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The branchial *Hox* code and its implications for gene regulation, patterning of the nervous system and head evolution

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Summary

In this study we have examined the expression of murine *Hox* homeobox containing genes by *in situ* hybridisation in the branchial region of the head. Genes from the *Hox* complexes display segmentally restricted domains of expression in the developing hindbrain, which are correlated with similar restricted domains in the neural crest and surface ectoderm of the branchial arches. Comparison of related genes from the different clusters shows that subfamily members are expressed in identical rhombomeres and branchial arches. These patterns suggest a combinatorial system for specifying regional variation in the head, which we refer to as a *Hox* code. The *Hox* genes also display dynamic dorso-ventral (D-V) restrictions in the developing neural tube which mirror the timing and spatial distributions of the birth of major classes of neurons in the CNS. Genes in the *Hox-2* cluster all have a similar D-V distribution that differs from that of genes from the other *Hox* clusters, and suggests that members of a subfamily may be used to specify positional values to different subsets of cells at the same axial level. These results are discussed in terms of a system for patterning the branchial regions of the vertebrate head, and evolution of head structures.

We have also examined aspects of the transcriptional regulation of *Hox-2* genes in transgenic mice using a *lacZ* reporter gene. We have been able to reconstruct the major pattern of the *Hox-2.6* gene on the basis of identical expression of the transgene and the endogenous gene with respect to timing, spatial restrictions and tissue-specific distributions. Deletion analysis has enabled us to identify three regions involved in generating this pattern. Two of these regions have the properties of enhancers which are capable of imposing spatially-restricted domains of expression on heterologous promoters. We have generated similar *Hox–lacZ* fusions that reconstruct the highly restricted patterns of the *Hox-2.1* and *Hox-2.8* genes in the developing nervous system, supporting our *in situ* analysis and the idea of a *Hox* code. These transgenic experiments are a useful step in examining regulation in the *Hox* cascade.

Key words: neural crest, homeoboxes, *Hox* genes, transgenic mice, *Hox* code, segmentation, rhombomeres, branchial arches, head evolution.

Introduction

The branchial area of the vertebrate head is one of the parts of the body whose early regional specification is thought to involve *Hox* genes, and the particular properties of its development suggest that it may be informative in determining the roles of the different *Hox* clusters. Recent studies have shown that the hindbrain is a segmented structure that may have an extended role in general patterning of regional diversity in the branchial region. The purpose of this paper is to review strategies for organisation of the head, to examine the expression patterns of the entire *Hox* network during the early morphogenesis of the hindbrain and branchial arches, evaluate the genes potential roles in specification of regional identity, and to discuss these results in terms of evolution and interactions required for head development. Experiments in transgenic mice designed to identify regulatory components required to establish the spatially-restricted domains of *Hox* expression will also be presented and discussed in relation to organisation of the *Hox* complexes.

Developmental strategies of the head and trunk

The basic embryonic morphology of the head is distinct from that of the trunk. This is particularly apparent for the head mesoderm, which is the first mesoderm to ingress in gastrulation (Noden, 1988). In the trunk there is, from the midline laterally, the notochord underlying the spinal cord, blocks of paraxial mesoderm forming the somites, a region of intermediate mesoderm that forms part of the kidney and the lateral plate, which splits to form a coelom. The lateral plate gives rise to an outer layer of body wall muscle, an inner layer of gut smooth muscle, and contributes skeletogenic tissue to the limb buds. In the anterior part of the head the notochord extends into the prechordal plate, formed from the first mesoderm to ingress through Henson's node, and is continuous with the paraxial mesoderm to either side of it. The head paraxial mesoderm does not undergo epithelialisation to form somites, although repeated patterns of cell arrangements known as somitomeres have been reported (Jacobson, 1988). There is no intermediate mesoderm in the head, and the lateral plate, which does not split to form a coelom, is also continuous with the paraxial mesoderm (reviewed in Noden, 1988).

There is also evidence that head development involves patterning strategies different from the trunk. The pattern forming ability of trunk paraxial mesoderm appears to be intrinsic, as somites will produce vertebrae and ribs appropriate to their site of origin when grafted to ectopic sites (Chevallier, 1975). They also influence the development of spinal motor nerves, as reversal of the antero-posterior (A-P) polarity of a group of somites causes changes in the position of nerve outgrowth (Keynes and Stern, 1985). In contrast, after neural tube reversal, outgrowth still occurs opposite the rostral halves of somites. Thus, in the trunk the paraxial mesoderm is an important source of patterning information. However, replacement of head paraxial mesoderm with either segmental plate or somites results in a normal head skeleton, with normal patterns of neural development (Noden, 1986), suggesting that head mesoderm is patterned in a different way from that of the trunk.

In the branchial region of the head it is the neural epithelium which plays an important role in patterning. Amphibian neural plate is able to form anterior parts of the brain after a neuralizing induction, while an additional transforming signal that may be propagated through the neuroepithelium is required to form the hindbrain and more posterior regions (Saxen, 1989). At the five somite stage, the hindbrain neural plate consists of a series of bulges, the rhombomeres (r), shown in Fig. 1A. Their relationships to the first branchial arch are also shown. Lineage analysis with vital dyes has suggested that rhombomeres are compartments (Fraser *et al.* 1990), and the demonstration that early patterns of neurogenesis also show rhombomeric organisation imply that they represent important units of developmental organisation (Lumsden and Keynes, 1989). Lineage-restricted processes in the hindbrain neural plate may also be controlling aspects of craniofacial skeletogenesis *via* the neural crest (Noden, 1983; Hunt *et al.* 1991*b*; Lumsden, 1990*a*). The margins of the neural plate produce neural crest which migrates ventrally into the branchial arches, giving rise to the bulk of the connective tissue; crest is also important in the formation of the cranial ganglia (Le Douarin *et al.* 1986), which show similar spatial relationships to particular arches in all vertebrates (Romer, 1971). The prepatterned neural crest that gives rise to specific branchial arch structures always arises from particular rhombomeres (Kuratani and Kirby, 1991; Lumsden *et al.* 1991), suggesting that the developmental processes of the hindbrain and branchial arches are linked (Lumsden, 1990*b*). The spatial relationships between the hindbrain and the second and third arches are shown in Fig. 1B. There is evidence suggesting that the neural crest is imprinted with its regional identity before migration, which is then transferred to the branchial arches (Noden, 1988). Crest may also be able to direct the development of other tissues within the head, so that they produce structures appropriate to the crest they are in contact with (Noden, 1988). We believe that these differences between head and trunk are reflected in the behaviour of subgroups of *Antennapedia* class homeobox genes.

Comparison of subfamily expression domains in the head

Previous studies have shown that *Hox* genes are evolutionarily related to homeotic genes in the *Drosophila ANT-C* and *BX-C* complexes, and that they have spatially-restricted domains of expression in many embryonic contexts (Kessel and Gruss, 1990, 1991; Hunt *et al.* 1991*b*; Izpisua-Belmonte *et al.* 1991; Nohno *et al.* 1991). These patterns have been used to suggest that *Hox* and *HOM-C* genes could provide part of a molecular combinatorial system for specifying positional values (Lewis, 1978; Duboule and Dolle, 1989; Graham *et al.* 1989). One feature of these expression domains is that there is a direct correlation between the position of a gene in a *Hox* cluster and its relative A-P boundary of expression along the embryonic axis, a property known as collinearity. On the basis of this conservation of collinearity it is thought that the *Antennapedia* class genes of vertebrates and insects are descended from a single common ancestral cluster. A summary of the organisation, conservation and collinear expression of the vertebrate *Hox* clusters is shown in Fig. 2. One major difference between the *Antennapedia* class genes of insects and vertebrates is that vertebrates have four independent *Hox* clusters (Simeone *et al.* 1991), while in insects there is only one (*HOM-C*) (Akam, 1987, 1989; Beeman, 1987; Beeman *et al.* 1989). Sequence analysis reveals that the vertebrate complexes are related by duplication and divergence from a common ancestor. This process generated multiple versions of the same original gene which then diverged from each other to form subfami-

