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Expression of the mouse *labial*-like homeobox-containing genes, *Hox 2.9* and *Hox 1.6*, during segmentation of the hindbrain

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Summary

The sequence of a mouse *Hox 2.9* cDNA clone is presented. The predicted homeodomain is similar to that of the *Drosophila* gene *labial* showing 80 % identity. The equivalent gene in the *Hox 1* cluster is *Hox 1.6* which shows extensive similarity to *Hox 2.9* both within and outside the homeodomain. *Hox 2.9* and *Hox 1.6* are the only two mouse members of the *labial*-like family of homeobox-containing genes as yet identified.

Hox 2.9 has previously been shown to be expressed in a single segmental unit of the developing hindbrain (rhombomere) and has been predicted to be involved in conferring rhombomere identity. To analyse further the function of *Hox 2.9* during development and to determine if the other mouse *labial*-like gene *Hox 1.6*, displays similar properties, we have investigated the expression patterns of these two genes and an additional rhombomere-specific gene, *Krox 20*, on consecutive embryonic sections at closely staged intervals. This detailed analysis has enabled us to draw the following conclusions:

(1) There are extensive similarities in the temporal and spatial expression of *Hox 2.9* and *Hox 1.6*, throughout the period that both genes are expressed in the embryo (7½ to 10 days). At 8 days the genes occupy identical domains in the neuroectoderm and mesoderm with the same sharp anterior boundary in the presumptive hindbrain. These similarities indicate a functional relationship between the genes and further suggest that the *labial*-like genes are responding to similar signals in the embryo.

(2) By 9 days the neuroectoderm expression of both genes retreats posteriorly along the anteroposterior (AP) axis. The difference at this stage between the expression patterns is the persistence of *Hox 2.9* in a specific region of the hindbrain, illustrating the capacity of *Hox 2.9* to respond to additional positional regulatory signals and indicating a unique function for this gene in the hindbrain.

(3) The restriction of *Hox 2.9* expression in the hindbrain occurs at 8½ days, approximately the same time as *Krox 20* is first detected in the posterior adjoining domain. The mutually exclusive expression of *Hox 2.9* and *Krox 20* demarcated by sharp expression boundaries suggest that compartmentalisation of cells within the hindbrain has occurred up to 6 h before rhombomeres (morphological segments) are clearly visible.

(4) *Hox 2.9* expression is confined to the region of rhombomere 4 that shows cell lineage restriction and, unlike *Krox 20*, is expressed throughout the period that rhombomeres are visible (to 11½ days). These data strengthen the evidence that *Hox 2.9* participates in conferring segment identity.

(5) Migrating neural crest cells that arise from rhombomere 4 are uniquely identified by the expression of *Hox 2.9* supporting the idea that neural crest cells are patterned according to their rhombomeric origin.

(6) The *Hox 1.6* gene product is differentially transcribed; only one of the two alternative transcripts codes for a homeodomain-containing protein. A comparison of the distribution patterns of the two transcripts shows that the relative proportion of homeodomain-producing message decreases as development proceeds.

Key words: homeobox, *Hox 1.6*, *Hox 2.9*, mouse development, rhombomere.

Introduction

The mouse *Antennapedia*-like homeobox genes reside within four tightly clustered multigene arrays in the mouse genome designated *Hox 1* which is on chromosome 6; *Hox 2* on chromosome 11; Hox 3 on chromosome 15; and *Hox 4* (formerly *Hox 5*, Kessel and Gruss, 1990) on chromosome 2 (Bucan *et al.* 1986; Hart *et al.* 1985; Breier *et al.* 1988; Featherstone *et al.* 1988). The clusters seem to have arisen from a common ancestral cluster by chromosomal duplication events. This is shown by the sequence comparison of the genes; for example, all but two genes in the *Hox 2* cluster have counterparts in the *Hox 1* cluster (Hart *et al.* 1987; Graham *et al.* 1989; Duboule and Dolle, 1989). The cognate genes within the clusters show a further level of

similarity in that the genes are organised in the same linear order along the chromosome. It is apparent that the mammalian clusters are remnants of an ancient ancestral cluster that predates the organisation of homeobox-containing genes in insects. The insect complex of homeobox-containing genes, the HOM-C, which includes the *Bithorax* (Lewis, 1978; Sanchez-Herrero *et al.* 1985) and *Antennapedia* (Kaufman *et al.* 1980) gene complexes of *Drosophila*, is known to be homologous to the mammalian clusters from sequence comparison and the organisation of the genes along the chromosome. At one end of the insect HOM cluster resides the gene referred to as *labial* (Diederich *et al.* 1989). At corresponding positions in the mouse *Hox 1* and *2* clusters are the homologous genes *Hox 1.6* (Baron *et al.* 1987; Mlodzik *et al.* 1988; LaRosa and Gudas, 1988) and *Hox 2.9* (Rubock *et al.* 1990); these form a subfamily of *labial*-like homeobox-containing genes. Counterparts in the other two mouse gene clusters have not been reported.

During *Drosophila* development, the embryo is divided into segments along the anteroposterior (AP) axis and *Drosophila* homeobox genes are involved in conveying positional information during the process of segmentation (Gehring, 1987 for review). In vertebrates repetitive structures in the embryonic hindbrain called rhombomeres reflect an underlying segmental organisation and act as units of cell lineage restriction (Lumsden and Keynes, 1989; Fraser *et al.* 1990). The correspondence of expression of *Hox 2.9* with a single rhombomere suggests that it plays an analogous role to *Drosophila* homeobox-containing genes in conveying positional information in the mouse (Murphy *et al.* 1989). The other members of the Hox 2 cluster occupy overlapping domains along the AP axis of the central nervous system (CNS), the anterior boundaries that lie within the hindbrain corresponding to segment boundaries (Wilkinson *et al.* 1989*b*). In general, the data available on other mouse homeobox genes show that they are expressed in the ectoderm, the mesoderm and, to a lesser extent, the endoderm of the developing embryo. Expression of the earliest of these genes is detected at the time of gastrulation (Gaunt, 1987) and a small number of genes are still expressed in the newborn mouse (Awgulewitsch *et al.* 1986; Utset *et al.* 1987). The majority of homeobox-containing genes are expressed during the process of gastrulation, when AP positional values are established in the amphibian (Ruiz i Altaba and Melton, 1990), and during morphogenesis in the embryo. Within particular developmental fields homeobox genes occupy characteristic, overlapping expression domains with different subsets of genes active in different spatial domains, consistent with a role in positional determination (Dolle *et al.* 1989; Holland and Hogan, 1988; Graham *et al.* 1989).

It appears that subfamily members in different clusters display similar, although not always identical, AP expression domains. This was observed for the domains of *Hox 3.3* (formerly *Hox 6.1*) and *Hox 1.2* (Gaunt *et al.* 1988) and for the domains of *Hox 1.4*, *Hox 2.6* and *Hox 5.1* (Gaunt *et al.* 1989) in the CNS and prevertebral column at 12.5 days. *Hox 1.5* and *Hox 2.7* both have anterior boundaries within the CNS that correspond to the same rhombomere boundary, the anterior boundary of rhombomere 5 (Gaunt *et al.* 1987; Wilkinson *et al.* 1989*a*). However, two separate studies on the related pair of genes *Hox 2.5* (Graham *et al.* 1989) and *Hox 5.2* (Duboule and Dolle, 1989) show that expression of the former extends more anteriorly. This has been interpreted as indicating that similarity between paralogues does not hold for genes expressed only in the posterior embryo (Gaunt *et al.* 1989). Some mouse homeobox-containing genes are not contained within clusters and these also have closely related genes on other chromosomes (Joyner and Martin, 1987; Davidson *et al.* unpublished data). Detailed studies of these genes, the *engrailed*-like mouse genes *En1* and *En2* (Davidson *et al.* 1988) and the *Msh*-like mouse genes *Hox 7.1* and *Hox 8.1* (Hill *et al.* 1989; Davidson *et al.* unpublished data) show that they have overlapping or complementary expression patterns.

Here we present the cDNA sequence of *Hox 2.9* and show its relationship to other *labial*-like genes. We present the results of a detailed analysis of *Hox 1.6* and *Hox 2.9* expression in the early embryo including the use of the *Krox 20* gene as a temporal and positional molecular marker in the developing hindbrain. The analysis was designed to compare the expression patterns of the *labial*-like genes in the mouse, to investigate how the segmental expression of *Hox 2.9* in rhombomere 4 becomes localised from an earlier more widespread domain and to determine how the onset of localised, segmental expression relates to the appearance of morphological segments.

Materials and methods

Isolation of cDNA clones

cDNA clones for both *Hox 1.6* and *Hox 2.9* were isolated from an 8.5 day mouse embryonic cDNA library in lambda gt10. The original clones were selected as weakly hybridising to the *Drosophila* gene *fushi tarazu*. The cDNAs were subcloned into suitable vectors for sequencing and transcription.

