p.c.* shows that all are uniformly expressed across the neural tube at this stage (Fig. 2). This uniform pattern of expression is observed at all points along the axis within the rostrocaudal domain of expression for each gene. The sections in Fig. 2 are from three different positions along the A–P axis and illustrate this point. Sections a and b are from the level of hind limb and have been probed with Hox-2.5 and 2.3 respectively; while sections c and d are from the prospective lung region (probed with Hox-2.4 and 2.2). Sections e–i are hindbrain sections that illustrate the expression of Hox-2.1, 2.6 and 2.7. From these results, it is clear that none of the Hox-2 genes analysed show dorsoventral restrictions and all are expressed uniformly across the neural tube at 10.5 days of gestation.

Strong lateral expression of Hox-2 genes at 11.5 days p.c.

The uniform pattern changes by 11.5 days *p.c.* Fig. 3 shows expression of six members of Hox-2 at various points along the A–P axis in the CNS at 11.5 days *p.c.* Sections a and d are from caudal regions while b–f are from the hindbrain region. The ventral motor horns have formed at this stage and expression is not detectable for any of the Hox-2 genes in this region. This is most obvious for Hox-2.2 (Fig. 3d). High levels of expression are observed in the extreme lateral regions where the relay neurons are born (Wentworth, 1984*b*). The remaining dorsoventral part of the spinal cord still exhibits low uniform expression of these genes.

Expression of Hox genes reappears ventrally at 14.5 days p.c.

The strong lateral expression of Hox-2 genes at 11.5 days *p.c.* changes to the sharp dorsally restricted patterns observed at 12.5 days *p.c.* (Fig. 1). We have analysed 14.5 days *p.c.* embryos to look for changes in the 12.5 day *p.c.* profile. Analysis at this later stage of development reveals that the pattern described for the rostral 12.5 day *p.c.* spinal cord is also observed caudally at 14.5 days *p.c.* This is illustrated for Hox-2.5, -2.4 and -2.2 in Fig. 4 (c,f,i). The 14.5 day *p.c.* pattern changes as one moves more anterior, where expression is again detectable in the ventral half of the neural tube (Fig. 4d,g). Hox-2.5 does not exhibit this new ventral pattern since these sections are beyond the anterior boundary of Hox 2.5 expression. The results show that the Hox-2 patterns are dynamically changing even at 14.5 days *p.c.* and that the only major difference between the Hox-2 genes relates to their differential expression along the A–P axis. This A–P difference in expression is not restricted solely to ectodermal tissues but is also found in mesodermal derivatives.

Hox-3.1 is expressed in ventral stripes

The sharp dorsal restriction in Hox-2 expression at 12.5 days *p c.* is particularly interesting and we wished to compare this pattern with that of a homeobox gene from another cluster. The Hox-3.1 gene was chosen since this gene had previously been described as exhibiting a ventral domain of expression in the 12.5 day *p.c.* nerve cord (Brier *et al.* 1988). Adjacent sections were probed with Hox-2.5 and Hox-3.1 and the expression in the neural tube analysed (Fig. 5). In rostral regions, Hox-2.5 displayed a sharp dorsal domain of expression while Hox 3.1 was most abundant ventrally and did not display a sharp demarcation in expression. Hox-2.5 displayed the typical 'M'-shape pattern, while Hox-3.1 exhibited a strikingly different pattern (Fig. 5e,f). Hox-3.1 is expressed in a stripe across each half of the neural tube projecting laterally from the edge of the ventricular zone. There is also Hox-3.1 expression along the sides of the ventricular layer, and no expression is detected in other regions. Thus, the expression pattern of at least two genes from different clusters are radically different at the same stage of development.

Reciprocal expression of Hox-2 genes and of CRBP at 12 5 days p.c.

We were interested in whether other genes displayed similar dorsoventral restrictions in the CNS which correlate with the Hox-2 patterns. Retinoic acid has received considerable attention as a potential signalling molecule in the nervous system and homeobox genes are known to respond to retinoic acid. Therefore, we have also compared the expression of Hox-2.5 with that of the cellular retinol binding protein (CRBP) which is found in the developing CNS (Maden *et al.* 1989). Since there were no Hox-2 antibodies or CRBP nucleic acid probes available we have compared Hox-2.5 RNA distribution (*in situ* hybridisation) with the CRBP protein distribution (immunohistochemistry). The results of this comparison are shown in Fig. 6. It is apparent that products from these two genes occupy reciprocal domains within the transverse plane of the nerve cord, with Hox-2.5 being dorsal and CRBP being ventral. It appears that these domains of expression directly abutt each other. A more detailed analysis of the expression of CRBP in the developing mouse embryo has demonstrated that this molecule is ventrally abundant with a sharp restriction at 10 to 11 days of