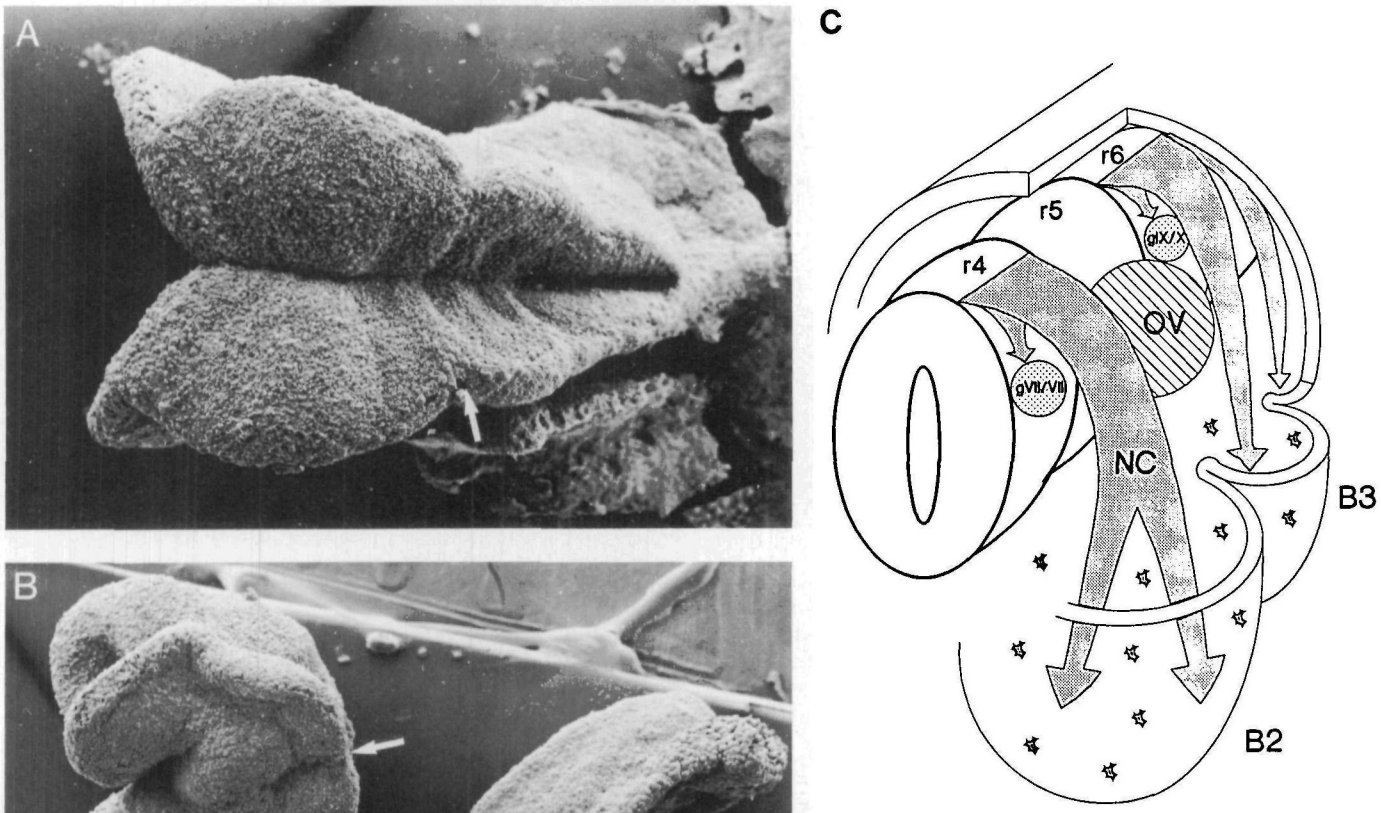


Fig. 1. The relationships between rhombomeres and branchial arches. A and B are Scanning electron micrographs of a 5 somite mouse embryo. (A) A dorsal view into the hindbrain and midbrain before closure of the neural tube illustrating the periodic undulations which correspond to hindbrain rhombomeres. (B) A lateral view of the same embryo showing a developing first branchial arch. The arrows in both panels indicate the same axial level and corresponds to the junction in the neural plate and surface ectoderm that delineates the first and second branchial arches. Anterior is to the left and posterior to the right. Magnification (A) 120× and (B) 75.2×. Photographs courtesy of Liz Hirst. C is a diagrammatic representation of neural crest migration into the second and third branchial arches between 8.0 and 9.0 days of embryogenesis based on Fig. 2, Hunt and Krumlauf, 1991. The large grey arrows represent mesenchymal crest which is the first to migrate from the rhombomeres into the branchial arches. The small grey arrows represent neurogenic crest which contributes to the cranial ganglia. Note that "based on the findings of Lumsden *et al.* 1991" no neural crest appears to emigrate from rhombomere 5 and so the crest in the second branchial arch is derived from r4. The major mesenchymal component in the arches is the neural crest, however, there is also a small contribution from head mesoderm indicated by the small grey shapes in the second (B2) and third (B3) branchial arches. (OV) otic vesicle, gVII/VIII facial–acoustic sensory ganglion complex, gIX/X glossopharyngeal–vagal sensory ganglion complex, (NC) neural crest, (r) rhombomeres.

lies or paralogous groups. These subfamilies are indicated in Fig. 2 as vertical rows with the *Drosophila* homologue at the top; thus *Hox-1.4*, *-2.6*, *-3.4* and *-4.2* are related to the *Drosophila* gene *Dfd* and they are known as the *Dfd* subgroup. Four clusters of *Hox* genes are present in all vertebrates, arguing for an important conserved function, but it is not clear why vertebrates require four complexes or four genes related to *Dfd*, while insects only require one. This opens the possibility of redundancy in some aspects of function between subfamily members, especially as their homeodomains are very similar, arguing for almost identical DNA binding specificity.

The expression domains of 3′ subgroups of *Hox* genes show similar anterior limits in the nervous system and the somites (Gaunt, 1987; Gaunt *et al.* 1989; Wilkinson *et al.* 1989; Chavrier *et al.* 1990). Detailed comparisons of somite domains reveal that subgroup members differ in which somites they are expressed in (Gaunt *et al.* 1989, 1990; reviewed in Kessel and Gruss, 1991), leading to the suggestion that each somite is uniquely specified by a particular combination of gene expression or *Hox* code (Kessel and Gruss, 1991). There are more somites than could be individually specified by an overlapping code of *Hox* genes from any one cluster. Members of the more posteriorly expressed

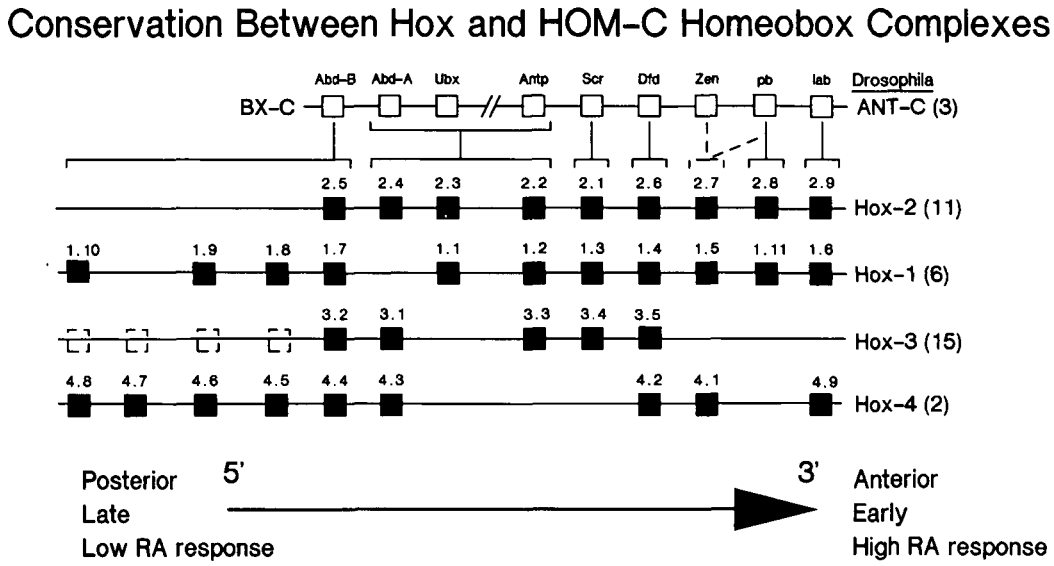


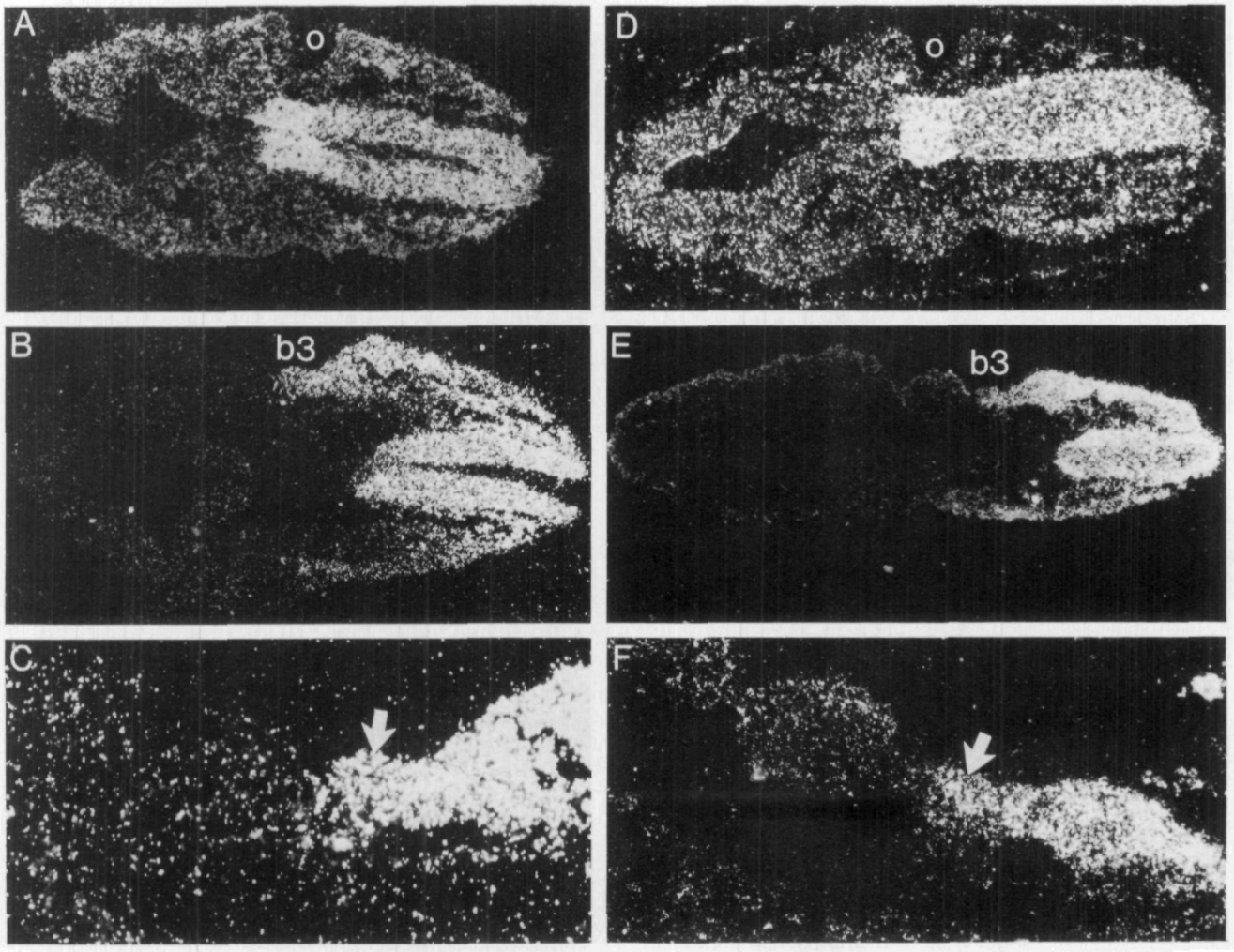
Fig. 2. The structure of the murine *Antennapedia* class homeobox gene complexes, illustrating the relationship between members of a subfamily of vertebrate genes and their *Drosophila* equivalents. Members of a subfamily or paralagous group of genes are vertically aligned with each other. Note that some subfamilies are not represented in all *Hox* clusters. The bracketed numbers to the right of the figure indicate the chromosomal location of their respective gene clusters. The arrow at the base of the diagram indicates that the various properties of genes listed vary with the positions of the genes within their clusters in the direction indicated; thus the most 3′ genes are the most anteriorly expressed, are expressed to these limits at the earliest times, and are most sensitive to retinoic acid. Solid boxes represent characterised genes, and the dashed boxes genes predicted on the basis of their presence in the human *HOX* clusters.

subfamilies such as the *Abd-B* group also show differences in A-P expression limits in the nervous system and somites (Izpisua-Belmonte *et al.* 1990). Therefore, part of the reason for the conservation of four *Hox* clusters in vertebrates may be to individually specify parts of the trunk nervous system and paraxial mesoderm.

The *Hox-2* genes have been shown to be candidates for an involvement in patterning the hindbrain (Wilkinson *et al.* 1989) and branchial arches (Hunt *et al.* 1991*b*,*c*), and there is a collinear relationship between the anterior limit of expression of a gene in branchial structures and its position in the *Hox-2* complex (Wilkinson *et al.* 1989; Hunt *et al.* 1991*c*). The hindbrain expression is characterised by a two rhombomere periodicity, yet each rhombomere has an identity distinct from the rest (Lumsden and Keynes, 1989), thus there are more rhombomeric units than could be individually specified by the genes of a given *Hox* cluster. Therefore we wanted to determine if genes from all four *Hox* clusters have the potential to generate the full range of rhombomere diversity. The branchial neural crest is able to give rise to a wide range of cell types (Le Douarin, 1983), and may be specified on the basis of axial level as to its morphogenetic capabilities (Noden, 1988; Hunt *et al.* 1991*b*), so genes of other clusters may be involved in different aspects of crest development as well. For these reasons we have examined the expression of 11 of the 12 *Hox* genes expressed in the hindbrain and branchial arches to define a *Hox* code for this region.

The relationships we have found in the expression of members from a subfamily are exemplified by the genes of the *Zen/pb* group. Expression of two members, *Hox-1.5* and *Hox-2.7*, is shown in Fig. 3. *Hox-1.5* is expressed with an anterior limit at the boundary between r5 and r4 in a 9½ day embryo, as shown in Fig. 3A. Although rhombomere 5 contributes to the facial nerve innervating the second arch, by analogy to the chick (Lumsden *et al.* 1991) it does not produce crest able to colonise the branchial arches, and so *Hox-1.5* is expressed in the third and more posterior branchial arches, not the second arch (Fig. 3B). There is embryological evidence for an instructive interaction between branchial crest and its overlying ectoderm (Noden, 1988; Hunt *et al.* 1991*c*), and this is supported by patterns of gene expression, which show identical limits in both tissues as indicated in Fig. 3C.