DNA sequence determination and analysis

Random subclones of a full-length Hox 2.9 cDNA were sequenced by dideoxynucleotide sequencing procedures (Sanger *et al.* 1987) using 'Sequenase' (US Biochemicals) as described by the manufacturers. The sequences were aligned using the Staden-plus computer package (Amersham). Sequence comparisons were carried out by the GAP program in the sequence analysis software package of the University of Wisconsin Genetics Computer Group.

Preparation of embryo sections

Embryos were obtained from outbred Swiss mice. For ageing purposes, midday on the day of detection of a vaginal plug was designated 0.5 days *post coitum*. Embryos within a litter were precisely staged by their morphology, the size and shape of the head fold and the appearance of rhombomeres being the most important criteria between 8 and 9 days. Embryos were fixed in 4% paraformaldehyde at 4°C overnight and

embedded in paraffin wax. 5–7 μm sections were cut and floated onto TESPA (3-aminopropyltriethoxysilane; Sigma) -treated slides.

In situ *hybridisation*

Sense and antisense RNA probes were produced by incorporating ^{35}S-UTP into the transcription products of selected *Hox 1.6*, *Hox 2.9* and *Krox 20* subclones inserted into T7- and T3-containing transcription vectors. The *Hox 1.6* probe used has been previously called cDNA 1 (Baron *et al.* 1987). The 3′ *Pst*I/*Eco*RI fragment of *Hox 2.9* (Fig. 1A) was used for preparations of the riboprobe. For *Krox 20* the probe was prepared from the 1.5 kb *Apa*I–*Eco*RI fragment (Chavrier *et al.* 1988). The *Hox 1.6* probe includes the homeobox sequence, but the characteristic pattern of expression observed indicates that there is no cross-reactivity under the conditions used. Sense (control) probes showed no specific labelling. The *in situ* hybridisation protocol has been previously described (Davidson *et al.* 1988) and included high-stringency washes. All probes were used at the same specific activity with $3–6\times10^5$ disints min^{-1} added per section, varied with section size.

Silver grain density estimation

The number of grains in at least two different areas of 177 μm^2 within each tissue were directly counted at times 1000 magnification. Three to six counts were taken in each area. Background counts were estimated in areas with comparable cell density and the average background count was subtracted from each count.

Results

Structure and sequence of the Hox 2.9 *gene*

A probe for the *Hox 1.6* gene, including the homeobox, was used to isolate a *Hox 2.9* cDNA from an 8.5 day mouse embryonic cDNA library by low-stringency hybridisation. We sequenced the 1780 bp *Hox 2.9* cDNA containing a single long open reading frame of 894 bp with an in-frame homeobox domain (Fig. 1). It is predicted to encode a 32×10^3 M_r protein. A *Hox 2.9* transcript of approximately 2 kb was detected in RNA prepared from 9 day embryos and F9 teratocarcinoma cells (data not shown), indicating that the sequenced cDNA clone represents an almost full-length transcript. The absence of a poly(A) stretch and recognisable polyadenylation site (Proudfoot and Brownlee, 1976) indicates that the cDNA insert is truncated at the 3′ end. The predicted ATG translational start codon is the first ATG in frame with the homeobox. No other in-frame ATG was found within 514 bp upstream of the proposed start site.

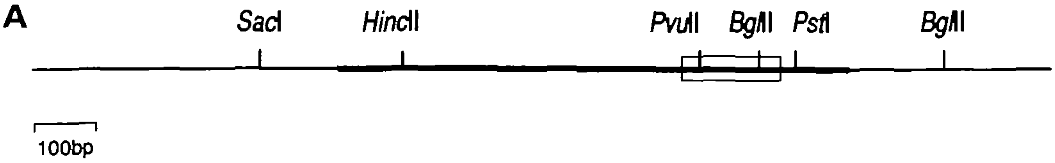
Sequence comparison reveals that *Hox 2.9* contains a homeodomain similar to that of the *Drosophila* gene *labial* (Fig. 2A). The *Drosophila labial* homeodomain has diverged significantly from the *Antennapedia* sequence (67 % amino acid identity) and is most closely related to its homologues in other species including *Hox 2.9* (Fig. 2A, Table 1). The *Hox 2.9* homeodomain shows 80 % amino acid identity to the *Drosophila labial* homeodomain and 87 % identity to that of *Hox 1.6*, which has previously been shown to be *labial*-like (85 % amino acid identity). Comparison of the *labial*-like homeodomains to other genes in the mouse shows at best 62 % identity. We therefore suggest that *Hox 2.9* is the second member of the *labial* subfamily in the mouse. No other mouse homeobox-containing gene has been reported that belongs to this subfamily; however, genes isolated from chicken (*Ghox.lab*) (Sundin *et al.* 1990) and human (*HOX 2I*) (Acampora *et al.* 1989) are *labial*-like.

Comparison of the mouse and chicken *labial*-like genes reveals that regions of similarity also exist outside the homeobox (Fig. 2B). These include a stretch of 22 amino acids at the amino-terminus of the proteins and regions that extend from both ends of the homeobox. The *Hox 1.6* gene has previously been shown to contain only two amino acids (Trp-Met) of the conserved hexapeptide (Ile/Val-Tyr-Pro-Trp-Met-Arg) found in many homeodomain proteins (Baron *et al.* 1987). However, the four replacements (Trp-Phe-Asp-X-X-Lys) in this region in *Hox 1.6* are conserved in both *Hox 2.9* and *Ghox lab* and interestingly mark the beginning of the extended region of homology around the homeodomain. Examination of the full coding region shows that *Ghox lab* is more similar to *Hox 2.9* (Fig. 2B, Table 1), particularly at the carboxy-terminal end, and we predict that this chicken gene is the *Hox 2.9* homolog; however, expression analysis is required to make definite conclusions.

Hox 2.9 *and* Hox 1.6 *expression between* $7\frac{1}{2}$ *and 9 days of development (formation of rhombomeres)*

Between $7\frac{1}{2}$ and 9 days of development, the expression patterns of *Hox 2.9* and *Hox 1.6* change rapidly and dramatically. To establish how the patterns evolve during this period, we have examined embryos at closely staged intervals of approximately 6 h, using the *Krox 20* (Wilkinson *et al.* 1989*a*) gene as a molecular marker for events occurring in the developing hindbrain. Expression of *Hox 2.9* and *Hox 1.6* is first detected at $7\frac{1}{2}$ days during the early stages of gastrulation (Fig. 3). Both genes are expressed within the primitive streak in newly formed mesoderm and overlying neuroectoderm. *Hox 2.9* expression is at a higher level and is more extensive than *Hox 1.6*. In the early 8 day embryo, *Hox 2.9* and *Hox 1.6* are expressed at a high level in extensive domains, extending from the posterior end of the embryo along the neuroectoderm and mesoderm tissue layers into the region of the developing hindbrain of the headfold (Fig. 4A–D). The two genes have identical, sharp anterior boundaries of expression in the neuroectoderm that coincide with the preotic sulcus (a characteristic groove in the surface of the presumptive hindbrain). An adjacent section probed with the *Krox-20* gene shows that it is expressed in a single domain in the hindbrain, the posterior boundary of which corresponds to the anterior boundary of *Hox 2.9* and *Hox 1.6* (Fig. 4D). As was previously described, *Krox 20* is first detected in a single domain in the hindbrain and later in its characteristic two-stripe pattern (Wilkinson *et al.* 1989*a*). Within the mesoderm of the 8-day embryo, both *Hox 2.9* and *Hox 1.6* expression is restricted to lateral plate mesoderm as