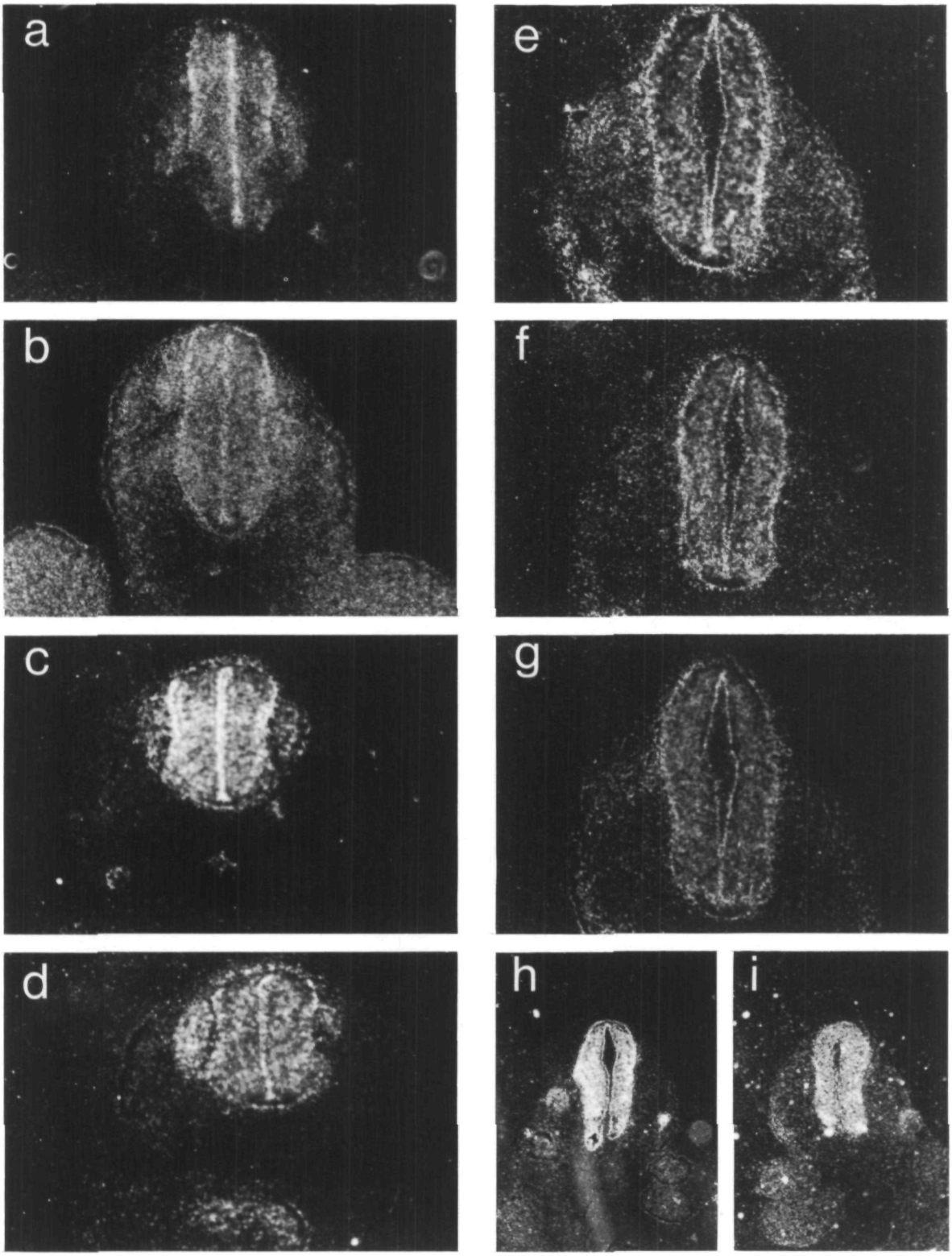

Fig. 2. The patterns of expression for seven members of the hox 2 cluster in the 10.5 day *p.c.* nerve cord. Transverse sections have been taken at various points along the rostrocaudal axis and have been probed with each of the seven probes and the results are shown in dark ground. The probes were as follows: (a) Hox-2.5, (b) Hox-2.3, (c) Hox-2.4, (d) Hox-2.2, (e) Hox-2.1, (f,h) Hox-2.6, (g,i) Hox-2.7.