The expression pattèrn of another subfamily member, *Hox-2.7*, is shown in Fig. 3D–F. It shows identical anterior expression limits to *Hox-1.5* in the rhombomeres (3D), branchial arches (3E), and the surface ectoderm (3F). The bottom of Fig. 3 schematically illustrates the expression of this subfamily. In addition to identical anterior boundaries of expression, *Hox-1.5* and *Hox-2.7* also show a similar patterns of expression within their rhombomeric domains. There is a high level in r5, and lower levels in more posterior parts of the neural tube. In contrast the third member of the group, *Hox-4.1*, shows expression at a uniform level up to the r5/6 boundary, then a lower level in r5 (Hunt *et al.* 1991*a*). These data show that members of the *Zen/pb* subfamily may differ in levels of expression within their overall domains, but the anterior boundaries and combinations of rhombomeres and branchial arches in which they are expressed are identical. A similar analysis with members of the *Dfd* and *pb* subfamilies shows that each member of a group also has



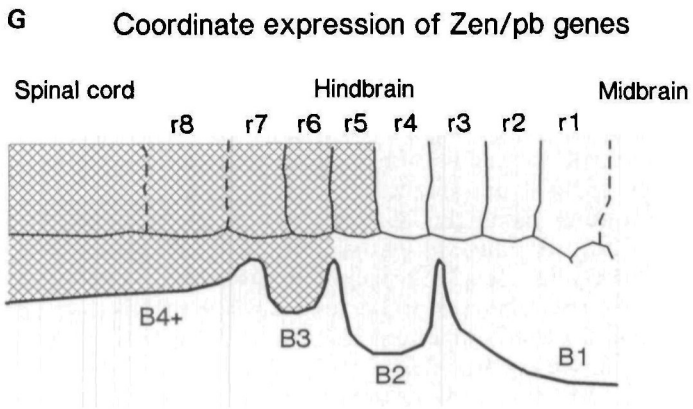


Fig. 3. Expression of *Hox-1.5* and *Hox-2.7* at 9½ d.p.c. in the hindbrain and branchial region. A–C show sections hybridised with *Hox 1.5*, and D–F shows sections hybridised with *Hox-2.7*. A and D are coronal sections through the hindbrain, and show expression with an anterior limit at the boundary of r4 and r5. Note highest levels of expression over r5. B and E are coronal sections through the branchial arches, showing expression confined to the third branchial arch and posterior regions. C and F are high magnification views of B and E respectively. The arrows indicate expression in the layer of surface ectoderm that overlies the largely neural crest-derived mesenchyme beneath. G summarises the expression data of the *Zen/pb* family at 9½ d.p.c., showing expression up to the third branchial arch and the r4/r5 boundary. o, otic vesicle; b3, third branchial arch. Magnification A,B,D,E (×73), C,F (×231).

identical boundary of expression in the hindbrain and branchial arches that is characteristic for its subfamily, but there are also regional variations in level (Hunt *et al.* 1991*a*). The expression of *Hox* genes in the hindbrain and the levels within these domains are summarised in Fig. 4, although it is not possible to make a direct comparison between the absolute levels of expression of two genes by *in situ* hybridisation.

The only exception to this rule of identical patterns among paralogues is in the *labial* group, illustrated in Fig. 5. Their expression does not persist into phases of embryogenesis later than 12½ d.p.c. in mouse, and at 9 d.p.c. the three genes exhibit domains of expression distinct from each other. For example *Hox-2.9* is the only gene in the family expressed within the neural tube at 9½ d.p.c., and this domain is restricted to a single

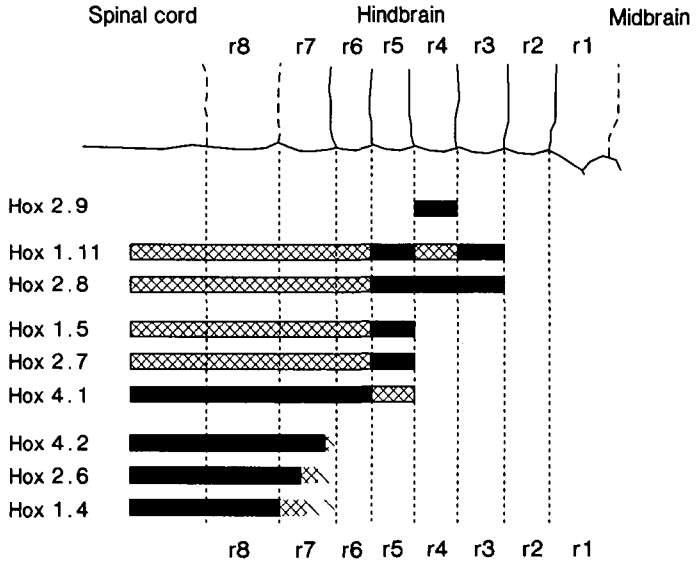


Fig. 4. Summary of the levels of *Hox* gene expression in the hindbrain of a 9½ d.p.c. mouse embryo. The darkest shading corresponds to the most intense expression. Expression levels are relative within the expression domains of a single gene.

rhombomere, r4 (Fig. 5A; Duboule and Dolle, 1989; Murphy *et al.* 1989; Wilkinson *et al.* 1989). In contrast, an equivalent section hybridised with *Hox-1.6* shows no expression above background (5B), and *Hox-4.9* expression is confined to the surface ectoderm overlying the hindbrain (5C). *Hox-1.6* is however, expressed elsewhere in the embryo at this stage (Murphy and Hill, 1991), as illustrated in Fig. 5D,E, where there is expression in the lining of the foregut (fg) and the mesenchyme posterior of the third branchial arch.

The sequence of establishment of the branchial *Hox* code

The timing of establishment of expression of *Hox* genes within the hindbrain also reflects their position within their clusters. Around the time of formation of the first somite the *pb* and *labial* groups of genes show expression domains that respect their final anterior limits in the hindbrain (Murphy and Hill, 1991), while more posteriorly expressed groups of genes have not yet reached their anterior limits (Fig. 6; Wilkinson *et al.* 1989). These domains of expression are segmentally-restricted before the morphological appearance of the rhombomeres and their relative positions can be defined in comparison with another gene, *Krox-20*, which marks the presumptive rhombomere 3. At 8½ d.p.c., two stripes of *Krox-20* are established (r3 and r5), and the more posterior groups such as *Zen/pb* and *Dfd* have also reached their final expression limits. These events are summarised in Fig. 6. The *labial* group also show differences in behaviour from other subgroups in the way in which their expression patterns develop. At an early stage *Hox-1.6* and *Hox-2.9* have been shown to have identical expression domains (Murphy and Hill, 1991), but by 8½ d.p.c. expression of *Hox-1.6* and part of the *Hox-2.9* domain recedes posteriorly. At the same time there is a marked upregulation of *Hox-2.9* expression in r4 domain. At 8 d.p.c. *Hox-4.9* is once again expressed differently than its two *labial* equivalents. Fig. 5F shows that *Hox-4.9* is posteriorly restricted at a stage in which the other two members are expressed up to the presumptive r4/r3 boundary (Murphy and Hill, 1991).

The *Hox* code of the head

Fig. 7 is a summary of the patterns of expression of all of the *Hox* genes we have examined at a stage when the rhombomeres have fully formed and neural crest has migrated into the branchial arches. It represents a branchial *Hox* code in which regional differences in expression could be used to specify positional differences in the various tissues.

With the exception of the *labial* subfamily, there do not seem to be any differences in which rhombomeres or branchial arches members of the same subfamily are expressed. In contrast, in the somites the expression limits of members of the same subfamilies can be offset from each other (Gaunt *et al.* 1989). The presence of areas of reduced crest emigration in rhombomeres 3 and 5 (Kuratani and Kirby, 1991; Lumsden *et al.* 1991) means that the expression of the *pb* and *Zen/pb* groups is one rhombomere out of phase with expression in the hindbrain. Because members of a subfamily apparently have identical patterns of expression it is unlikely that the rhombomeres and the branchial arch structures derived from them are further spatially subdivided at the time of their formation by differential expression of members of a paralogous group. However, expression patterns at the later stages of development when branchial arches are undergoing morphogenesis have not been investigated.

Based on our findings, we suggest that an early event in the patterning of the head is the establishment of patterns of *Hox* expression in the neural plate that will give rise to the hindbrain. Because the crest that forms the ganglia and branchial arches arises at particular positions along the A-P axis of the neural plate, the arches will have a pattern of *Hox* expression that reflects their level of origin. Rhombomeres r3 and r5 do not contribute crest to the arches which means that the neural crest component in arches 2 and 3 is derived from one rhombomere.

In the *Hox* code presented in Fig. 7 we assume the genes are acting in a simple combinatorial way, in which presence or absence of gene expression is the important property in terms of morphogenesis. However, we have shown that subfamily members can have different levels of expression within the same rhombomeric domains, as summarised in Fig. 4. It is possible that expression of subfamily members above a specific threshold may be necessary for morphogenetic function, and that specific subsets of the domains of expression are the only ones involved in combinatorial patterning. These regional