A



B

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  10                  30                  50                  70                  90                  110
AACCGCAGGTTCAGACATTTGGTGTATGTGCTTGGCTGAGGAGCCAATGGGGCGAAGCTACCATCTGTGGGATTATGACTGAACGCCTCTAAGTCAGAATCCCGCCCAGGGCAACGATAC
  130                 150                 170                 190                 210                 230
GGCAGCGGCAAAGGAGCCTCGGTTGGCCCCGGATAGCCGGTCCCCGTCCGTCCCCGCTCGGGTGCCCCCGCGTCGTCCCCGCGCGGCGCTCTTCCCCCCGGGCCGGCTCGGACCGGGGTC
  250                 270                 290                 310                 330                 350
GTGCGGAGAGCCGTTCGTCTTGGGAAACGGGGTGCGGCCGGAAAGGGGGCCGCCCTCTCGCCCGTCACGTTGAACGACCGTTCGTGTGGAACCTGGCGCTAAACCATTCGTAGACGACCT
  370                 390                 410                 430                 450                 470
GCTTCTGGGTCGGGGTTTCGTACGTAGCAGAGCTCCCTCGCTGCGATCTATTGAAAGTCAGCCCTCGACACAAGGGTTTGTGACATACTGCCGAAAGGTTGTAGGGCAAGAGGGTGTCTC
  490                 510                 530                 550                 570                 590
CCCCAAACGGCCCGACCCTCCTTCGGCCTCTACATGGACTATAATAGGATGAGTTCCTTTTTAGAGTACCCACTTTGTAACCGGGGACCCAGCGCCTACAGCGCCCCAACCTCTTTTCCC
                                   MetAspTyrAsnArgMetSerSerPheLeuGluTyrProLeuCysAsnArgGlyProSerAlaTyrSerAlaProThrSerPhePro
  610                 630                 650                 670                 690                 710
CCCTGCTCAGCTCCGGCCGTTGACACGTACGCAGGGGAGAGCCGCTATGGTGGAGGGCTGCCTAGCTCAGCGCTCCAACAAAACTCGGGGTATCCTGTCCAGCAGCCGCCGTCATCCCTG
ProCysSerAlaProAlaValAspThrTyrAlaGlyGluSerArgTyrGlyGlyGlyLeuProSerSerAlaLeuGlnGlnAsnSerGlyTyrProValGlnGlnProProSerSerLeu
  730                 750                 770                 790                 810                 830
GGGGTGTCCTTTCCCAGCCCCGCTCCCTCGGGGTACGCCCCAGCCGCCTGCAACCCCAGCTATGGGCCTTCTCAGTATTATTCTGTGGGTCAGTCGGAAGGAGATGGAAGCTATTTTCAT
GlyValSerPheProSerProAlaProSerGlyTyrAlaProAlaAlaCysAsnProSerTyrGlyProSerGlnTyrTyrSerValGlyGlnSerGluGlyAspGlySerTyrPheHis
  850                 870                 890                 910                 930                 950
CCGTCGAGCTACGGAGCCCAGCTAGGGGGGTTGCCCGACAGCTATGGAGCGGGTGGAGTCGGCTCAGGGCCATATCCTCCGCCGCAGCCCCCATACGGAACTGAGCAGACCGCAACCTTT
ProSerSerTyrGlyAlaGlnLeuGlyGlyLeuProAspSerTyrGlyAlaGlyGlyValGlySerGlyProTyrProProProGlnProProTyrGlyThrGluGlnThrAlaThrPhe
  970                 990                 1010                1030                1050                1070
GCATCAGCCTACGACCTCCTCTCTGAGGACAAGGAATGCCCTTGCTCGTCAGAACCCAGCACTCTCACTCCCCGGACCTTCGACTGGATGAAGGTCAAGAGAAACCCACCTAAGACAGCG
AlaSerAlaTyrAspLeuLeuSerGluAspLysGluCysProCysSerSerGluProSerThrLeuThrProArgThrPheAspTrpMetLysValLysArgAsnProProLysThrAla
  1090                1110                1130                1150                1170                1190
AAGGTGTCCGAGCTGGGACTGGGGCCTCCCCGCGGTCTCCGCACAAACTTCACCACGCGCCAGCTGACGGAGCTGGAGAAGGAATTTCATTTCAACAAATACCTGAGCCGTGCCCGGAGG
LysValSerGluLeuGlyLeuGlyProProArgGlyLeuArgThrAsnPheThrThrArgGlnLeuThrGluLeuGluLysGluPheHisPheAsnLysTyrLeuSerArgAlaArgArg
  1210                1230                1250                1270                1290                1310
GTGGAGATCGCGCCCACCCTGGAGCTCAATGAAACGCAGGTGAAGATCTGGTTCCAGAACCGGCGCATGAAGCAGAAGAAACGCGAGCGAGAGGGGGGCAGGATGCCTGCAGGCCCCCCA
ValGluIleAlaProThrLeuGluLeuAsnGluThrGlnValLysIleTrpPheGlnAsnArgArgMetLysGlnLysLysArgGluArgGluGlyGlyArgMetProAlaGlyProPro
  1330                1350                1370                1390                1410                1430
GGTTGCCCAAAGGAAGCCGCTGGAGATGCCTCTGACCAGTCCGCGTGCACCTCCCCAGAAGCCTCGCCCAGTTCCATCACCTCTTGAATTGAACTTCCTAAGTAACTGGGCTTCCAACGT
GlyCysProLysGluAlaAlaGlyAspAlaSerAspGlnSerAlaCysThrSerProGluAlaSerProSerSerIleThrSerEnd
  1450                1470                1490                1510                1530                1550
TGACCAGTTCTCTCGAAGACTTTCCCAAACTTCACAGCCTTGGTGATCCCTCTCAAGGCCGAGGCACCAGTTTAGAGCTTGTCCCAGGAAACTGGGCAGGAGTTGGGCCCTGTATTTTTC
  1570                1590                1610                1630                1650                1670
TCTCTCTCAGATCTAGGGGTGGAGGGATGATTGATGGCTGGGGATCCTACAGGTCTTGGGACCTGGGGAACACTCAACTCATCAGAGGTCGAAGGAAGGCCTTTTGGCTTTGATCTGGAG
  1690                1710                1730                1750
TCAGCCCATCCTTTCGGGCTTCTCCTTTCCCTTCCAACTCAGTTCAGTGCCTTTGAGCTTAGAGAGTTCTTCTTTCGAA
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Fig. 1. The *Hox 2.9* cDNA clone. (A) A line diagram of the 1759 bp *Hox 2.9* cDNA. The heavy line represents the predicted coding sequence. The open box represents the homeobox. Key restriction enzyme sites are indicated. (B) The sequence of the *Hox 2.9* cDNA illustrated in A. The amino acid sequence of the longest open reading frame with the homeodomain in frame (boxed) is given below the DNA sequence. The conserved hexapeptide is underlined. The arrowhead shows the predicted splice site based on comparison with other *labial*-like gene sequences.

far anterior as the headfold (Fig. 5D–F) and to presomitic mesoderm with expression decreasing as somites condense.

By 8½ days of development, *Hox 2.9* expression in the hindbrain has become localised (Fig. 4G). The anterior boundary is at the same position as at 8 days (the preotic sulcus) with *Hox 2.9* and *Krox 20* continuing to share this boundary, but now there is a new posterior boundary also within the hindbrain. We simultaneously detect the initiation of the second band of *Krox 20* expression, the anterior boundary of which coincides with the posterior boundary of *Hox 2.9*. The expression

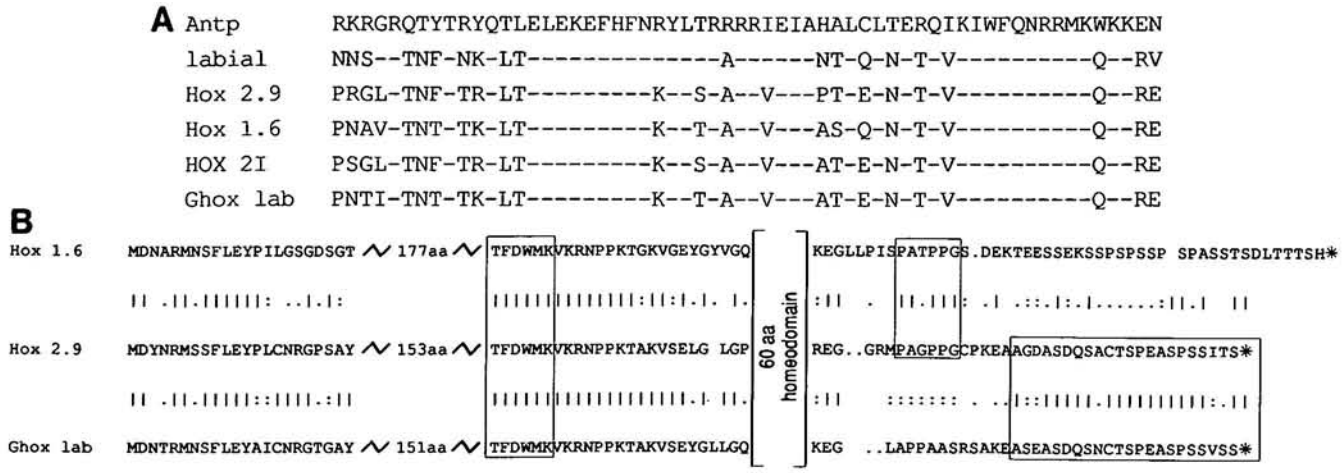

Fig. 2. Alignment of the amino acid sequences of *labial*-like genes. (A) The amino acid sequences of the homeodomains of *Drosophila labial* and the vertebrate *labial*-like genes (*Hox 2.9* and *Hox 1.6*: mouse, *HOX 2I*: human, *Ghox lab*: chicken, see text for references) compared to *Drosophila Antennapedia*. Differences from the *Antennapedia* sequence are noted revealing the characteristic features of the *labial* family of genes. (B) A representation of the full-coding region of *Hox 1.6*, *Hox 2.9* and *Ghox lab*. A line between two sequences represents an amino acid identity. Dots represent conservative changes (as judged by the GAP program). The conserved hexapeptide is boxed. A divergent stretch 5′ of the hexapeptide, and the homeodomain shown in 2A, are omitted from this diagram.