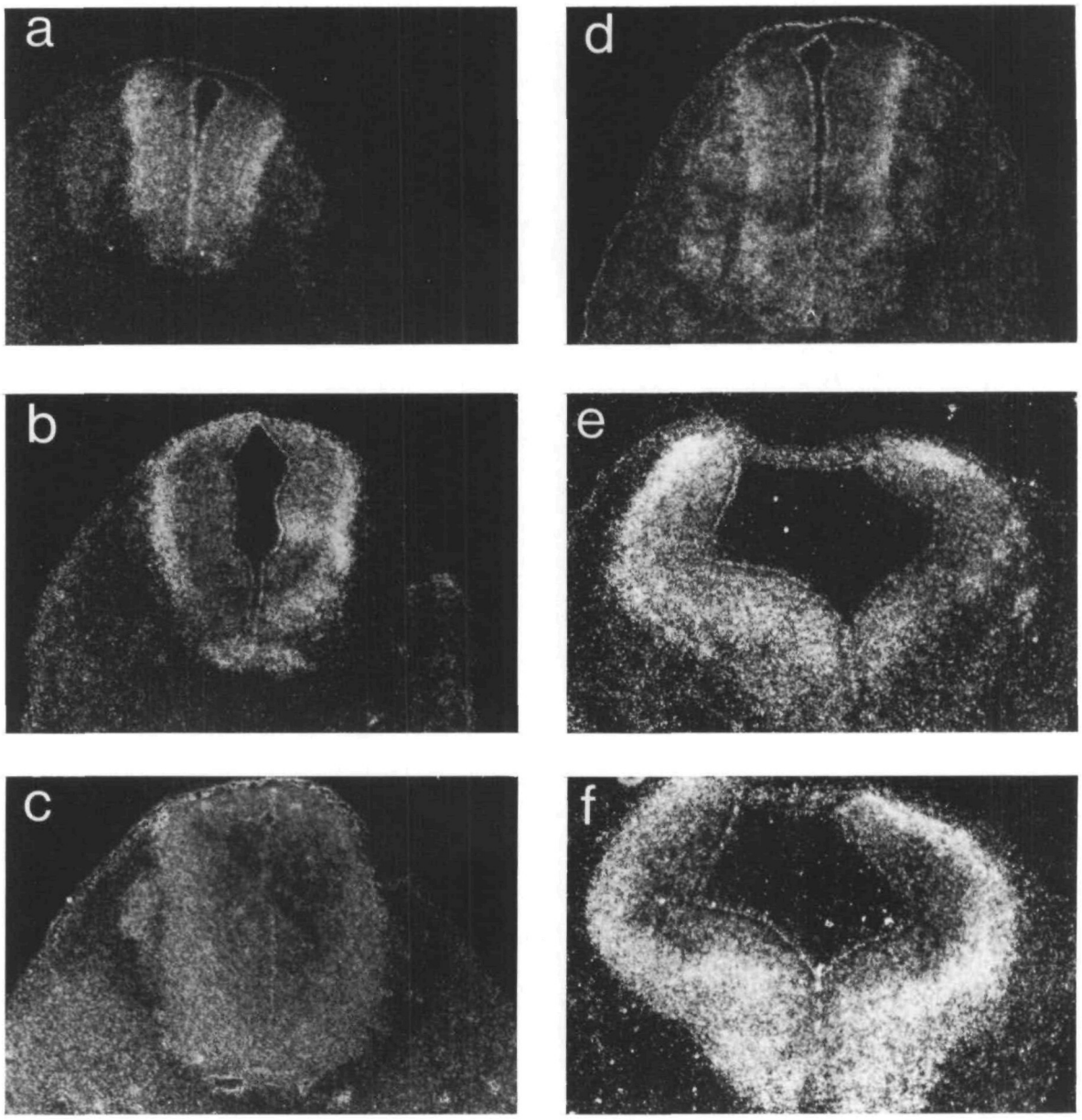

Fig. 3. Transverse sections of the 11.5 day *p.c.* mouse nerve cord probed with Hox-2 genes. The results are shown in dark ground. The probes used were as follows: (a) Hox-2.5, (b) Hox-2.4, (c) Hox-2.2, (d) Hox-2.1, (e) Hox-2.6, (f) Hox-2.7.

gestation, at a time when Hox-2.5 is uniformally expressed (Maden *et al.* 1990). The temporal differences in the appearance of sharp dorsal restrictions for each of these products makes an exact comparison of the early and late boundaries impossible. Yet, it is clear that Hox-2.5 is expressed dorsally while CRBP is expressed ventrally and that these two domains of expression do appear to be reciprocal at 12.5 days *p.c.*

Discussion

The expression patterns, in transverse sections of the nerve cord, for seven members of Hox-2 (Hox-2.1 to 2.7) have been compared on serial sections. This comparison has used embryos at different stages and sections have been taken from numerous points along the rostrocaudal axis. The results show temporally dynamic patterns of expression during the development of the central nervous system.

At the earliest times analysed, 9 days of gestation, these genes show uniform expression across transverse sections of the neural tube and in the closing neural folds (data not shown). At 10.5 days of gestation, the expression remains uniformly distributed across transverse sections of the neural tube (Fig. 2). A day later, members of this cluster show strong lateral expression, and non-detectable levels in the motor horns, with low uniform expression in other regions of the nerve cord. By 12.5 days *p.c.*, the caudal regions of the spinal cord exhibit a more dorsalised pattern. Expression is found