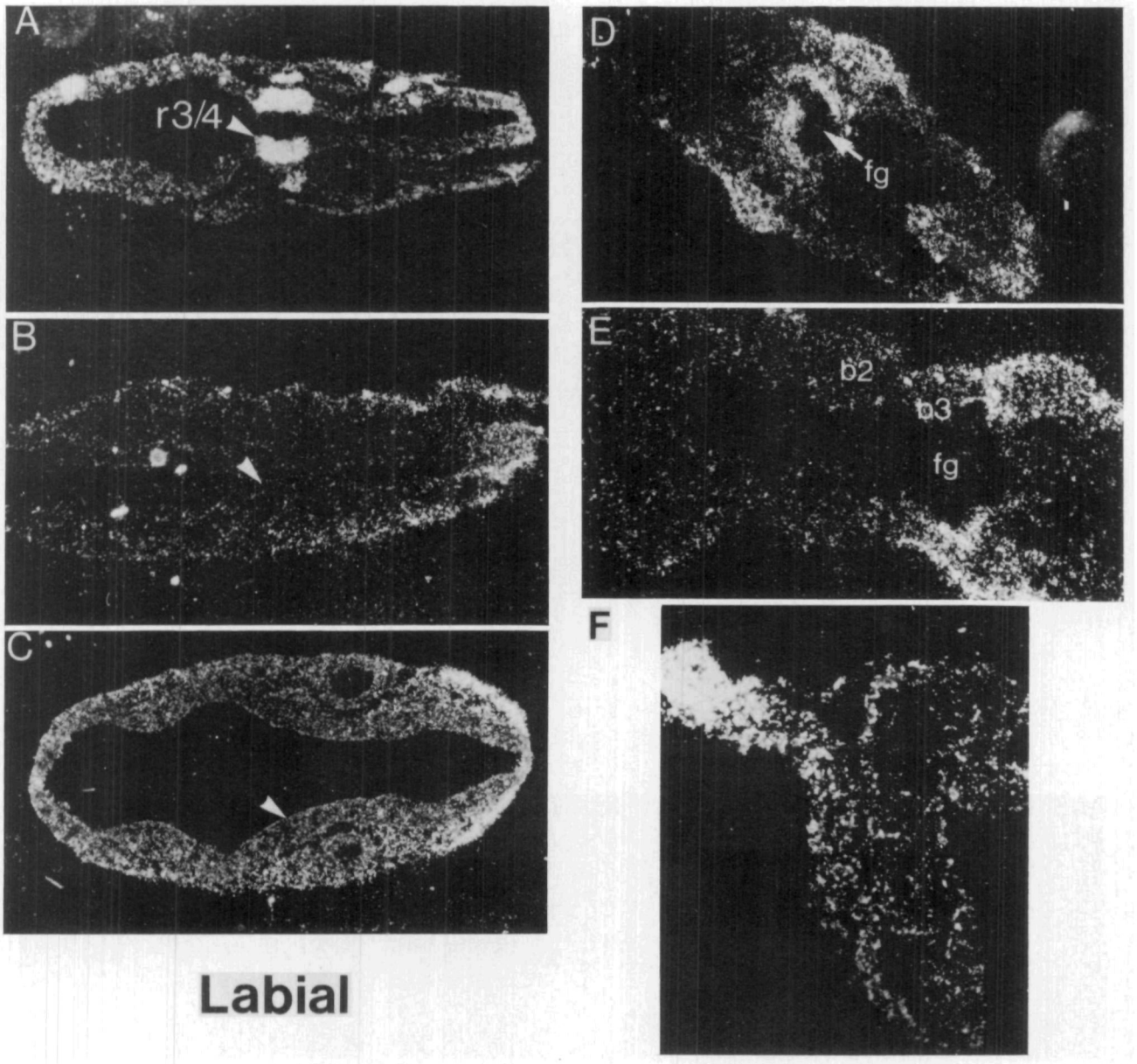


Fig. 5. Expression of the *labial* family of genes in 8 and 9½ day mouse embryos. A–E are coronal sections of 9.5 d.p.c. embryos and F is sagittal. (A) shows the expression of *Hox-2.9* in r4 and the adjacent vii/viii cranial ganglia. (B) A similar section with *Hox-1.6* shows no expression in neural tube or cranial ganglia, but more ventral sections of the same embryo show expression in the foregut (fg) and ectoderm and mesenchyme of the posterior branchial arches (D,E). An equivalent section with *Hox-4.9* (C) shows expression in specific regions of surface ectoderm, but none in the neural tube. F shows the expression of *Hox-4.9* in an 8 d.p.c., 1 somite embryo that expressed *Hox-2.8* up to the presumptive r2/r3 boundary. At this stage the two other *labial* family genes show r3/r4 boundary restricted expression in the hindbrain. Sections A–C (×100), Section D (×200), Section E (×240), Section F (×200).

variations could also be important for late stage patterning in the neural tube and not implicated in the neural crest specification. The timing of axogenesis, the first morphological criterion of rhombomere identity occurs sufficiently late for this to be a possibility.

Hox expression in the dorso-ventral axis

The similarities in early domains of *Hox* subfamily expression in the branchial region do not suggest unique roles for individual genes at this stage of development. However, consideration of the way in which *Hox* expression develops at later stages of development suggests potential roles for different members of the same subfamily of genes (Graham *et al.* 1991). Fig. 8 illustrates the expression of *Hox-2.5* at different stages of the development of the spinal cord. At 9 days, the stage at which we have examined expression in detail in the branchial region, expression is homogenous throughout the dorso-ventral extent of the spinal cord. At 10.0 and 11.0 d.p.c. expression is

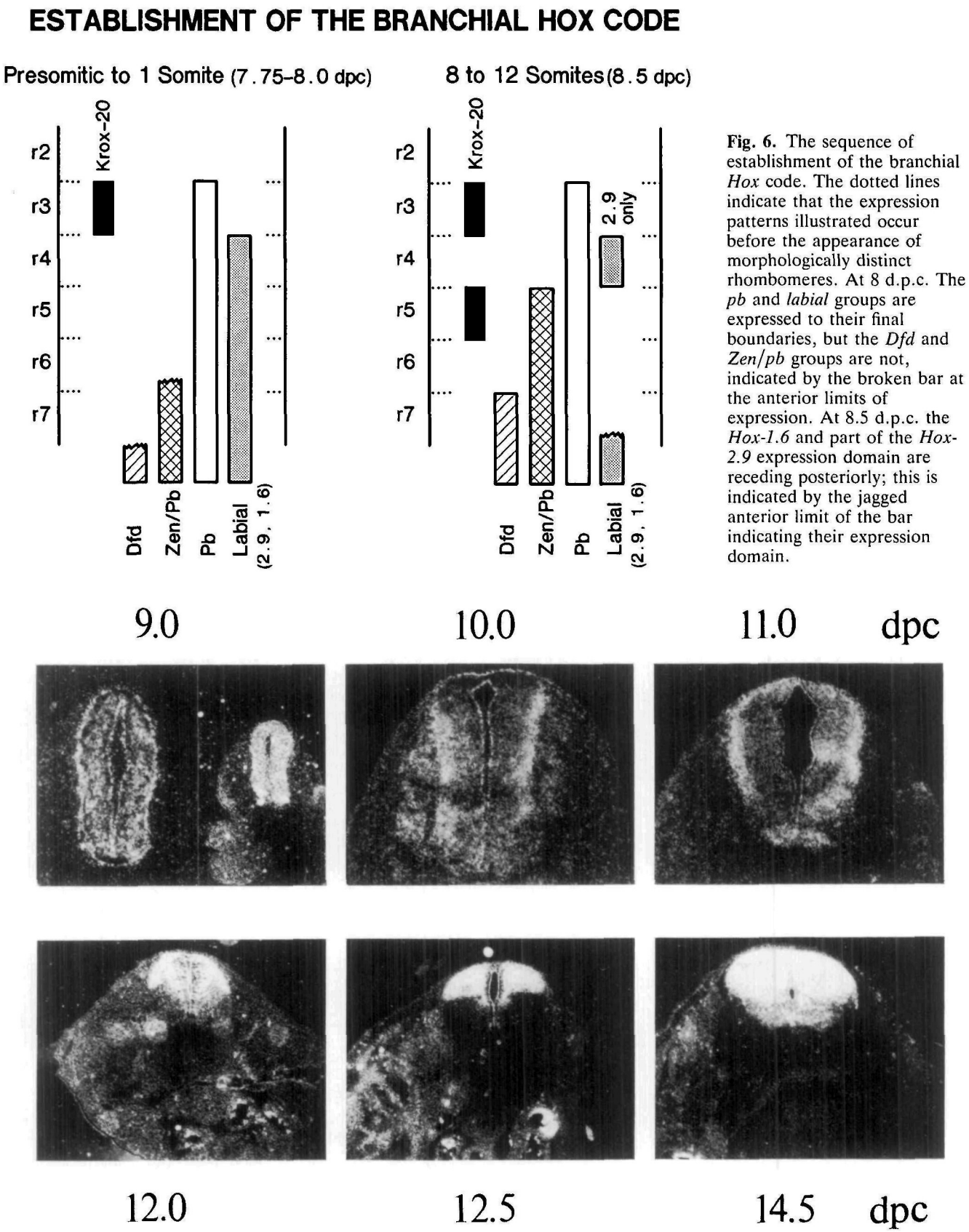


Fig. 6. The sequence of establishment of the branchial *Hox* code. The dotted lines indicate that the expression patterns illustrated occur before the appearance of morphologically distinct rhombomeres. At 8 d.p.c. The *pb* and *labial* groups are expressed to their final boundaries, but the *Dfd* and *Zen/pb* groups are not, indicated by the broken bar at the anterior limits of expression. At 8.5 d.p.c. the *Hox-1.6* and part of the *Hox-2.9* expression domain are receding posteriorly; this is indicated by the jagged anterior limit of the bar indicating their expression domain.

Fig. 8. Progressive dorsal restriction of the expression of the *Hox-2.5* gene in the spinal cord of mouse embryos. A series of transverse sections, with dorsal uppermost. At 9 days there is an even distribution, while at 10 and 11 days expression is lost from the ventrolateral motor horns and increases over the lateral commissural neurons. At 12 days and 12.5 days expression is confined to dorsal regions of the spinal cord. At 14.5 days expression is extending ventrally again.

subsequently lost from the ventral motor horns, while at the same time there is strong expression in the lateral commissural neurons, which have already performed their final round of cell division. At later stages, 12.0–12.5 d.p.c. RNA distributions are limited to the dorsal sensory regions, without expression in the commissural cells (Graham *et al.* 1991). Finally, at 14.5 d.p.c. expression reappears in the ventral region. This pattern of expression is the same for all members of the *Hox-2* complex. Thus there is evidence that *Hox-2* expression reflects the birth of particular populations of neurons within the spinal cord and could be used to specify their positional values (Sims and Vaughin, 1979; Altman and Bayer, 1984; Wentworth, 1984*a*,*b*). Genes of other *Hox* clusters also show dorso-ventral restrictions within the spinal cord (Graham *et al.* 1991), but they differ from those of the *Hox-2* genes. This raises the possibility that different clusters have distinct roles in particular types of tissues. This type of behaviour may also occur in the branchial region of the head.

Analysis of the control regions necessary for *Hox* expression

The conservation of the vertebrate *Hox* complexes and the similarity in the domains of expression within a subfamily suggests that the regulatory regions involved in generating these patterns may also be conserved. It will be important to examine the regulatory hierarchy of factors and signals required to establish the patterns of *Hox* expression and specify regional variation. In the absence of convenient genetic screens in mammals it will be important to use alternative methods of dissecting the regulatory cascade. In this regard we have begun to examine the *cis-acting* regulatory requirements for imposing spatially-restricted domains of expression using *Hox–lacZ* reporter constructs in transgenic mice. This has allowed the identification of regions which can be used to search for upstream regulatory factors and to alter the expression of *Hox* genes *in vivo* for perturbing the *Hox* code.

The basic strategy for transgenic analysis is shown in Fig. 9 for the *Hox-2.6* gene. A region of genomic DNA containing the gene and its putative regulatory regions is modified by the insertion of a *lacZ* reporter gene into the coding sequence of the *Hox-2.6* gene. This construct is the used to generate transgenic mice by microinjection of fertilised eggs. The expression pattern of the *Hox-2.6–lacZ* fusion protein is analysed by whole-mount staining of embryos for β-galactosidase activity at different stages of development.