Table 1. *Comparison of* labial *and* labial-*like genes*

	HOX 2I		*Hox 1.6*		*Ghox lab*		*labial*	
(a) *Homeobox/homeodomain comparisons*								
	DNA	amino acid	DNA	amino acid	DNA	amino acid	DNA	amino acid
Hox 2.9	87%	96.6%	79.0%	86.6%	80.6%	90.0%	72.3%	80.0%
HOX 2I			82.8%	88.3%	82.8%	91.7%	74.5%	81.6%
Hox 1.6					81.1%	93.3%	76.2%	85.0%
Ghox lab							74.5%	85.0%
(b) *Whole protein comparisons*								
	Similarity	Identity	Similarity	Identity	Similarity	Identity		
Hox 2.9	90.3%	85.6%	62.5%	45%	71.1%	55.1%		
Hox 1.6					58.8%	46.5%		

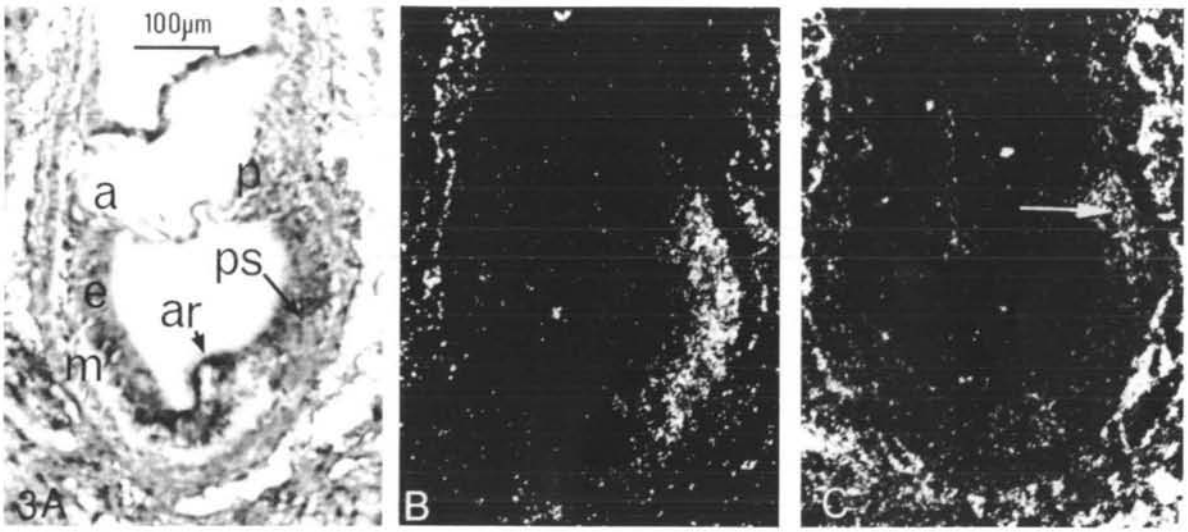

Fig. 3. Adjacent sagittal sections through a 7½ day mouse embryo. A and B show bright- and dark-field images of the same section probed with *Hox 2.9*. C was probed with *Hox 1.6*. a, anterior; p, posterior; ps, primitive streak; e, ectoderm; m, mesoderm; ar, archenteron. The arrows indicate the labelled cells in C and D.

of *Hox 2.9* in the anterior neural tube seems to retreat posteriorly at this time with expression persisting in more posterior regions. *Hox 1.6* expression also appears to retreat posteriorly along the neural tube in the same way; however, in contrast to *Hox 2.9* no expression of *Hox 1.6* remains in the hindbrain (Fig. 4F). Within the mesoderm both *Hox 2.9* and *Hox 1.6* remain expressed in lateral plate mesoderm up to the level of the posterior hindbrain and in presomitic mesoderm. We now first detect expression of both genes in an endodermal derivative, the epithelium of the foregut pocket.

The rhombomeres, which are the morphological representation of segments within the hindbrain, are visible at 8¾ days (Fig. 4I). The rhombomeres are small and more evenly shaped at this stage than at later stages. We can now see that the expression domains of *Hox 2.9* and *Krox 20* within the developing hindbrain are perfectly coincident with rhombomere 4 in the case of *Hox 2.9* and rhombomeres 3 and 5 in the case of *Krox 20*. These results show that the expression of *Hox 2.9* and *Krox 20* becomes localised within the hindbrain in an anterior-to-posterior order up to 6 h before segments are visible.

At 8¾ days, labelling with *Hox 2.9* is also detected in the mesoderm lateral to rhombomere 4 in the region where sensory ganglia are condensing (Fig. 4J). Migrating neural crest cells that originate from rhombomere 4 also express *Hox 2.9* (Fig. 5A,B).

Hox 2.9 *and* Hox 1.6 *expression between 9 and 11 days*

Between 9 and 10 days of development *Hox 2.9* and *Hox 1.6* are expressed within the neural tube in posterior regions only, in a way that is consistent with the expression domains retreating posteriorly, since there is a posterior-to-anterior gradient (Fig. 5J–L). This may relate to the process of maturation in the neural tube. A dorsoventral gradient of *Hox 2.9* expression within the neural tube is also visible (not shown) and this relates to a period of cytodifferentiation in which sensory neurons are being produced in the dorsal region of the neural tube where *Hox 2.9* is most abundantly expressed. Dorsoventral sublocalisation of homeobox gene expression within the neural tube has previously been described (Bogarad *et al.* 1989). *Hox 2.9* is expressed most heavily within rhombomere 4 of the hindbrain (Fig. 5G,H). We have previously described how sharply defined this domain is at the cellular level (Murphy *et al.* 1989). A series of sections through a 10-day embryo shows that a very narrow band of cells in the floor plate of rhombomere 4 does not express *Hox 2.9* (Fig. 6), this complements the fact that rhombomere boundaries do not extend into the floor plate.

Within the mesoderm, expression of both genes is now seen in gut-associated mesoderm at and below the level of the heart and in remaining presomitic mesoderm in posterior regions (Fig. 5G–L). In addition, *Hox 2.9* is expressed in the nephrogenic duct of the developing kidney (Fig. 5K). The domains of the two genes in the gut-associated mesoderm have the same AP restrictions, although *Hox 1.6* expression appears to be more extensive laterally, but this may simply reflect differences in the efficiencies of the two probes. There is also expression in the surface ectoderm adjacent to the labelled gut-associated mesoderm (Fig. 5J–L). Both genes are expressed in gut epithelium at the level of the forelimb bud (Fig. 5G–I). This is a derivative of the endoderm and is therefore one of the few examples of endodermal expression of homeobox-containing genes (Holland and Hogan, 1988; Duprey *et al.* 1988).

By 10½ days the mesodermal expression of *Hox 2.9* and *Hox 1.6* has been down-regulated (Fig. 7A–C) and is not detectable at 11½ days (Fig. 7D–F). At 11½ days, the rhombomeres are no longer visible and the expression of *Krox 20* is no longer detectable but *Hox 2.9* expression persists at a reduced level in the hindbrain (Fig. 7E). By 12½ days no expression of *Hox 2.9* or *Hox 1.6* is detectable in the embryo.

Expression of the differential transcripts of Hox 1.6

Hox 1.6 is differentially spliced to give two transcripts that differ by a 203 bp region 5′ of the homeobox (LaRosa and Gudas, 1988). The transcripts that contain this region code for a full-length protein with a homeodomain whereas the transcripts that lack this region code for a truncated protein with the same amino-terminal half but no homeodomain. In F9 teratocarcinoma cells both transcripts are produced, the relative amount of the shorter transcript increasing from 10% to as high as 56% after treatment with retinoic acid; a treatment that induces the cells to differentiate (LaRosa and Gudas, 1988). We isolated 17 different *Hox 1.6* cDNA clones from an 8½ day cDNA library and found that only 10 contained the differentially spliced region, showing that both forms of transcript are produced in the early embryo. Following specific amplification by polymerase chain reaction, the differentially spliced region was subcloned into a transcription vector to produce antisense and sense RNA for *in situ* hybridisation. This probe is referred to as the differential probe *Hox 1.6.d* and hybridises to only full-length transcripts encoding the homeodomain. The expression pattern detected by *Hox 1.6d* was compared to that observed with a 3′ probe, which hybridises to both transcripts, by analysis of consecutive embryo sections.