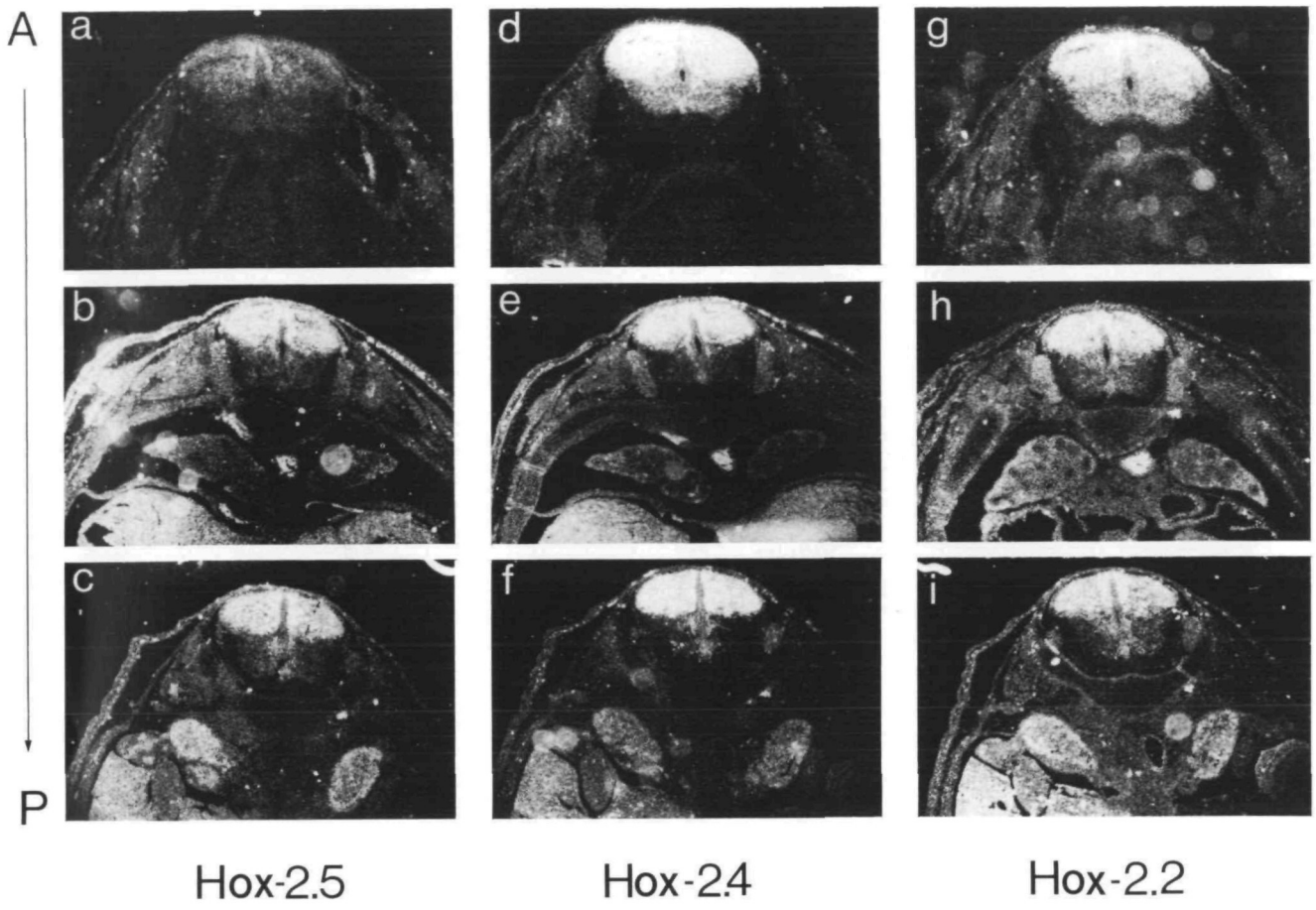

Fig. 4. Expression of three Hox 2 genes at three different points along the rostrocaudal axis of the 14.5 day *p.c* mouse embryo. Each column of sections have been probed with the same gene as is indicated at the base. Each horizontal row of sections is from the same position along the rostrocaudal axis The results are shown in dark ground. The A (anterior), P (posterior) and arrow at the left of the figure indicate the relative anteroposterior region of the embryo from which the rows of sections were derived.

in lateral dorsal regions, but not in the ventricular zone or ventral areas, resulting in an 'M'-shaped pattern. In rostral sections of the 12.5 day CNS, the transcripts of these genes show dorsal restriction with a sharp demarcation. A dorsal restriction has also been described for the Hox-1.4 gene and, at 12.5 days *p.c.*, this gene was found not to be expressed in the ventricular zone (Toth *et al.* 1987). The rostral 12 5 day *p.c.* Hox-2 patterns can still be observed in caudal sections of the 14.5 day *p.c.* spinal cord, but not in more rostral regions. In rostral regions of the 14.5 day *p.c.* CNS, new domains of expression can be found in the neural tube in ventral regions. Since the neural tube matures in a rostral-to-caudal direction, we would suggest that the differences between caudal and rostral sections at 12.5 days *p.c.* reflects the temporal difference in development along the A–P axis. This suggestion is also supported by the fact that the rostral 12.5 day *p.c.* pattern is still observed in caudal portions of older 14.5 days *p.c.* embryos.

These results on seven genes of the Hox-2 cluster are consistent with an earlier report on aspects of the transverse expression pattern of Hox-2.1 (Holland and Hogan, 1988) and in particular confirm the results of Bogarad *et al.* (1989) with the Hox-2.5 gene. The uniform expression across the spinal cord that we previously described for Hox-2.6 (Graham *et al.* 1988) represents an intermediate stage between 11.5 and 12.5 days *p.c.*, because in this more complete study Hox-2.6 is dorsally restricted at 12.5 days *p.c.* The expression of Ghox-2.1 in the CNS, the chicken homologue of Hox 2.1, has been shown to be predominantly dorsally located at stage 25 (Wedden *et al.* 1989), which is approximately equivalent to a 12- to 13-day *p.c.* mouse embryo (Hamburger and Hamilton, 1951) and shows the observed patterns are not unique to the mouse. Thus, in two vertebrate species, the Hox-2.1 gene shows similar patterns of expression in the CNS, suggesting that these genes are performing homologous functions and responding to similar signals in the developing CNS.