Figs 10 and 11 show the expression domains of a number of constructs that recreate the normal pattern or particular subsets of the pattern of the *Hox-2.6* gene and two other *Hox-2* genes. In Fig. 10A the expression pattern produced under the control of a 17 kb region of genomic DNA surrounding the *Hox-2.6* gene is shown. This transgene produces an expression domain identical to that of the endogenous *Hox-2.6* gene, in that it has the appropriate rhombomere and prevertebral boundaries, is expressed in the correct tissues and temporal stages of development, and displays a sharp dorso-ventral restriction in the neural tube (Whiting *et al.* 1991). On the basis of these extensive comparisons between expression of the endogenous gene and that produced by the transgenic construct, we believe that we have accurately reconstructed the pattern of *Hox-2.6* expression. These mice have been useful in examining the expression of the gene at the cellular

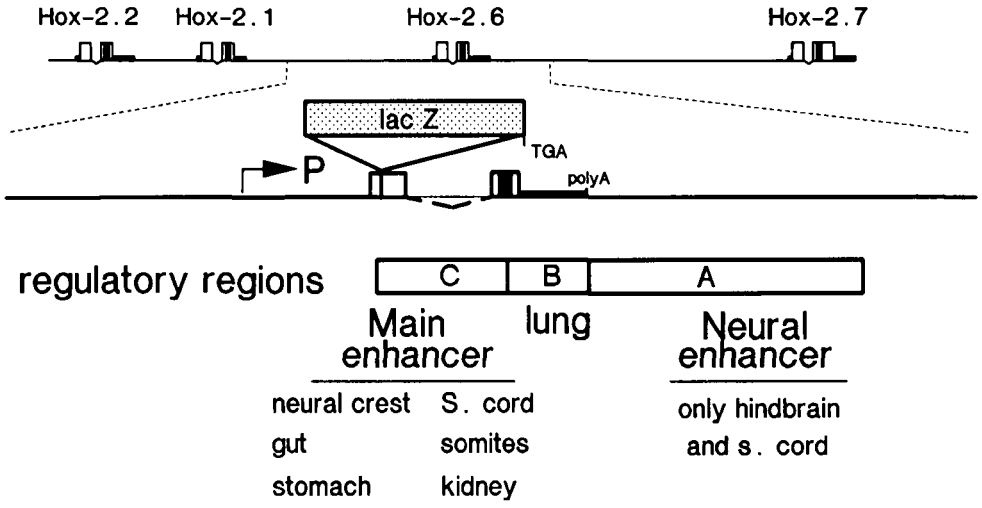


Fig. 9. The strategy used to generate constructs for analysis of the expression of genes in transgenic mice, illustrated for the *Hox-2.6* gene. The upper part shows a portion of the *Hox-2* complex around the *Hox-2.6* gene, and indicates the position of the 17 kb genomic fragment with respect to surrounding genes. The middle part of the diagram indicates how a *LacZ* reporter construct is inserted in frame in the first exon of *Hox-2.6*. Transcripts initiating at the 2.6 promoter in the construct (P) will contain in frame coding sequence for β-gal protein, at the end of which is a TGA translation termination codon. A fusion protein will be made containing the first amino acids of *Hox-2.6* fused on to the coding sequences of *LacZ*. The lower part of the diagram indicates the control regions identified by this analysis, and their positions within the *Hox-2.6* genomic region. The aspects of the *Hox-2.6* expression domain they direct are listed.

level. Our data indicate that within a domain of expression not all cells are positively stained. This suggests that some genes may be involved in patterning limited regions of a tissue.

Deletion analysis was then carried out to further define the location and nature of the regulatory regions capable of imposing this pattern of expression. Three different regions of the *Hox-2.6* gene were identified and their properties and positions in the gene are summarised in Fig. 9. For example 10B shows the expression pattern of an element, region A, able to produce a neural restricted pattern of expression on the *Hox-2.6* gene which maps to the rhombomere r6/7 boundary identical to the endogenous gene. This region is capable of working on heterologous promoters and has the properties of an enhancer. A second region, C, is able to generate a large part of the *Hox-2.6* pattern, with staining in the mesoderm and neural ectoderm. This is shown in Fig. 11C, with expression due to the 17 kb construct shown for comparison in Fig. 11A. The boundary of expression in the neural tube is posterior to that of the *Hox-2.6* normal limit, demonstrating that there is some degree of redundancy in the regulatory regions that can drive expression in the CNS. This region also behaves as a spatially restricted enhancer on heterologous promoters. The difference in expression domains produced by regions A and C can more clearly be seen in the dorsal views of the same embryos shown in Fig. 11B and D, where the position of the otocyst allows comparison of anterior limits of expression. This data suggests that the normal expression domain is built up from a combination of control elements, which in isolation confer domains of expression that partially overlap with each other. A combination of all elements is needed to fully reconstruct expression however.

In an analogous manner it has been possible to isolate regions able to confer the normal expression domains of the *Hox-2.8* gene, as shown in Fig. 10C. Expression in this $10\frac{1}{2}$ day embryo respects the r2/r3 boundary in the hindbrain and the second branchial arch, which can be orientated by their relationship with the otic vesicle. The relationship between expressing regions of hindbrain and branchial arch described earlier is very clear in this whole-mount preparation. Because of the lack of spatial resolution with *in situ* hybridisation, it has not been possible before to determine the nature of the anterior limits of expression at the cellular level. The expression of the β-gal protein shown in Fig. 10C suggests an anterior limit that is sharp at the cellular level. There is no evidence for a graded decrease in expression in cells at the boundary, or for a mixed population of expressing and non-expressing cells there. The boundary appears to be between a population of cells expressing protein at a particular level and a population of non-expressing cells.

Fig. 10D also shows a transverse section of the spinal cord of an embryo expressing a construct that recreates part of the *Hox-2.1* pattern. The progressive dorsoventral restriction of expression of *Hox-2* genes in the spinal cord during development has been described above (Graham *et al.* 1991), and this is reflected in the behaviour of this construct. Expression is initially homogenous throughout the spinal cord, and then is lost from the ventral motor horns. At the same time as the loss of expression from the ventral horns, there is strong expression in the lateral commissural neurons, which have already performed their final round of cell division. This is the situation illustrated by the transgene shown in Fig. 10D, which shows little expression in the ventral regions of the spinal cord. There is expression medial of the motor horns due to the projection of axons from strongly expressing commissural cells that are visible in the lateral regions of the neural tube. It is also clear that not all cells in the dorsal region are positively stained.

Discussion

The development of the vertebrate head is thought to involve a series of interactions between neural plate, neural crest, mesoderm, surface ectoderm and pharyngeal endoderm. Despite this complexity, it is possible to define a sequence of events by which some components of this system are spatially specified. The developmental and spatial relationships between neural tube and branchial arches suggest that primary patterning events in the neural plate could be transmitted to other parts of the head by migrating neural crest. We believe that the vertebrate *Hox* genes are a component of the process that achieves this spatial specification. The similarity of expression domains between members of a subfamily suggests that there is redundancy in the earliest aspects of the specification of morphological units in the branchial region by *Hox* genes. It may be useful to think in terms of an entire subfamily specifying a pair of rhombomeres and their branchial arch (Fig. 2G). The specification of rhombomeres in pairs would be consistent with the two segment-periodicity in patterns of both branchial and somatic motor nerve development in the hindbrain (Lumsden and Keynes, 1989). There are sufficient subfamilies to uniquely specify each branchial arch. This would suggest that there are additional systems for specifying odd and even rhombomeres acting in combination with the *Hox* network. In this regard *Krox-20* could be an example of such a gene (see paper by Nieto *et al.* this volume). In support of this, there is evidence from rhombomere grafting experiments that odd and even rhombomeres differ in their cellular properties, as the apposition of part of an odd rhombomere with part of an even one results in the formation of a new rhombomere boundary, while the other possibilities result in the formation of a large, compound rhombomeres (Guthrie and Lumsden, 1991). The specific roles for which particular subfamily members are required occur after primary specification of rhombomeres and branchial arches.

These ideas are supported by the phenotype of mice lacking normal Hox-1.5 (Chisaka and Capecchi, 1991) and Hox-1.6 proteins (Lufkin *et al.* 1991). The rhombomere morphologies of *Hox-1.5* and *Hox-1.6* mutant mice appear normal, supporting the idea that

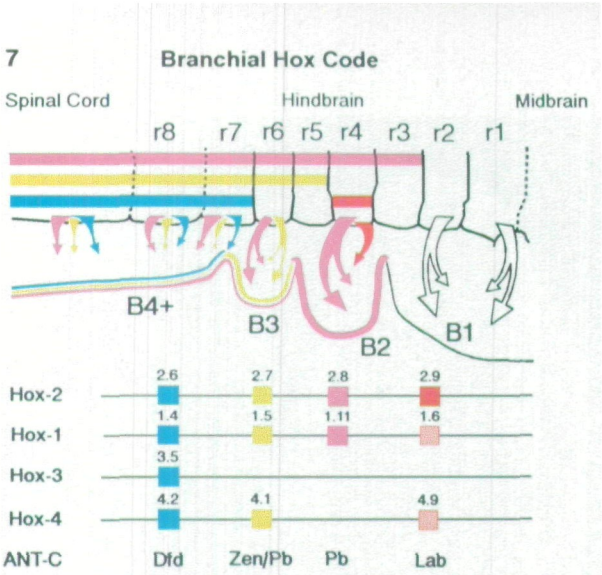


Fig. 7. The complete branchial *Hox* code based on gene expression. The diagram indicates the patterns of *Hox* subfamilies expressed in the branchial region after neural crest migration is complete, when distinct rhombomeres are apparent. The coloured arrows indicate the migration of mesenchymal and neurogenic crest from specific rhombomeres, resulting in a transfer of a combinatorial code to the branchial arches. The branchial arch ectoderm subsequently adopts an identical pattern of *Hox* subfamily expression indicated by its colour of shading, presumably as a result of an interaction with underlying neural crest. *Hox-2.9* expression is confined to the ganglionic crest as indicated by the short red arrow. *Hox* subfamily expression in the hindbrain is out of phase with the branchial arches by one rhombomere as a result of the lack of contribution to branchial arch crest by r3 and r5 by analogy to the chick embryo (Lumsden *et al.* 1991). This is represented by the absence of arrows emanating from r3 and r5. The large open arrows on the right of the diagram represents first arch crest that does not have a *Hox* label. The chromosomal relationship of the relevant subfamilies is shown at the bottom of the diagram. This diagram is based on that in Hunt *et al.* (1991*a*).