At 8 days of development both *Hox 1.6* probes detected the same widespread domain of expression (Fig. 8A–C). The labelling with *Hox 1.6.d* was at a lower level (53–65%, Table 2) but it shows that the full-length transcript is being produced in the embryo at 8 days. At 9 days of development, however, when the *Hox 1.6* 3′ probe detects transcripts in a broad region of gut-associated mesoderm and gut epithelium, presomitic mesoderm, and posterior neural tube, the full-length transcript is only detectable above background with *Hox 1.6.d* in the gut epithelium (Fig. 8D–G). The labelling of the gut epithelium with *Hox 1.6d* is too low to be visible in the photographs, but the grain counts show that it is labelled above background. Estimates

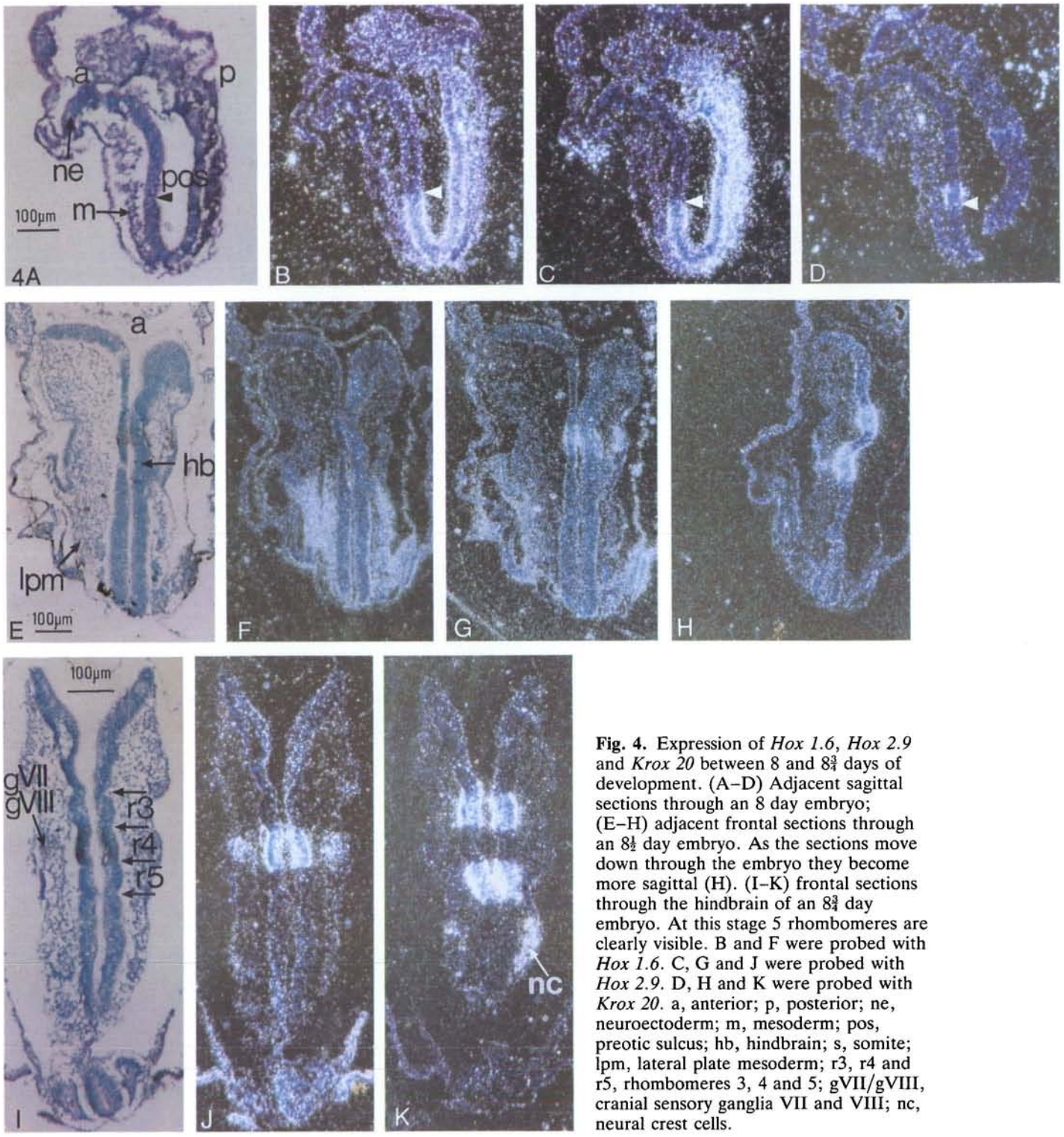

Fig. 4. Expression of *Hox 1.6*, *Hox 2.9* and *Krox 20* between 8 and 8¾ days of development. (A–D) Adjacent sagittal sections through an 8 day embryo; (E–H) adjacent frontal sections through an 8½ day embryo. As the sections move down through the embryo they become more sagittal (H). (I–K) frontal sections through the hindbrain of an 8¾ day embryo. At this stage 5 rhombomeres are clearly visible. B and F were probed with *Hox 1.6*. C, G and J were probed with *Hox 2.9*. D, H and K were probed with *Krox 20*. a, anterior; p, posterior; ne, neuroectoderm; m, mesoderm; pos, preotic sulcus; hb, hindbrain; s, somite; lpm, lateral plate mesoderm; r3, r4 and r5, rhombomeres 3, 4 and 5; gVII/gVIII, cranial sensory ganglia VII and VIII; nc, neural crest cells.

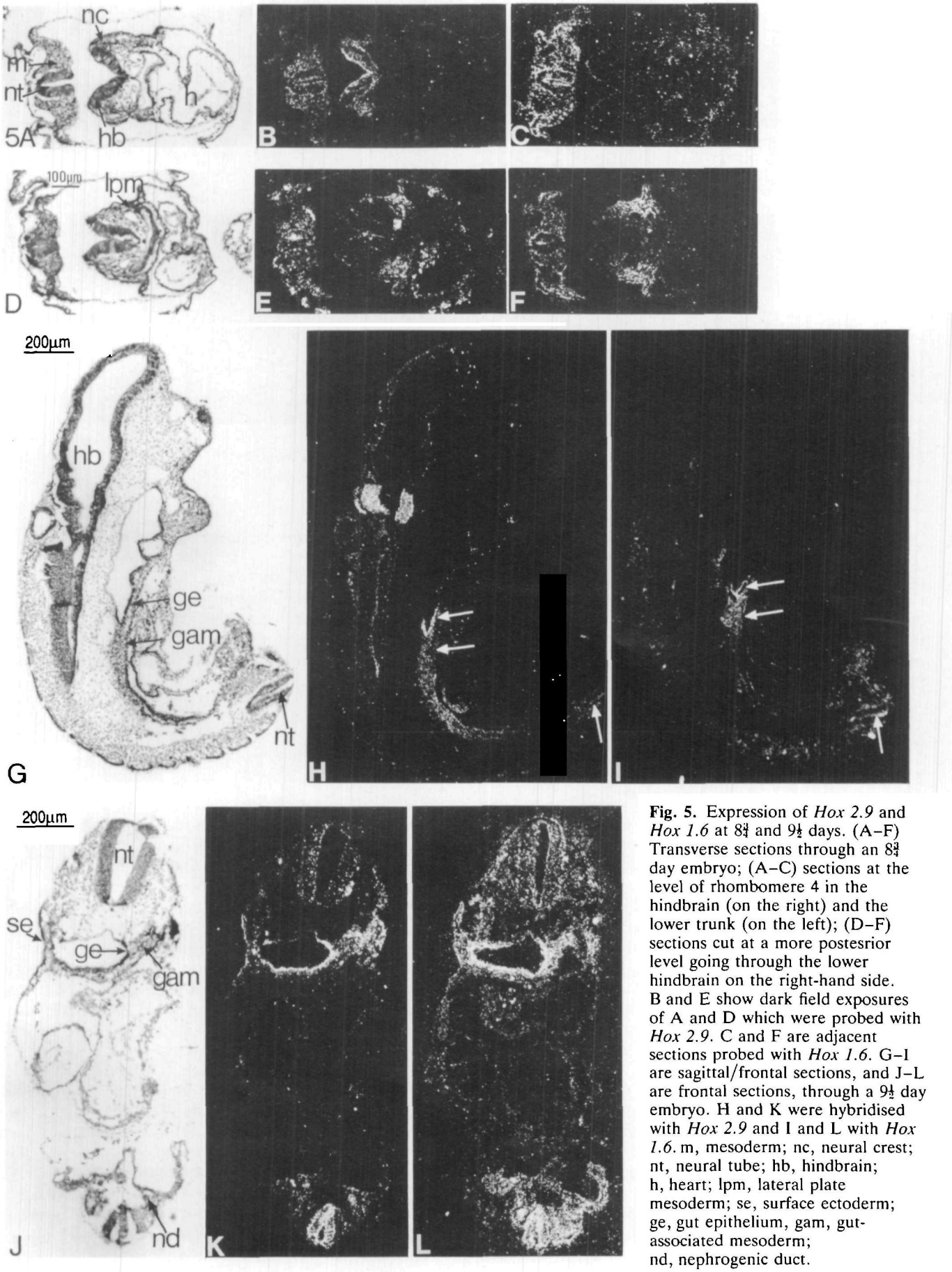

Fig. 5. Expression of *Hox 2.9* and *Hox 1.6* at 8¾ and 9½ days. (A–F) Transverse sections through an 8¾ day embryo; (A–C) sections at the level of rhombomere 4 in the hindbrain (on the right) and the lower trunk (on the left); (D–F) sections cut at a more postesrior level going through the lower hindbrain on the right-hand side. B and E show dark field exposures of A and D which were probed with *Hox 2.9*. C and F are adjacent sections probed with *Hox 1.6*. G–I are sagittal/frontal sections, and J–L are frontal sections, through a 9½ day embryo. H and K were hybridised with *Hox 2.9* and I and L with *Hox 1.6*. m, mesoderm; nc, neural crest; nt, neural tube; hb, hindbrain; h, heart; lpm, lateral plate mesoderm; se, surface ectoderm; ge, gut epithelium, gam, gut-associated mesoderm; nd, nephrogenic duct.