One important finding is that each of the seven Hox-2 genes analysed showed the same expression pattern with respect to both changes during the development of the CNS and restricted distribution in the transverse plane. The only major difference observed between any

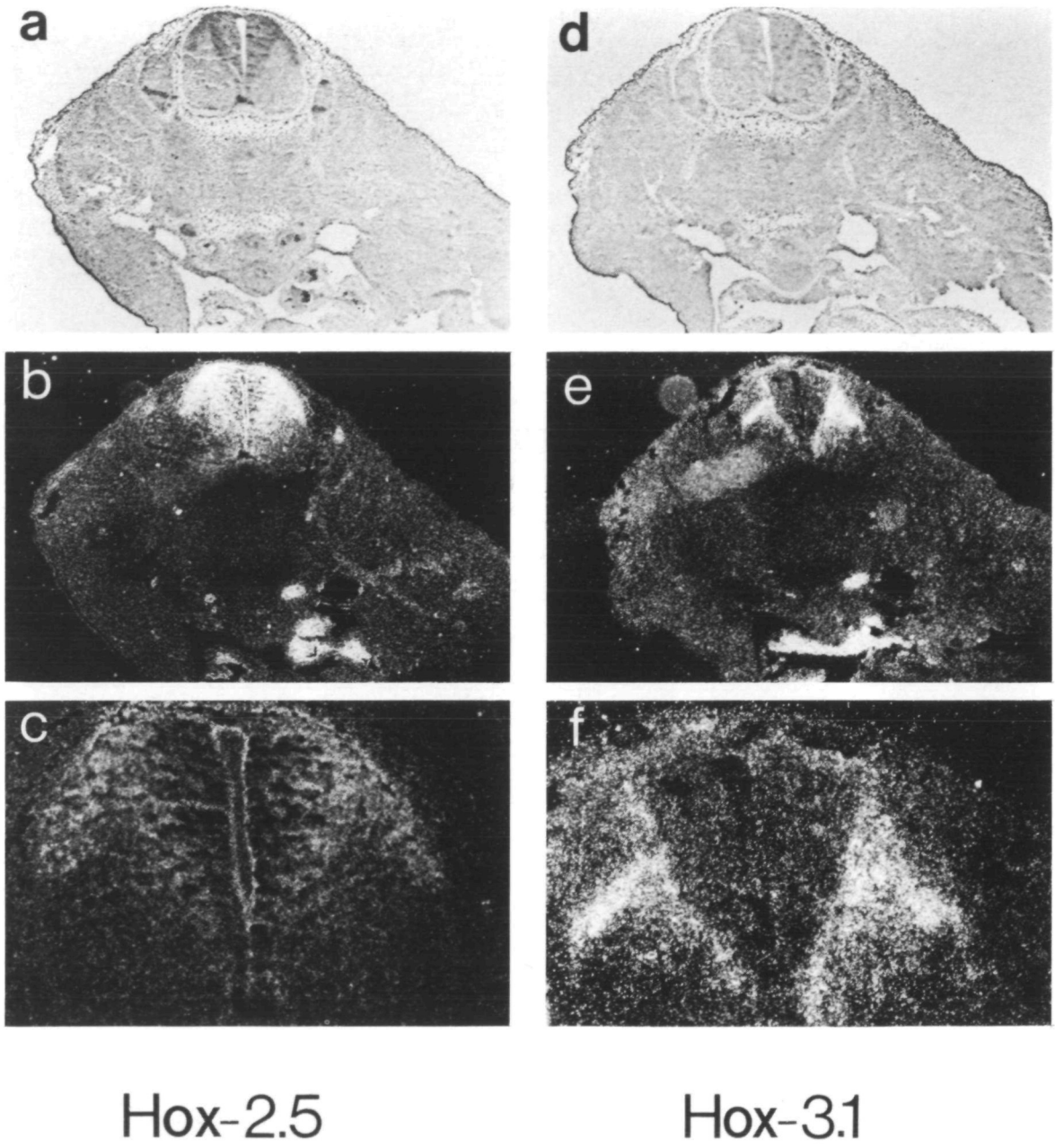

Fig. 5. A comparison between the expression patterns of the Hox-2 5 and the Hox-3.1 genes in the caudal 12 5-day mouse spinal cord. The bright-field photographs (a,d) show the regions that were probed, and the hybridisation patterns are shown at low (b,e) and high (c,f) power in dark ground. The probes used are indicated at the base of each column, Hox-2 5 (a,b,c) and Hox-3.1 (d,e,f).

of these genes in the transverse sections analysed, from 10.5 to 14.5 days of gestation, was in their expression along the rostrocaudal axis As has been previously reported, the genes of the Hox-2 cluster show differential rostrocaudal expression such that there is a correlation between the physical order of the genes and their expression along this axis. This differential rostrocaudal expression of the Hox-2 genes has been used to suggest that they may play a role in rostrocaudal patterning. Since none of these Hox-2 genes show any differences in their transverse patterns of expression in the developing spinal cord, we would predict that individual members of the Hox-2 cluster would not have a differential role in the transverse patterning of the CNS. These results suggest that the dynamic patterns observed instead reflect the response of Hox-2 to developmental events in the spinal cord.