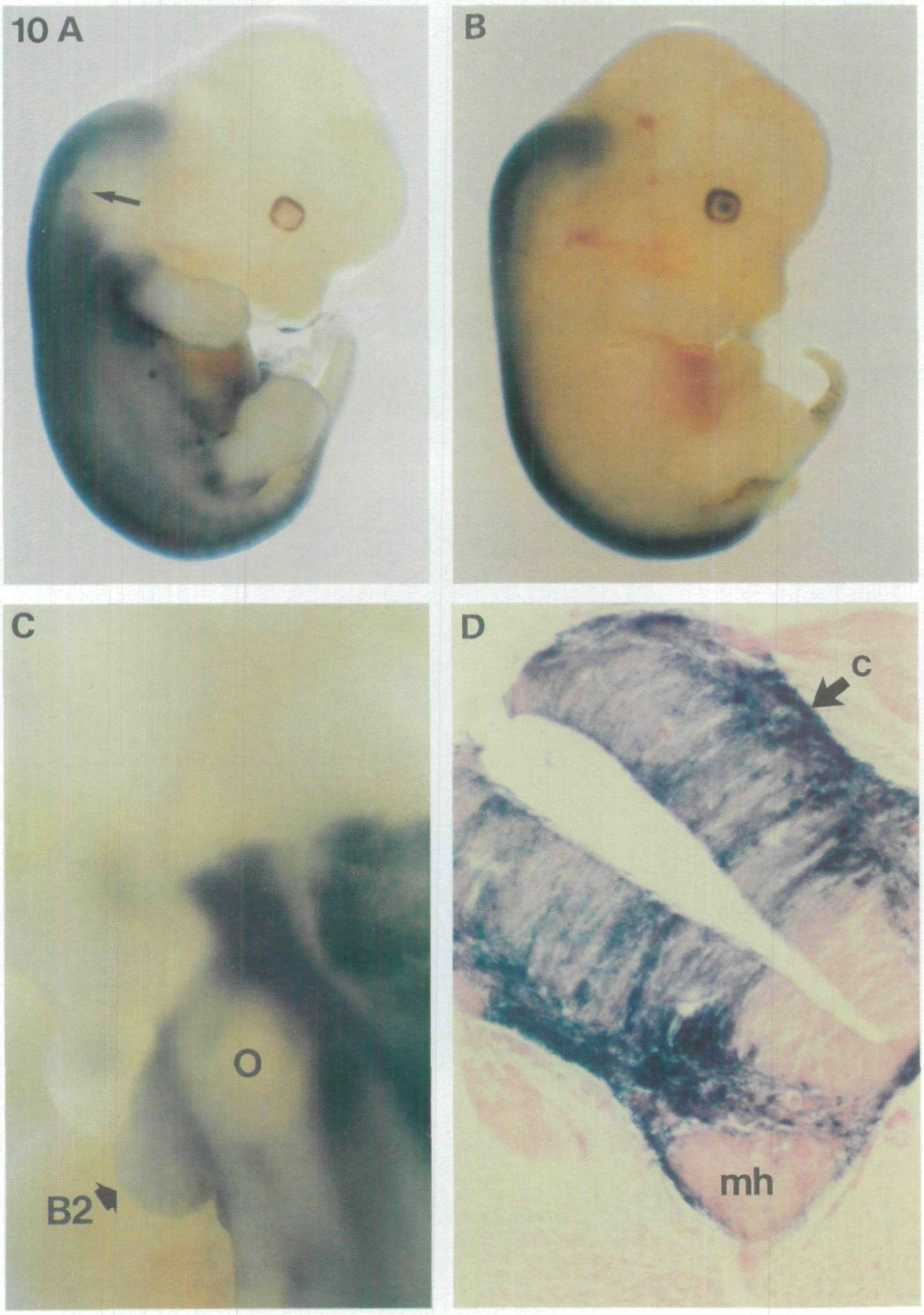


Fig. 10. Expression in transgenic mouse embryos of constructs that reconstruct the normal patterns of *Hox* gene expression. A and B show constructs generating all (A) or part (B) of the *Hox-2.6* pattern in 12½ d.p.c. and 13½ d.p.c. mouse embryos respectively. A shows the pattern produced by the 17 kb genomic fragment, B shows that of region A acting on the 2.6 promoter. Both constructs produce the normal anterior limit of expression in the hindbrain, and the 17 kb fragment produces the rest of the normal pattern, including the normal anterior limit in the mesoderm and DRG indicated by the black arrow. C shows a construct recreating the normal *Hox-2.8* pattern of expression in a 10½ day embryo. Note the relationship of the anterior limit of expression in the neural tube at r2/r3 with the expression in the second branchial arch (B2). D shows a construct producing the normal 2.1 pattern of expression in the spinal cord. In a transverse section of an 11.0 day embryo it is possible to see the lack of expression in the motor horns (mh) and the most intense expression in the commisural neurons (c) and the axons they have projected ventromedially.

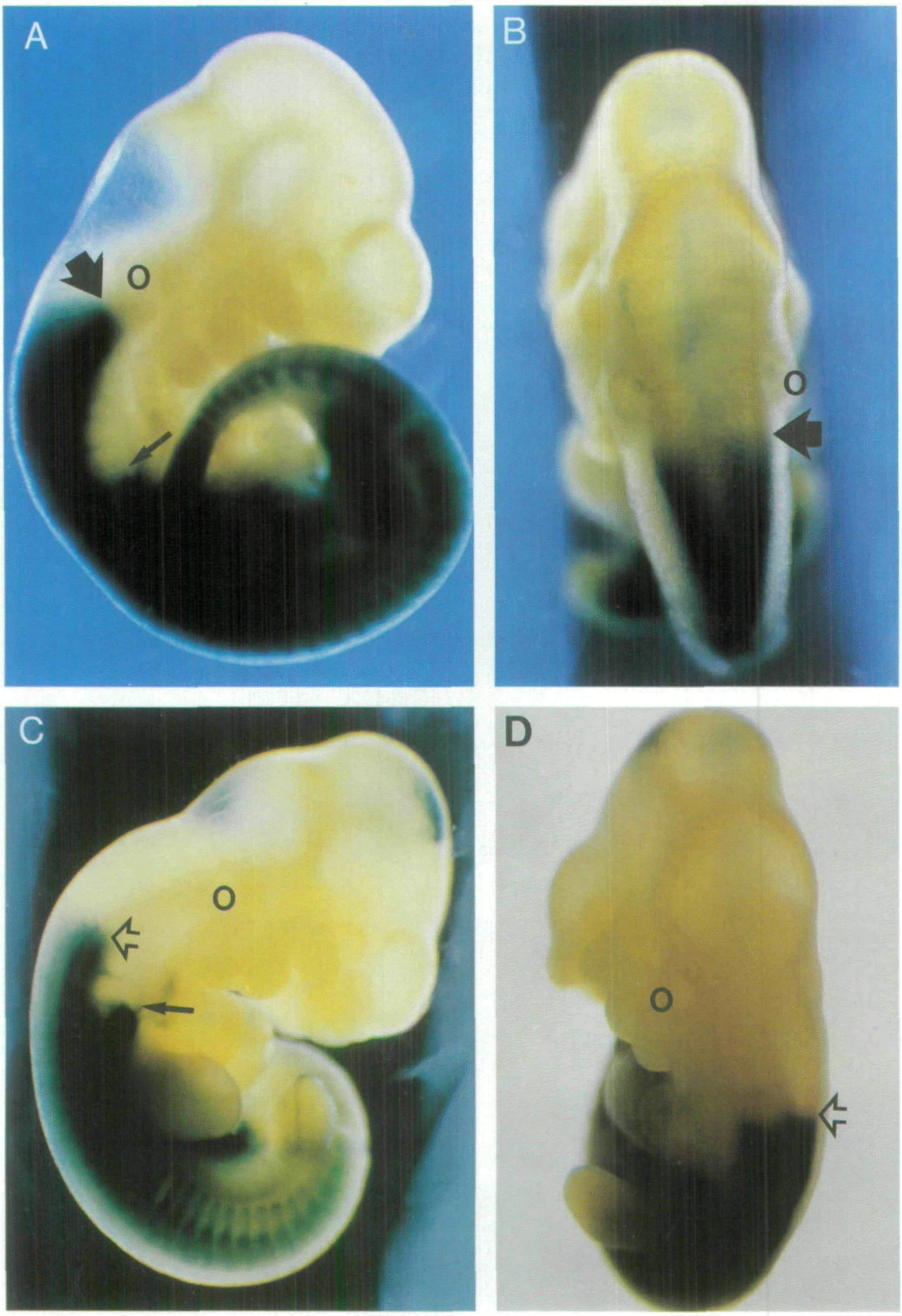


Fig. 11. Dissection of the elements generating the *Hox-2.6* pattern in 10½ d.p.c embryos. A and B are two views of the same embryo expressing the 17 kb construct, while C and D are two views of a different embryo expressing a construct from which the 3′ regions, including region A, have been deleted. A and B show the normal anterior limit of expression in the neural tube, indicated by the solid arrowhead, relative to the otocyst (o). Without region A, expression does not extend so far anteriorly in the neural tube, as indicated by the open arrowheads. Deletion of region A does not alter the anterior limit of expression in the somites, indicated by the small black arrow in A and C.

Hox genes are involved in spatial specification of units rather than their initial establishment. The *Hox-1.5* mice have normal cranial ganglia and defects in tissues dependent upon mesenchymal neural crest for their development such as the heart outflow tract and the glands of the neck (Kirby, 1989; Chisaka and Capecchi, 1991), some of which correspond to the normal domain of *Hox-1.5* expression. The fact that normal ganglia are formed suggest that there is sufficient information to spatially specify them in the absence of *Hox-1.5*, supporting the idea of redundancy in the early role of *Hox* genes within the neural epithelium. The defects could mean that *Hox-1.5* is required to maintain spatial specification in mesenchymal crest derivatives, or that *Hox-1.5* has a specific role in some other aspect of development of mesenchymal crest. In contrast the *Hox-1.6* mice show normal mesenchymal structures, but disrupted nuclei of branchial nerves VII, VIII, IX and X, and their associated ganglia (Lufkin *et al.* 1991). The fact that two members of the same *Hox* complex produce defects in different ranges of tissues argues against a simple model where a particular cluster of Hox genes is solely responsible for spatial specification and/or differentiation in a particular group of tissues.

It is interesting that cranial ganglia which do not normally express the gene are disrupted in *Hox-1.6* mutant mice, although the neural plate from which both the ganglia and the rhombomeres derive did express this gene at 8 d.p.c. (Murphy and Hill, 1991). It may be that in the absence of *Hox-1.6* it is not possible to establish or maintain normal patterns of expression of other genes, which may result in defects later in development in tissues that do not normally express *Hox-1.6*. It is not clear how the other 3′ *Hox* genes are expressed in the branchial region of mice lacking *Hox* genes. In *Drosophila*, posterior genes are known to repress the expression of genes expressed in more anterior domains (Akam, 1987), thus it is possible that interactions between vertebrate *Hox* genes are necessary to maintain appropriate patterns of gene expression. It is also known that the transcription patterns within vertebrate *Hox* complexes are complex (Simeone *et al.* 1988; M-H. Sham, *et al.* 1992), and that transcripts for one gene may originate within another. It may be that deletion of one gene may alter expression patterns of other genes, perhaps by disturbing the genetic circuitry necessary to establish and maintain gene expression by removing one of its components. Alternatively the large genomic insertions used to disrupt a gene may destroy transcription start sites of other genes, and hence alter their patterns of expression. This is a general problem in the interpretation of the phenotype of mice lacking a specific *Hox* gene; until the effects of the loss of a specific gene on the expression of the other members of the cranial *Hox* gene network are known it is not possible to interpret phenotypes in terms of alterations in the cranial *Hox* code. Homologous disruption of a *Hox* gene could also perturb the expression of other genes important for head development.

The *Hox-1.5* gene is normally expressed in an identical way to its homologue *Hox-2.7* in the crest at early stages (Fig. 2), which populates the third and posterior branchial arches. There are skeletal abnormalities in neural crest derivatives which do not normally express *Hox-1.5*, such as the first branchial arch derived mandibles and maxillae, and absence of the lesser wing of the hyoid bone, a second arch derivative (Chisaka and Capecchi, 1991). However, the greater wing of the hyoid bone, derived from the *Hox-1.5* expressing third arch, appears more normal. The defects seen with *Hox-1.6* lie within a smaller region, but also show evidence of perturbations of structures which never express *Hox-1.6* (Lufkin *et al.* 1991; Murphy and Hill, 1991). These are mainly associated with the bony parts of the ear, some of which are derived from the otocyst, a structure which is produced by an induction from the underlying hindbrain. There is much evidence of the importance of interactions between different tissues in the head for normal morphogenesis (Moody and Heaton, 1983*a*,*b*; Hall, 1987; Thorogood, 1988; Ruiz-i-Altaba, 1990), and the role of *Hox* genes in the endoderm and head paraxial mesoderm are not clear. The defects in more anterior arches beyond the normal regions of *Hox-1.5* and *Hox-1.6* gene expression may be due to interactions between structures of different arches necessary to produce normal development.