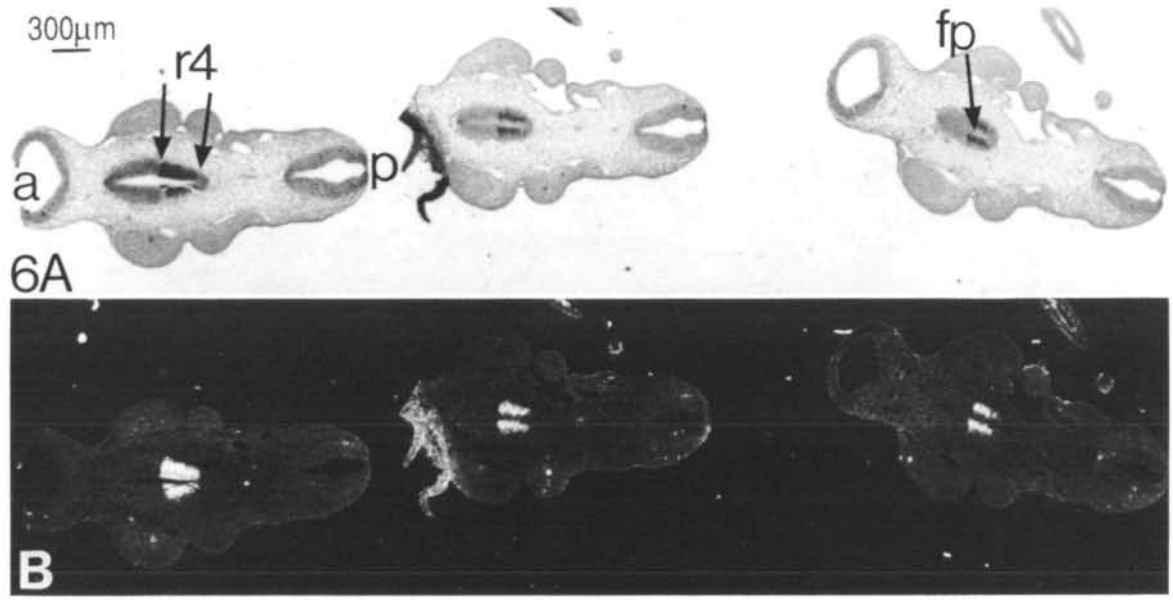

Fig. 6. *Hox 2.9* expression in a series of consecutive frontal sections through a 10½ day embryo. The sections to the right are progressively more ventral and include the floor plate of the hindbrain which is not labelled with *Hox 2.9*. a, anterior; p, posterior; r4, rhombomere 4; fp, floorplate.

from direct silver grain counts show that there is an overall drop in the proportion of full-length transcripts produced at 9 days. The three areas that were examined (Table 2); gut-associated mesoderm, gut epithelium and posterior neural tube, all show dramatic decreases in the level of labelling. The counts for gut-associated mesoderm and posterior neural tube were not above background counts.

Discussion

Evolution of the labial *family of genes*

Hox 2.9 and *Hox 1.6* (Mlodzik *et al.* 1988; LaRosa and Gudas, 1988) are the mouse homologs of the *Drosophila* gene *labial*. *labial*-like genes have also been identified in the human and the chicken. The homeodomains of the vertebrate genes are very similar to that of *labial* (80–85% identical, Table 1), but there is little similarity throughout the rest of the protein. The *Drosophila* protein is 629 amino acids long whereas the vertebrate proteins are much shorter varying between 298 and 336 amino acids. Vertebrates also lack the intron that interrupts the homeodomain of the *Drosophila* gene (Diederich *et al.* 1989; LaRosa and Gudas, 1988; Acampora *et al.* 1989). The vertebrate genes are more similar to each other in structure and sequence with homology extending outside the homeodomain (Fig. 2B). Comparing the full-length proteins, it appears that the chicken gene *Ghox-lab* is more similar to *Hox 2.9* than *Hox 1.6* although the homeodomains of all three genes are very similar. It seems therefore that *Ghox-lab* is the homolog of *Hox 2.9*. The expression pattern of *Ghox-lab* has not been fully described and it remains to be seen if it is segmentally expressed in the hindbrain. This information will be valuable since the chicken is a useful system for developmental manipulation.

The genetics of the *Drosophila* gene *labial* have proven difficult to interpret but a homeotic role for *labial* has been concluded from clonal studies (Merrill *et al.* 1989). The *labial* protein has been found in neural and epidermal cells of a very distinct region of the head that is thought to represent an ancestral segment (Diederich *et al.* 1989). The fact that *labial* and one of its mouse homologs, *Hox 2.9*, are expressed in single anterior segments is striking. Although it is likely that there are differences in the systems for determining position in two such distinct and specialised organisms, these highly conserved genes are involved in both.

An interesting general feature of the expression of clustered homeobox genes, which is shared by vertebrates and *Drosophila*, is that position within the cluster is reflected in position along the body axis at which the gene is expressed (Akam, 1987; Scott and Carroll, 1987; Harding *et al.* 1985; Graham *et al.* 1989; Duboule and Dolle, 1989). In this respect, *Hox 2.9* represents a special case in that it is positioned at the end, termed the 3′ end, of the cluster, but the neighbouring gene to the 5′ side, *Hox 2.8*, is expressed more anteriorly (Wilkinson *et al.* 1989*b*). *Hox 2.8* has no equivalent gene in the *Hox 1* cluster and so *Hox 1.6* is the most anteriorly expressed (Duboule and Dolle, 1989). Both mouse *labial*-like genes have exceptional expression patterns within the hindbrain at 9 days. The expression of *Hox 1.6* in the hindbrain is more transient than that of the other homeobox-containing genes in that no expression is detectable at 9 days. *Hox 2.9* expression disrupts the pattern observed with other *Hox 2* cluster members of sequential genes possessing anterior boundaries at two-segment intervals (Wilkinson *et al.* 1989*b*). *Hox 2.9* is the only *Hox 2* cluster gene