Hox-2 gene expression and the birth of the major classes of spinal neurons

An important question now emerges as to the nature of the developmental events modulating Hox-2 expression. They do not appear to be correlated with

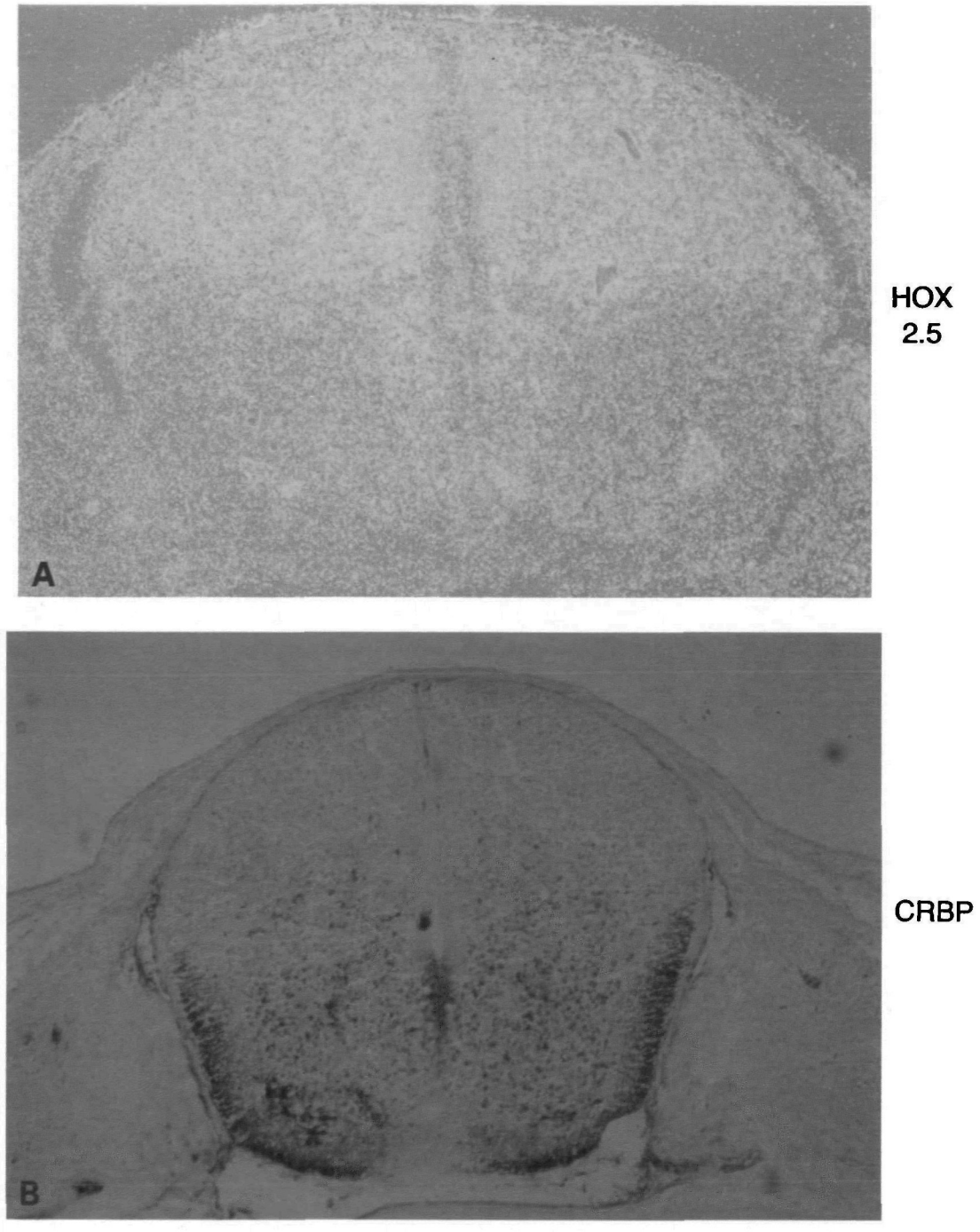

Fig. 6. Comparison of the dorsoventral boundaries of Hox-2.5 and the cellular retinol binding protein (CRBP) in the 12.5 day *p.c.* neural tube Adjacent sections were analysed for Hox-2.5 expression by *in situ* hybridisation (A) and for CRBP by immunohistochemistry (B). In A the pink grains represent Hox-2 5 expression.

changing patterns of cell proliferation, based on short term thymidine labelling studies of the mouse neural tube (Nornes and Cary, 1978). Nor does it seem likely that these genes are involved in basic neuronal differentiation, since neurons of the same class located at different points along the rostrocaudal axis do not express the same Hox-2 genes. The dynamic patterns of Hox-2 expression are more similar, in both temporal and spatial aspects, to that observed for the birth of the major classes of spinal neurons (Sims and Vaughin, 1979; Altman and Bayer, 1984; Wentworth, 1984*a*, 1984*b*).

We would like to suggest that the early pattern of expression across transverse sections of the neural tube are temporally and spatially modified as each of the major classes of neuron are born. First, we see expression in the ventral motor neurons, followed by a drop in expression in these ventral cells by 11.5 days *p.c.* Expression becomes high in the forming commissural neurons whose cells bodies should lie lateral at about 11.5 days. Expression then recedes dorsally, but is still lateral, as the next major class, the association neurons, are born. Finally, one sees general dorsally restricted expression as the interneurons of the dorsal horn are born between 12 and 14 days *p.c.* The transient expression of these Hox-2 genes could allow the differentiating neuronal classes to mark their position along the rostrocaudal axis. Once the cells are fully differentiated, they have already determined their A–P position and Hox-2 expression may no longer be required.