It is interesting that there is no evidence for transformation of structures in either in these mutant mouse lines. In contrast misexpression of a *Hox-1.1* gene in transgenic mice does result in vertebral transformations (Kessel *et al.* 1990). This may reflect both the more interactive nature and complexity of head development, and that different types of *Hox* codes are used in the two contexts.

For what sort of processes is a particular Hox *gene an absolute requirement?*

One possibility is that each gene in a subfamily has a distinct role at the earliest stage of structure specification, with a different range of structures specified by each member, for example cranial ganglia, rhombomeres or branchial cartilages. Thus members of a subfamily would act in parallel and independently to specify regional identity in the different derivatives of the hindbrain neural plate, hence coincident expression domains. The apparent similarities in expression domains could reflect the lack of cellular resolution in the radioactive *in situ* hybridisation technique and it is not yet established that a single cell can express more than on gene at the same time. Antibodies against the proteins and techniques with higher resolution may reveal differences between subpopulations of cells. The *lacZ* transgenic mice seem to indicate that not all cells in a region are positively stained for the protein product, suggesting that there may be some restricted differences.

The lineage relationships between cells of the cephalic neural plate are not as clear as in the trunk, where evidence suggests that there is multipotency in cells while within the neural plate, in that descendants

of a single cell can contribute to the neural tube, dorsal root ganglia, the adrenal medulla and pigment cells (Bronner-Fraser and Fraser, 1988). In the head also there is some preliminary *in vivo* data (Bronner-Fraser and Fraser, 1988) and *in vitro* data that suggests that both mesenchymal and neurogenic derivatives can derive from a single crest cell precursor (Baroffio *et al.* 1991). Given this apparent level of plasticity in crest differentiation potential, it is hard to imagine how cells belonging to subpopulations of crest lineages can be defined before crest emigration occurs. Thus if *Hox* subfamily members are supplying information in parallel to different lineages of crest from the same axial level they must be doing so after the crest precursors have left the neural tube and have made the decisions as to which lineage they will represent.

There cannot be a simple relationship between the genes of a particular *Hox* complex and spatial specification of particular crest lineages. Crest cells contributing to the first four branchial arches are able to give rise to the same range of cell types, yet there are no *Hox* genes expressed in the first arch, no *Hox*-3 or *Hox*-4 members in the second arch, and no *Hox*-3 member expressed in the third arch. This suggests that *Hox* genes are not a requirement for the establishment of early crest lineages, unless there are great differences in how particular crest lineages arise at different axial levels. For the same reason it is hard to imagine why the different components of more posterior branchial arches require independent *Hox* complexes for their early spatial patterning while more anterior arches employ fewer genes to perform apparently the same task.

Subgroup members probably have distinct roles in the head later in development as part of the mechanism causing cells to follow particular differentiation pathways, hence the specific problems in particular crest derivatives in the mutant mice. We feel that there is functional redundancy in the early morphogenetic developmental events with unique functions arising later in development.

Hox *genes and the evolution of the neural crest*

The differences between head and trunk may reflect that they and the mechanisms giving rise to them in development evolved at different times, an idea previously suggested on the basis of morphological evidence. Vertebrates are thought to have evolved from chordates similar to *Branchiostoma*, which possesses a notochord, gill slits, a dorsal nervous system and trunk somites (Jefferies, 1986). The most striking difference between such animals and vertebrates in body plan lie in the head, where in vertebrates there are concentrations of sensory receptors and a brain to interpret the information they supply. It has been suggested that the evolution of the neural crest and cephalic placodes was the key step in allowing these specialisation to occur, as in modern vertebrates they give rise to the special sense organs and the associated supporting skeletal capsules (Gans and Northcutt, 1983; Gans, 1989). The neural crest may have produced the first characteristicly vertebrate skeleton, either dermal bone in association with bilateral sense organs or elastic cartilage in the branchial arches to permit more efficient energy utilisation in ventilation (Gans, 1989). The advantages of more efficient sense organs, improved ventilation of the pharyngeal slits and the possibility of innervation of structures further from the central nervous system by ganglion formation may have permitted increases in body size that allowed further vertebrate specialisations (Gans, 1989).

The general similarity of structure and expression of genes within *Hox* clusters between insects and vertebrates suggests conservation of a cluster of genes from a common ancestor. The similarities are very strong for the more 3′ subfamilies of genes, as *Zen* and *pb* and the subfamilies containing *Hox-2.7* and *Hox-2.8* are thought to be derived from a single ancestral gene, and single subfamilies homologous to *lab*, *Dfd* and *Scr* exist in vertebrates (Akam, 1989; Duboule and Dolle, 1989; Graham *et al.* 1989). The extent of these similarities suggests that the direct precursors of these four gene subfamilies already existed in vertebrate ancestors prior to the evolution of neural crest, rather than being produced by tandem duplication of genes subsequently. We suggest that at the time of the appearance of the neural crest, the existing developmental specification system of the anterior neural plate, involving *Antennapedia* class homeobox genes, was coopted into a new role in patterning the neural crest. The patterning system of the first 'new vertebrate head' was intimately dependent upon *Hox* genes where, as now, a single *Hox* gene or subfamily of genes was involved in the association between a region of anterior neural tube and a specific branchial arch.

In these animals there were fewer *Hox* clusters than in modern vertebrates, perhaps two. If the structure of one of these clusters resembled *Hox*-3 or *Hox*-4 this would mean that some branchial units would only have one gene involved in their specification. Subsequently the *Hox* clusters duplicated, with the extra sets of genes able to take on distinct roles in development. We suggest that members of a subgroup of *Hox* genes are expressed in identical groups of rhombomeres and branchial arches because the *Hox* cluster duplication event or events leading to four clusters of genes occurred when the patterning system of the branchial region was well established, and not able to increase the range of structures that could be specified by employing extra *Hox* genes to specify new morphological units. The conservation of the relationships between hindbrain ganglia, rhombomeres and branchial arches in all vertebrates may be evidence for the constraints operating in the branchial patterning system. The increased number of 3′ genes that cluster duplication provided were subsequently used in the development of particular groups of tissues.

Hox *genes in the head and trunk*

On the basis of the likely biology of prevertebrates it is thought that the mineralised cranial skeleton evolved before that of the trunk (Gans and Northcutt, 1983;

Gans, 1989). It is more parsimonious to envisage the single evolutionary event of the appearance of a group of related tissues, the neural crest and placodes, than to require the evolution of the neurogenic crest and placodes and separately the acquisition of skeletogenic potential in the somites, followed by the transfer of this potential to the neural crest. The earliest vertebrate fossils show evidence of cranial neural crest-derived skeletal structures such as dermal armour and branchial cartilages (Elliott, 1987; Smith and Hall, 1990), but no traces of trunk skeleton have been found. This is not necessarily proof that the head skeleton preceded that of the trunk, as a cartilaginous trunk skeleton would not fossilise if the conditions of preservation were such that soft parts did not survive.

Originally the somites are thought to have consisted of dermomyotome, on the basis of their fates in *Branchiostoma*. We speculate that the appearance of skeletogenic sclerotomes in the trunk may be connected to the duplication event to give rise to four *Hox* clusters. The ability to make cartilage and bone would increase the range of structures that a somite could make, and the increased number of possible elements could utilise the greater range of *Hox* genes now available to differentially specify them. For this reason the *Hox* code of the somites involves offsets between members of a subfamily rather than the overlapping code of the branchial region, hence the more dramatic effects of perturbing the expression patterns of a single gene in the trunk (Kessel *et al.* 1990).

It is intriguing that where there is evidence that a structure has some kind of intrinsic fate specification, either the trunk somites (Chevallier, 1975) or the neural crest of the branchial arches (Noden, 1988), there is an overlapping code of *Hox* gene expression that could be involved in this specification. The anterior parts of the head do not express *Antennapedia* class *Hox* genes however, so some other mechanisms must be involved in their specification.

In *Drosophila*, the anterior parts of the head are also beyond the domains of expression of the HOM-C genes, and seem to be specified by an independent patterning system (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). In vertebrates there is emerging molecular evidence for groups of genes distinct from the four clusters of *Antennapedia* class *Hox* genes involved in the specification of the anterior parts of the head. The murine homologue of the cell signalling molecule, *Wnt-1* is expressed in the mesencephalon (Wilkinson *et al.* 1987), which is deleted in homologous recombinant mice lacking the gene function (McMahon and Bradley, 1990). The expression patterns of other genes suggests an involvement in the patterning of brain and neural crest. In more anterior parts of the mouse neuroepithelium there is evidence for a gene related to the *Distal-less* gene of *Drosophila* showing spatially restricted domains of expression consistent with regional patterning (Price *et al.* 1991). Some of these genes may be involved in the actual establishment of pattern in the head anterior of the hindbrain, others may be early markers of regionally restricted differentiation events. A more detailed comparison of the onset of expression of such genes coupled with the emerging technology of directed mutagenesis in mice will elucidate the relative positions of such genes in the developmental hierarchy of the head.

There is also evidence for head patterning strategies that do not involve genetic specification of the neural plate followed by transfer of patterning information to other parts of the head by imprinted neural crest. Evidence suggests that crest prespecification may be less important in more anterior parts of the head (McKee and Ferguson, 1984; Noden, 1988). The distribution of type II collagen suggest that aspects of craniofacial morphogenesis may be controlled by the distribution of molecules on cranial epithelia that arrest crest migration and induce chondrogenesis at specific sites (Thorogood *et al.* 1986; Wood *et al.* 1991). In this 'flypaper' model patterning information resides in the neural plate but also in other head epithelia such as the primordia of the paired sense organs (Thorogood, 1988), while there is little intrinsic information in the crest. These developmental mechanisms probably evolved during the elaboration of the anterior vertebrate head caused by the increasing concentration and importance of the brain and sense organs, anterior of the more primitive branchial region. These epigenetic mechanisms are now likely to be the more important in craniofacial morphogenesis, as the bulk of cranial and facial structures are derived from this region.

Thus the vertebrate body axis appears to be divided into three zones in terms of *Hox* expression; the anterior head, without any expression by *Antennapedia* class genes, the branchial region, with an overlapping code of rhombomere and branchial arch specification, and the trunk, where offsets in subfamily expression mark particular somites and parts of the spinal cord.

Conclusions

There seems little doubt that the *Hox* family is an important part of the molecular mechanisms for specifying regional variation. The descriptive studies suggested they played an important role in the head, which has clearly been directly demonstrated by the phenotypes observed in mice bearing mutated *Hox* genes. It will now be essential to identify primary and secondary roles of the *Hox* genes, and to determine how the signals for establishing the patterns are established. In this regard the identification of transcriptional regions that regulate a particular aspect of the expression pattern will be useful to dominantly alter expression in transgenic mice and test the *Hox* code. They would be good targets for disrupting specific subsets of *Hox* expression in the germline to examine the role of a gene in an isolated region. Finally they provide a means of purifying factors that bind to the genes which are involved in the upstream parts of the regulatory cascade. Studies on development of the

vertebrate head appear to be rapidly approaching an unveiling of some of the basic molecular components necessary for pattern formation.

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References

AKAM, M. (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, J. (1984). The development of the rat spinal cord. *Adv. Anat. Embryol. Cell Biol.* **85**, 1–166.

BAROFFIO, A., DUPIN, E. AND LE DOUARIN, N. (1991). Common precursors for neural and mesenchymal derivatives in the cephalic neural crest. *Development* **112**, 301–305.

BEEMAN, R. (1987). A homeotic gene cluster in the red flour beetle. *Nature* **327**, 247–249.