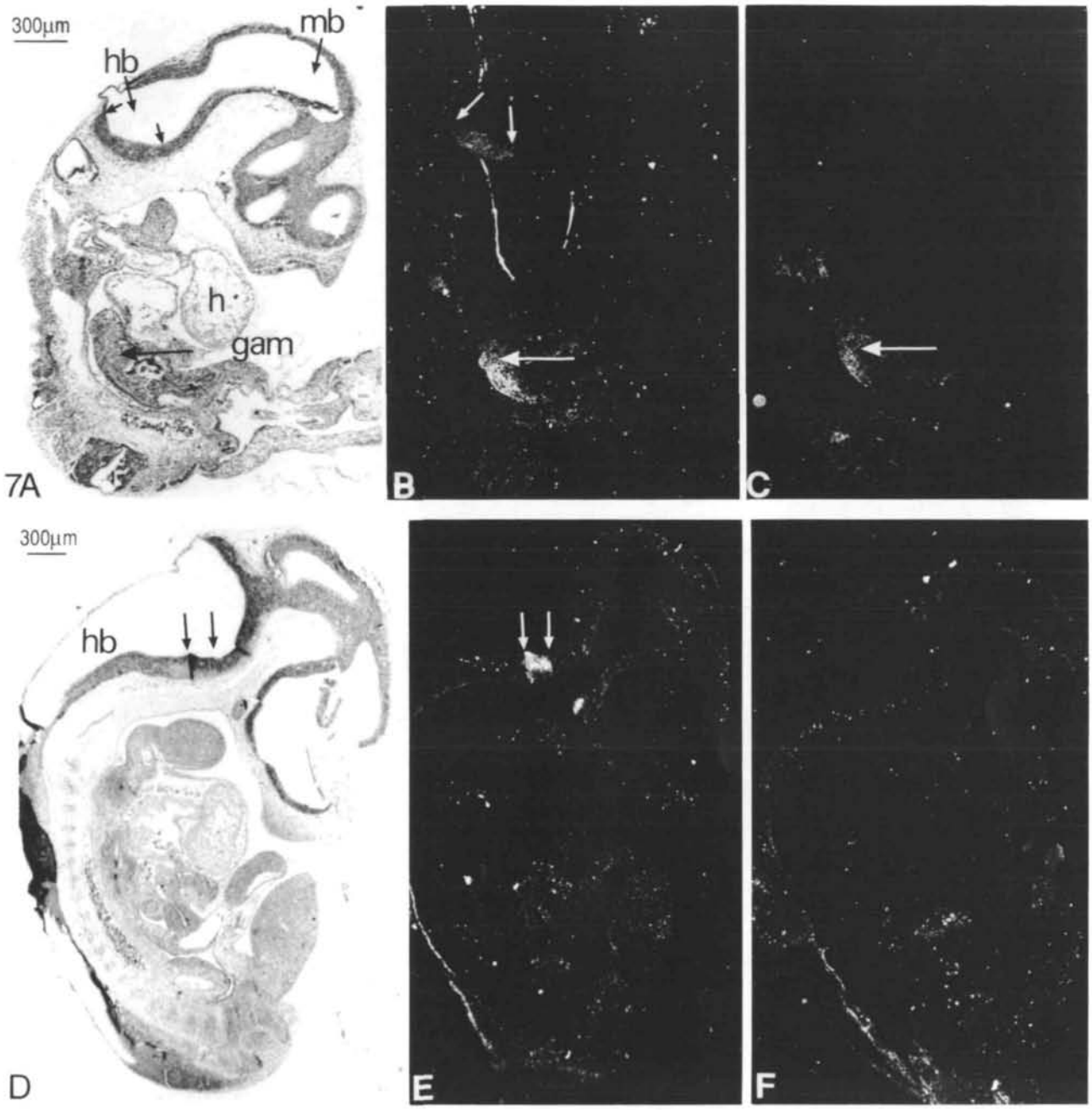

Fig. 7. Expression of *Hox 2.9* and *Hox 1.6* between 10½ and 11½ days of development. A–C show sagittal sections through a 10½ day embryo. D–F show sagittal sections through an 11½ day embryo. B and E show labelling with *Hox 2.9*; C and F have been probed with *Hox 1.6*. a, anterior; p, posterior; hb, hindbrain; mb, midbrain; h, heart; gam, gut-associated mesoderm.

to be uniquely expressed in a single rhombomere. Unlike other *Hox* genes which are generally expressed in overlapping domains in the somites and later the prevertebral column (Holland and Hogan, 1988; Graham *et al.* 1989; Duboule and Dolle, 1989), mouse *labial*-like genes are only expressed in the presomitic mesoderm with expression decreasing as somites condense.

As a result of duplication there are at least two *labial*-like genes in the mouse, and most likely also in other vertebrates. The duplicated genes have diverged but have maintained to a remarkably high degree certain features in common; this in itself would indicate a conservation of function at some level. This study has also shown that the two mouse genes share several features of their expression patterns, which extends the theory of functional similarity. The duplication of these genes must have been necessary to accommodate the

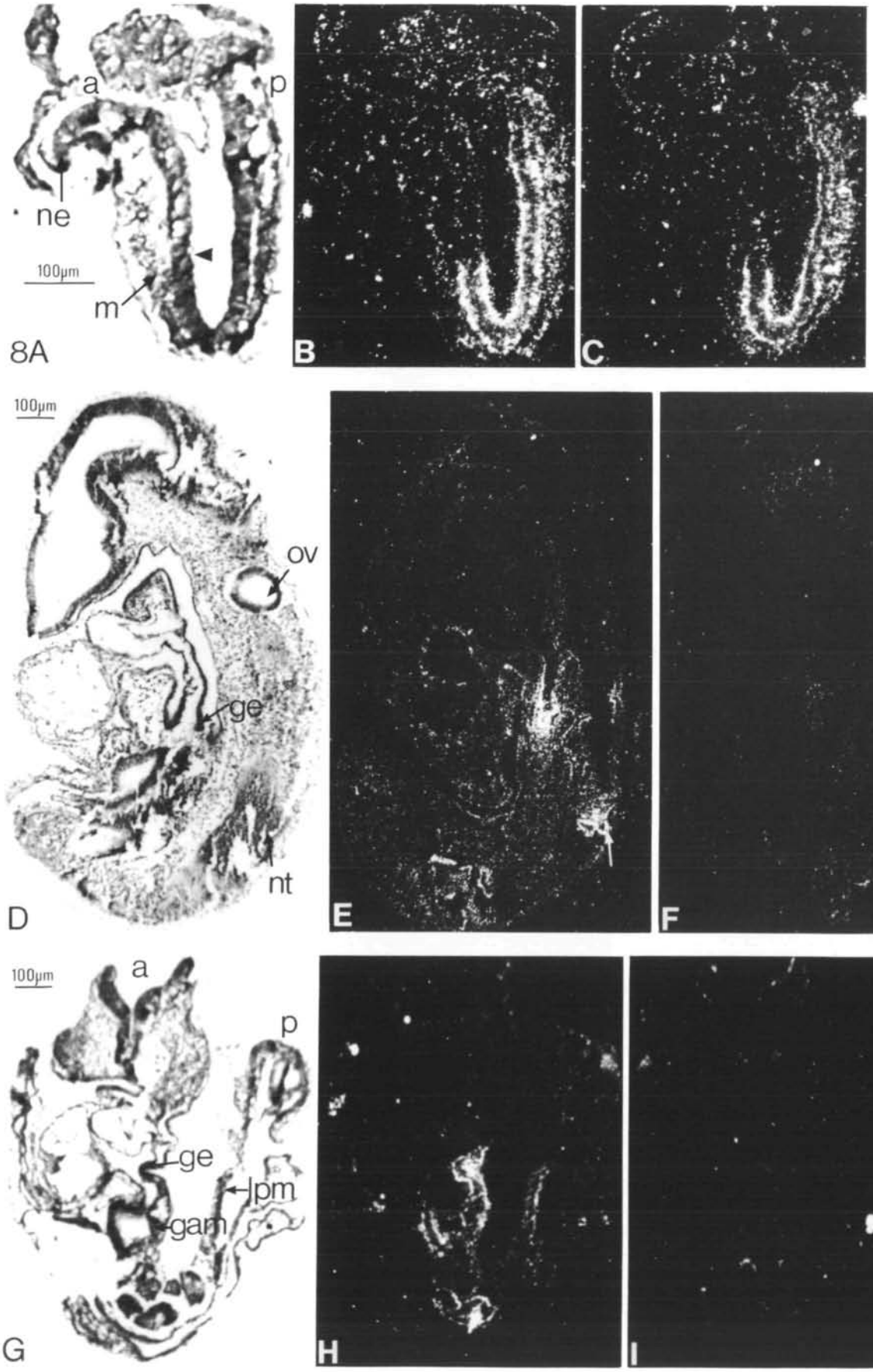
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Fig. 8. Expression of the differential transcripts of *Hox 1.6* at 8 and 9 days of development. (A–C) Sagittal sections through an 8 day embryo. (D–F) Sagittal sections, and (G–I) frontal sections through a 9 day embryo. B, E and H were probed with a fragment from the 3′ end of *Hox 1.6* which detects both transcripts. C, F and I were probed with *Hox 1.6d* which detects only the full-length transcript. The arrowhead denotes the anterior boundary of expression; a, anterior; p, posterior; ne, neuralectoderm; m, mesoderm; ov, otic vesicle; ge, gut epithelium; nt, neural tube; gam, gut-associated mesoderm; lpm, lateral plate mesoderm.

establishment of the more complex body plan of the vertebrate in which members of subfamilies maintain similar although specialised functions.

Hox 2.9 *and* Hox 1.6 *expression: temporal and spatial similarities*

Hox 2.9 and *Hox 1.6* share several features of their temporal and spatial expression patterns. Expression of both genes is initiated at the posterior end of the 7½ day gastrulating embryo. By 8 days, the expression patterns are indistinguishable with both genes occupying domains that extend from the primitive streak into the presumptive hindbrain, with identical anterior boundaries within the neuroectoderm at the preotic sulcus. At a relatively early stage (before 8½ days), the neural tube expression retreats along the AP axis so that at 9½ days only very posterior regions of the neural tube express *Hox 2.9* and *Hox 1.6*, with the additional persistent expression of *Hox 2.9* in a distinct region of the hindbrain. There are also parallels in the mesodermal and ectodermal expression of *Hox 2.9* and *Hox 1.6*; in the presomitic and lateral plate mesoderm at 8½ days where transverse sections reveal that they have the same AP limits; in the modified mesodermal expression at 9½ days when both genes are expressed in gut-associated mesoderm; and in the lack of detectable mesodermal expression after 10 days. The timing, the extent and the transient nature of the expression of these two genes indicate that they are responding to the same or similar signals in the embryo. The rapid loss of *Hox 2.9* and *Hox 1.6* RNA from the neural tube is either due to a loss of this stimulating signal or to simultaneous active repression of the genes. It also indicates that the transcripts have a rapid turnover rate, which has been shown for *Drosophila ftz* RNA with a half-life of 6–8 min (Edgar *et al.* 1986). The persistence of *Hox 2.9* expression in the hindbrain indicates that it can respond to an additional specific signal related to the segmentation of the hindbrain. For this reason, it will be important to investigate the control regions of these genes and to compare the binding sites for regulators that are present.