The correlation between Hox-2 expression and neurogenesis does not account for all aspects of the observed dorsoventral patterns. At 14.5 days *p.c.*, the genes show new ventral domains of expression, which cannot be easily linked to a new class of neurons. However, the ventral re-expression could reflect the process of gliogenesis. The sharp dorsal restriction observed from 12.5 to 14.5 days *p.c.* is also difficult to resolve solely on the basis of neurogenesis, and together these findings suggest that a number of different processes are patterning the homeobox genes in the spinal cord at the later stages.

Differential distribution of the Hox-2 genes and the Hox-3.1 gene

It has been demonstrated that genes that share similar positions within different Hox clusters exhibit high degrees of sequence identity and it has been suggested that these gene clusters all arose from a common ancestral cluster (Duboule and Dolle, 1989; Graham *et al.* 1989). In one case related genes within different Hox clusters have been shown to display identical rostral limits of expression in the CNS (Gaunt *et al.* 1989). However, the Hox 3.1 pattern of expression (Fig. 5) is obviously very different from that of members of Hox-2. Not only is Hox-3.1 ventrally abundant in the rostral portion of the nerve cord at 12.5 days of gestation, while the Hox 2 genes display a sharp dorsal domain of expression, but in caudal regions Hox 3.1 is expressed in a stripe across each side of the spinal cord extending laterally from the edge of the ventricular zone. At this level it is also expressed along the side of the ventricular zone. The difference between the dorsoventral expression in the CNS of Hox 3.1 and Hox 2.4, its closest Hox 2 gene, indicates that, while genes may have the same anterior boundary in expression, they can display very different patterns of expression in the dorsoventral plane. Recently Gaunt *et al.* (1990) have found that there are differences in the dorsoventral patterns of related mouse Hox genes. Hence, it seems likely that Hox genes from different clusters gene must be responding to different dorsoventral cues than those that modulate the A–P domains of expression. It is possible that during deutrostome evolution as the central nervous system became more complex the duplicated Hox gene clusters may have provided the potential to specify different positions.

Sharp dorsoventral restrictions to gene expression in the spinal cord

The dorsal restrictions that we observe do not clearly correspond to any obvious morphological feature in our sections. However, one morphological marker that lies in a similar position is the sulcus limitans, which has been described in rat (Altman and Bayer, 1984) and human (Muller and O'Rahilly, 1988) embryos. This structure marks the division in the lateral plate neuroepithelium between the alar and basal plates. The sulcus limitans forms in rat and human embryos at stages that are comparatively earlier than 12.5 days *p.c.*, when we see the dorsally restricted Hox-2 patterns, and is not as obvious a structure in the mouse. Therefore, it is not known whether the sulcus limitans plays a prominent role in early neural development and represents the same boundary as Hox-2 expression.

The Hox genes are not the only genes to shown a sharp dorsoventral restriction in the developing spinal cord. We have shown that CRBP displays a strong ventral band of staining, opposite to the pattern observed with Hox-2.5 in adjacent sections (Fig. 6). The two genes seem to respect the same dorsoventral boundary at 12.5 days *p.c.* Maden *et al.* (1990) have shown that the ventral CRBP stripe appears at day 10 *p.c.* in the mouse neural tube and is one of the earliest markers for the onset of differentiation. CRBP remains restricted in later stages, particularly in the motor neurons and motor axons. Since the CRBP becomes restricted at earlier stages than the Hox-2 and Hox-3.1 genes, it implies that the CRBP may have an early patterning role in the neural tube, and that the homeobox genes must be involved in later events.

Conclusions

While this study actually focuses on dynamic spatial and temporal transverse patterns of Hox-2 genes in the spinal cord, it serves to reinforce the importance of their differential expression along the rostrocaudal axis. The patterns are consistent with Hox 2 genes appearing to act in conferring rostrocaudal position on newly

formed classes of neurons. The expression pattern of Hox-3.1 is clearly different from all of the Hox-2 genes, which suggests that it is responding to different developmental cues. Therefore, each of the Hox clusters may play different roles in dorsoventral patterning. The observation that CRBP is dorsoventrally restricted prior to Hox-2 implies that other genes must be involved earlier in dorsoventral patterning. Finally, the roles of murine homeobox genes can be seen to be strikingly similar to their *Drosophila* counterparts, and in both organisms these genes could be involved in the specification of A–P position and regulating correct anteroposterior differentiation in the CNS.

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References

AKAM, M E (1987) The molecular basis for metameric pattern in the *Drosophila* embryo *Development* **101**, 1–22

AKAM, M (1989) Hox and HOM: Homologous gene clusters in insects and vertebrates. *Cell* **57**, 347–349

ALTMAN, J AND BAYER, S A (1984) The development of the rat spinal cord *Adv Anat Embryol Cell Biol* **85**, 1–166

BOGARAD, L D., UTSET, M F , AWGULEWITSCH, A , MIKI, T , HART, C. P. AND RUDDLE, F. H (1989). The developmental expression pattern of a new murine homeobox gene Hox 2 5 *Devl Biol* **133**, 537–549

BRIER, G , DRESSLER, G. R AND GRUSS, P (1988) Primary structure and developmental expression of Hox 3.1, a member of the Hox 3 homeobox cluster *EMBO J* **7**, 1329–1336

DOE, C Q , HIROMI, Y , GEHRING, W. J AND GOODMAN, C. S (1988*a*) Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis *Science* **239**, 170–175