BEEMAN, R., STUART, J., HAAS, M. AND DENELL, R. (1989). Genetic analysis of the homeotic gene complex (HOM-C) in the beetle *Tribolium castaneum*. *Devl Biol.* **133**, 196–209.

BRONNER-FRASER, M. AND FRASER, S. (1988). Cell lineage analysis reveals multipotency of some avian neural crest cells. *Nature* **335**, 161–164.

CHAVRIER, P., VESQUE, C., GALLIOT, B., VIGNERON, M., DOLLE, P., DUBOULE, D. AND CHARNAY, P. (1990). The segment-specific gene *Krox-20* encodes a transcription factor with binding sites in the promoter of the *Hox 1.4* gene. *EMBO J.* **9**, 1209–1218.

CHEVALLIER, A. (1975). Role du mesoderme somitique dans le developpement de la cage thoracique de l'embryon d'oiseau. I. origine du segment sternal et mecanismes de la differenciation des cotes. *J. Embryol. exp. Morph.* **33**, 291–311.

CHISAKA, O. AND CAPECCHI, M. (1991). Regionally restricted developmental defects resulting from targetted disruption of the mouse homeobox gene *Hox 1.5*. *Nature* **350**, 473–479.

COHEN, S. AND JURGENS, G. (1990). Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature* **346**, 482–485.

DUBOULE, D. AND DOLLE, P. (1989). The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. *EMBO J.* **8**, 1497–1505.

ELLIOTT, D. (1987). A reassessment of *Astraspis desiderata*, the oldest North American vertebrate. *Science* **237**, 190–192.

FINKELSTEIN, R. AND PERRIMON, N. (1990). The orthodenticle gene is regulated by *bicoid* and *torso* and specifies *Drosophila* head development. *Nature* **346**, 485–488.

FRASER, S., KEYNES, R. AND LUMSDEN, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431–435.

GANS, C. (1989). Stages in the origin of vertebrates: analysis by means of scenarios. *Biol. Rev.* **64**, 221–268.

GANS, C. AND NORTHCUTT, R. (1983). Neural crest and the origin of vertebrates: a new head. *Science* **220**, 268–274.

GAUNT, S. J. (1987). Homeobox gene *Hox 1.5* expression in mouse embryos: earliest detection by *in situ* hybridization is during gastrulation. *Development* **101**, 51–60.

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**, 329–339.

GAUNT, S. J., KRUMLAUF, R. AND DUBOULE, D. (1989). Mouse homeo-genes within a subfamily, Hox-1.4, -2.6 and -5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation. *Development.* **107**, 131–141.

GRAHAM, A., MADEN, M. AND KRUMLAUF, R. (1991). The murine Hox-2 genes display dynamic dorsoventral patterns of expression during central nervous system development. *Development* **112**, 255–264.

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

GUTHRIE, S. AND LUMSDEN, A. (1991). Formation and regeneration of rhombomere boundaries in the developing chick hindbrain. *Development* **112**, 221–229.

HALL, B. (1987). Tissue Interactions in Head Development and Evolution. In *Developmental and Evolutionary Aspects of the Neural Crest* (ed. P. F. A. Maderson), pp. 215–259. New York: John Wiley.

HUNT, P., GULISANO, M., COOK, M., SHAM, M., FAIELLA, A., WILKINSON, D., BONCINELLI, E. AND KRUMLAUF, R. (1991*a*). A distinct *Hox* code for the branchial region of the head. *Nature* **353**, 861–864.

HUNT, P. AND KRUMLAUF, R. (1991). Deciphering the Hox code: Clues to patterning the branchial region of the head. *Cell* **166**, 1075–1078.

HUNT, P., WHITING, J., MUCHAMORE, I., MARSHALL, H. AND KRUMLAUF, R. (1991*b*). Homeobox genes and models for patterning the hindbrain and branchial arches. *Development* **112 Supplement**, 187–196.

HUNT, P., WILKINSON, D. AND KRUMLAUF, R. (1991*c*). Patterning the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating neural crest. *Development* **112**, 43–51.

IZPISUA-BELMONTE, J.-C., DOLLE, P., RENUCCI, A., ZAPPAVIGNA, V., FALKENSTEIN, H. AND DUBOULE, D. (1990). Primary structure and embryonic expression pattern of the mouse *Hox-4.3* homeobox gene. *Development* **110**, 733–745.

IZPISUA-BELMONTE, J.-C., TICKLE, C., DOLLE, P., WOLPERT, L. AND DUBOULE, D. (1991). Expression of homeobox *Hox-4* genes and the specification of position in chick wing development. *Nature* **350**, 585–589.

JACOBSON, A. (1988). Somitomeres: mesodermal segments of vertebrate embryos. *Development* **104**, 209–220.

JEFFERIES, R. (1986). Living acraniates – amphioxus and its relatives. In *The Ancestry of the Vertebrates*, pp. 55–86. London: British Museum (Natural History).

KESSEL, M., BALLING, R. AND GRUSS, P. (1990). Variations of cervical vertebrae after expression of a *Hox-1.1* transgene in mice. *Cell* **61**, 301–308.

KESSEL, M. AND GRUSS, P. (1990). Murine developmental control genes. *Science* **249**, 374–379.

KESSEL, M. AND GRUSS, P. (1991). Homeotic transformations of murine prevertebrae and concommitant alteration of Hox codes induced by retinoic acid. *Cell* **67**, 89–104.

KEYNES, R. AND STERN, C. (1985). Segmentation and neural development in vertebrates. *Trends Neurosci.* **8**, 220–223.

KIRBY, M. (1989). Plasticity and predetermination of the mesencephalic and trunk neural crest transplanted into the region of cardiac neural crest. *Devl Biol.* **134**, 402–412.

KURATANI, S. AND KIRBY, M. (1991). Initial migration and distribution of the cardiac neural crest in the avian embryo: An introduction to the concept of the circumpharyngeal crest. *Am. J. Anat.* **191**, 215–227.

LE DOUARIN, N. (1983). *The Neural Crest*. Cambridge: Cambridge University Press.

LE DOUARIN, N., FONTAINE-PERUS, J. AND COULY, G. (1986). Cephalic ectodermal placodes and neurogenesis. *Trends Neurosci.* **9**, 175–180.

LEWIS, E. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**, 565–570.

LUFKIN, T., DIERICH, A., LEMEUR, M., MARK, M. AND CHAMBON, P. (1991). Disruption of the Hox-1.6 homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66**, 1105–1119.

LUMSDEN, A. (1990*a*). The cellular basis of segmentation in the developing hindbrain. *Trends in Neurosci.* **13**, 329–335.

LUMSDEN, A. (1990*b*). The Development and Significance of Hindbrain Segmentation. In *Seminars in Developmental Biology*:

The Evolution of Segmental Patterns, vol. 1, issue 2 (ed. C. Stern), pp. 117–125. Philadelphia: W.B. Saunders.

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

LUMSDEN, A., SPRAWSON, N. AND GRAHAM, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281–1291.

MCKEE, G. AND FERGUSON, M. (1984). The effects of mesencephalic neural crest cell extirpation on the development of chicken embryos. *J. Anat.* **139**, 491–512.

MCMAHON, A. AND BRADLEY, A. (1990). The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of mouse brain. *Cell* **62**, 1073–1085.

MOODY, S. AND HEATON, M. (1983*a*). Developmental relationships between trigeminal ganglia and trigeminal motorneurons in chick embryos. I. ganglion development is necessary for motorneuron migration. *J. comp. Neurol.* **213**, 327–343.

MOODY, S. AND HEATON, M. (1983*b*). Developmental relationships between trigeminal ganglia and trigeminal motorneurons in chick embryos. II. ganglion axon ingrowth guides motorneuron migration. *J. comp. Neurol.* **213**, 344–349.

MURPHY, P., DAVIDSON, D. AND HILL, R. (1989). Segment-specific expression of a homeobox-containing gene in the mouse hindbrain. *Nature* **341**, 156–159.

MURPHY, P. AND HILL, R. (1991). Expression of mouse *labial*-like homeobox-containing genes, *Hox 2.9* and *Hox 1.6*, during segmentation of the hindbrain. *Development* **111**, 61–74.

NODEN, D. (1983). The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Devl Biol.* **96**, 144–165.

NODEN, D. (1986). Patterning of avian craniofacial muscles. *Devl Biol.* **116**, 347–356.

NODEN, D. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103 Supplement**, 121–140.

NOHNO, T., NOJI, S., KOYAMA, E., OHYAMA, K., MYOKAI, F., KUROIWA, A., SAITO, T. AND TANAGUCHI, S. (1991). Involvement of the Chox-4 chicken homeobox genes in determination of anteroposterior axial polarity during limb development. *Cell* **64**, 1197–1205.

PRICE, M., LEMAISTRE, M., PISCHETOLA, M., DI LAURO, R. AND DUBOULE, D. (1991). A mouse gene related to *Distal-less* shows a restricted expression in the developing forebrain. *Nature* **351**, 748–751.

ROMER, A. (1971). *The Vertebrate Body, Shorter Version*, Fourth edn. Philadelphia: W. B. Saunders Company. (pp 163–167)

RUIZ-I-ALTABA, A. (1990). Neural expression of the *Xenopus* homeobox gene Xhox3, evidence for a patterning neural signal that spreads through the ectoderm. *Development.* **108**, 595–604.

SAXEN, L. (1989). Neural induction. *Int. J. Devl Biol.* **33**, 21–48.

SIMEONE, A., ACAMPORA, D., NIGRO, V., FAIELLA, A., D'ESPOSITO, M., STORNAIUOLO, A., MAVILIO, F. AND BONCINELLI, E. (1991). Differential regulation by retinoic acid of the homeobox genes of the four HOX loci in human embryonal carcinoma cells. *Mechanisms of Development* **33**, 215–227.

SIMEONE, A., PANNESE, M., ACAMPORA, D., D'ESPOSITO, M. AND BONCINELLI, E. (1988). At least three human homeoboxes on chromosome 12 belong to the same transcription unit. *Nucl. Acids Res.* **16**, 5379–5390.

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

SMITH, M. M. AND HALL, B. (1990). Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues. *Biol. Rev.* **65**, 277–373.

THOROGOOD, P. (1988). The developmental specification of the vertebrate skull. *Development* **103**, 141–153.

THOROGOOD, P., BEE, J. AND VON DER MARK, K. (1986). Transient expression of collagen type II at epitheliomesenchymal interfaces during morphogenesis of the cartilaginous neurocranium. *Devl Biol.* **116**, 497–509.

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*). The development of the cervical spinal cord of the mouse embryo. II. A golgi analysis of the sensory, commisural and association cell differentiation. *J. Neurol.* **222**, 96–115.

WHITING, J., MARSHALL, H., COOK, M., KRUMLAUF, R., RIGBY, P., STOTT, D. AND ALLEMANN, R. (1991). Multiple spatially-specific enhancers are required to reconstruct the pattern of *Hox-2.6* gene expression. *Genes Dev.* **5**, 2048–2059.

WILKINSON, D., BAILES, J. AND MCMAHON, A. (1987). Expression of the proto-oncogene *int*-1 is restricted to specific neural cells in the developing mouse embryo. *Cell* **50**, 79–88.

WILKINSON, D., 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.

WOOD, A., ASHHURST, D., CORBETT, A. AND THOROGOOD, P. (1991). The transient expression of type II collagen at tissue interfaces during mammalian craniofacial development. *Development* **111**, 955–968.