During the early phase of *labial*-like gene expression (7½–8½ days), there appears to be coordinate expression in the ectodermal and mesodermal tissue layers resulting in corresponding AP limits in these two tissues. As development proceeds and the complexity of the embryo increases, expression in both tissue layers becomes modified and there is little correspondence between the two layers. We therefore suggest that in the early stages of development basic AP positional domains are being similarly defined in the embryo as a whole whereas in the later embryo developmental fields become more independent.

The unique expression of Hox 2.9 within rhombomere 4 must represent a specialised function for *Hox 2.9*. It is possible that the earlier more widespread domain, from which this domain is derived and which is shared with *Hox 1.6*, is only functional in priming the later restricted expression. This is reminiscent of *Drosophila* homeotic genes, which have early widespread expression domains that become restricted to the corresponding functional domains (for review Akam, 1987). Alternatively the broad expression of *Hox 2.9* and *Hox 1.6* at 8 days may be involved in positional signalling that is important prior to segmentation.

The relationship between the expression of Hox 2.9 *and hindbrain segmentation*

The localised expression of *Hox 2.9* and *Krox 20* within specific rhombomeres has previously been described (Murphy *et al.* 1989; Wilkinson *et al.* 1989*a*). The analysis presented here focuses on earlier *Hox 2.9* expression and details further the role that this gene plays in hindbrain segmentation. Furthermore, together with *Krox 20*, these genes provide useful molecular markers in studying the process of segmentation of the hindbrain. *Krox 20* is first expressed in two domains within the hindbrain that will become rhombo-

Table 2. *Comparison of grain counts from embryo sections representing differential transcripts of* Hox 1.6

	Hox 1.63′ probe*	Hox 1.6d†	Hox 1.6d/Hox 1.63′
8 day neuroectoderm	91.3±2.9	59.1±8.4	0.65
8 day mesoderm	84.1±5.1	44.6±4.3	0.53
9 day gut epithelium	130.0±6.9	26.1±12.2	0.20
9 day neural tube	60.3±4.9	4.5±2.7‡	0.08
9 day mesoderm	40.2±10.6	4.3±4.6‡	0.10

Silver grain counts from *in situ* hybridised embryo sections (see Materials and methods) and the ratio of mean counts with two Hox 1.6 probes.

*Hox 1.63′ probe detects both differential transcripts of Hox 1.6.

†Hox 1.6d only detects full length transcripts. The differences between the mean counts for the two probes were found to be statistically signficant at less than the 1% level in all cases. With the exception of the values marked (‡) all were significantly above background estimations.

meres 3 and 5. This expression is first initiated in the more anterior domain, followed by initiation in the more posterior domain with both domains expressing *Krox 20* prior to the appearance of rhombomeres (Wilkinson *et al.* 1989*b*). The results presented here (Fig. 9) show that *Hox 2.9* is expressed in the hindbrain, with a defined anterior boundary, at the time that *Krox 20* is expressed in a single domain and before rhombomeres are visible. This is in contrast to the findings of Wilkinson *et al.* (1989*b*) who suggested that *Hox 2.9* is not expressed in the hindbrain when *Krox 20* is first detected. We further find that *Hox 2.9* expression becomes localised to the region of the hindbrain that will form rhombomere 4 at approximately the same time that *Krox 20* expression is initiated in the second domain.

At no time did we observe an overlap in the expression of *Hox 2.9* and *Krox 20* and once the domains are established they have sharp planar boundaries indicating that there is little or no cell mixing occurring between the domains. The expression pattern of these genes would therefore indicate that compartmentalisation of the hindbrain begins in the 8 day embryo and progresses in an anterior-to-posterior direction. By 8½ days the segmental units represented by rhombomeres 3, 4 and 5 have been defined. After rhombomeres are visible, we show that in rhombomere 4 *Hox 2.9* is not expressed in the floorplate. Fraser *et al.* (1990) have demonstrated that there are no rostrocaudal cell lineage restrictions in the floor plate of the chick hindbrain and that the floor plate also lacks visible rhombomere boundaries. *Hox 2.9* is therefore only expressed in the part of rhombomere 4 that is obviously segmented. Rhombomeres are transient structures that disappear by day 12. The *Hox 2.9* rhombomere 4 expression is not detectable after 11½ days and therefore expression persists throughout the period that rhombomere 4 exists. These data further suggest that *Hox 2.9* is involved in specifying the identity of the developmental compartment defined as rhombomere 4.

Hox 2.9 is also expressed in the sensory ganglia associated with rhombomere 4 and in the neural crest cells that migrate from rhombomere 4. The expression of *Hox 2.9* specifically in the neural crest cells that arise from rhombomere 4 supports the idea of neural crest cells being patterned according to their rhombomeric origin (Couly and LeDouarin, 1990). Neural crest cells are known to follow specific migratory pathways maintaining the AP order in which they arise (Tan and Morriss-Kay, 1986; Noden, 1975). Patterning of neural crest cells according to rhombomeric origin would therefore extend the segmental unit to regions outside the neuroectoderm.

It appears that the development of the hindbrain is under a complex regime of regulatory controls since the expression patterns of *Hox 2.9*, *Hox 1.6* and *Krox 20* within the developing hindbrain follow different modes as segmentation occurs. Both *Hox 2.9* and *Hox 1.6* are expressed early in broad domains with sharp anterior boundaries within the hindbrain; *Hox 2.9* later becomes localised to a single segment at which time *Hox 1.6* is no longer expressed. *Hox 2.9* and *Krox 20* are expressed in a segmental pattern; however, *Hox 2.9* results from the modification of a broad region of expression and *Krox 20* is initiated in distinct domains. Whereas the segmental expression of *Hox 2.9* and *Krox 20* appears to be established at the same time, *Hox 2.9* expression persists for a longer period. These genes will be important in understanding the positional signalling events and the regulatory elements involved in the process of hindbrain segmentation.

Hox 1.6 *differential transcripts; a change in their relative proportions as development proceeds*

The alternate *Hox 1.6* transcripts produced in the embryo code for a homeodomain protein and a truncated non-homeodomain protein. It is possible that the truncated protein has an independent function in the developing embryo or production of the spliced RNA may simply be a means of silencing the *Hox 1.6* direct DNA-binding function. It is not possible at present to tell if the truncated protein is functional and if so what that function might be. Although it does not

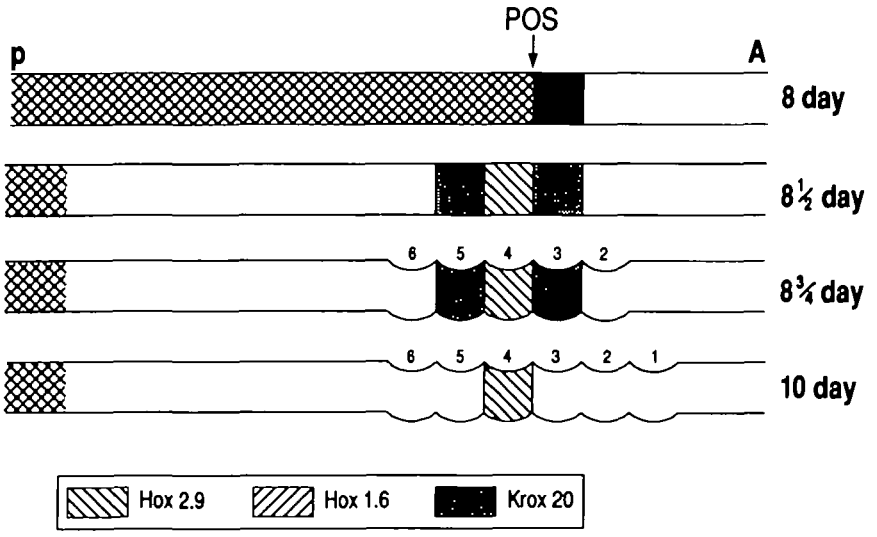

Fig. 9. A diagrammatic representation of the expression patterns of *Hox 2.9*, *Hox 1.6* and *Krox 20* in the CNS during segmentation of the hindbrain. The first appearance of rhombomeres is represented at 8¾ days, at which time at least 5 rhombomeres are visible. The diagram does not represent the relative size of the CNS at the various stages.

have the capacity to bind DNA directly, it may maintain the capacity to interact with other regulators and in this way may be involved in a complex regulatory network. Alternatively the splicing mechanism may be involved in removing functional *Hox 1.6* protein without shutting down the transcription of the gene. This paper describes extensive similarities in the expression patterns of *Hox 2.9* and *Hox 1.6* indicating that they may be similarly controlled in the embryo. This offers an explanation for the presence of such an additional control mechanism to down-regulate *Hox 1.6* without affecting *Hox 2.9*. Our results show that the relative amount of homeodomain-producing transcript drops dramatically between 8 and 9 days and that at 9 days it is only detectable by *in situ* hybridisation in the gut epithelium. This would imply that homeobox-containing protein from both genes is required to pattern the 8 day embryo but there is a greater requirement for *Hox 2.9* as a transcription factor at later stages.

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