DOE, C Q AND SCOTT, M P. (1988) Segmentation and homeotic gene function in the developing nervous system of *Drosophila* *TINS* **11**, 101–106

DOE, C Q., SMOUSE, D. AND GOODMAN, C S. (1988*b*) Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376–378

DONY, C AND GRUSS, P. (1987) Specific expression of the Hox 1 3 gene in murine embryonic structures originating from or induced by mesoderm *EMBO J* **6**, 2965–2975

DUBOULE, D AND DOLLE, P. (1989) The murine Hox gene network its structural and functional organisation resembles that of *Drosophila* homeotic genes *EMBO J* **8**, 1507–1508

GAUNT, S J (1988) Mouse homeobox gene transcripts occupy different but overlapping domains in embryonic germ layers and organs a comparison of Hox 3 1 and Hox 1 5 *Development* **103**, 135–144

GAUNT, S J., COLETTA, P L., PRAVTCHEVA, D AND SHARPE, P T (1990) Mouse Hox-3.4 homeobox sequence and embryonic expression patterns compared with other members of the Hox gene network. *Development* **109**, 329–337

GAUNT, S. J , KRUMLAUF, R AND DUBOULE, D. (1989) Mouse homeobox 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 *Development* **107**, 131–141

GAUNT, S J , SHARPE, P. T AND DUBOULE, D (1988) Spatially restricted domains of homeogene transcripts in mouse embryos relation to a segmented body plan *Development* **104 Supplement**, 169–179

GRAHAM, A , PAPALOPULU, N AND KRUMLAUF, R (1989) The murine and *Drosophila* gene complexes have common features of organisation and expression *Cell* **57**, 367–378

GRAHAM, A , PAPALOPULU, N , LORIMER, J , MCVEY, J H , TUDDENHAM, E G. D. AND KRUMLAUF, R. (1988) Characterisation of a murine homeobox gene, Hox 2 6, related to the *Drosophila Deformed* gene *Genes Dev* **2**, 1424–1438

HAMBURGER, V. AND HAMILTON, H L (1951) A series of normal stages in the development of the chick embryo *J Morph* **88**, 49–92

HARDING, K , WEDEN, C , MCGINNIS, W AND LEVINE, M (1985) Spatially regulated expression of homeotic genes in *Drosophila* *Science* **229**, 1236–1242

HOLLAND, P W. H AND HOGAN, B. L M (1986) Phylogenetic distribution of *Antennapedia* like homeoboxes *Nature* **321**, 251–253

HOLLAND, P. W H AND HOGAN, B L M (1988) Spatially restricted patterns of expression of the homeobox containing gene Hox 2 1 during mouse embryogenesis *Development* **102**, 159–174.

KRUMLAUF, R , HOLLAND, P W H , MCVEY, J H AND HOGAN, B L M (1987) Developmental and spatial patterns of expression of the mouse homeobox gene Hox 2 1 *Development* **99**, 603–617

LUMSDEN, A AND KEYNES, R (1989) Segmental patterns of neuronal development in the chick hindbrain *Nature* **337**, 424–428

MADEN, M , ONG, D E AND CHYTIL, F (1990). Retinoid binding protein distribution in the developing mammalian nervous system *Development* **109**, 75–80

MADEN, M , ONG, D E , SUMMERBELL, D , CHYTIL, F AND HIRST, E. A (1989) Cellular retinoic acid-binding protein and the role of retinoic acid in the development of the chick embryo *Devl Biol* **135**, 124–132

MULLER, F AND O'RAHILLY, R (1988) The development of the human brain from a closed neural tube at stage 13 *Anat Embryol* **177**, 203–224

NORNES, H AND CARY, M (1978) Neurogenesis in the spinal cord of the mouse: an autoradiographic analysis *Brain Res* **159**, 1–16

RUGH, R. (1968) *The Mouse its Reproduction and Development* Burgess

SIMS, T J AND VAUGHIN, J E (1979) The generation of neurons involved in an early reflex pathway of embryonic mouse spinal cord *J Neurol.* **183**, 707–720.

TOTH, L E , SLAWIN, K L., PINTAR, J E AND CHI NGUYEN-HUU, M. (1987) Region specific expression of mouse homeobox genes in the embryonic mesoderm and central nervous system *Proc Natn Acad Sci U S A* **84**, 6790–6794

WEDDEN, S E , PANG, K AND EICHLE, G (1989) Expression pattern of homeobox containing genes during chick embryogenesis *Development* **105**, 639–650

WENTWORTH, L E (1984*a*) The development of the cervical spinal cord of the mouse embryo I A golgi analysis of the ventral root neuron differentiation *J Neurol* **222**, 81–95

WENTWORTH, L E (1984*b*) Development of the cervical spinal cord of the mouse embryo II A golgi analysis of the sensory, commissural and association cell differentiation. *J Neurol.* **222**, 96–115

WILKINSON, D G , BAILES, J A , CHAMPION, J E AND MCMAHON, A P (1987) A molecular analysis of mouse development from 8 to 10 days post coitum detects changes only in embryonic globin gene expression *Development* **99**, 493–500

WILKINSON, D G , BHATT, S , COOK, M , BONCINELLI, E AND KRUMLAUF, R (1989) Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain *Nature* **341**, 405–409

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