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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. 1991b; Lumsden, 1990a). 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, 1990b). 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. 1991b; 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-
The branchial Hox code

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
Therefore, part of the reason for the conservation of subfamilies such as the four system and somites (Izpisua-Belmonte et al. 1990) 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.

**Conservation Between Hox and HOM-C Homeobox Complexes**

Fig. 2. The structure of the murine Antennapedia class homebox gene complexes, illustrating the relationship between members of a subfamily of vertebrate genes and their Drosophila equivalents. Members of a subfamily or paralogous 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. 1991b,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. 1991c). 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 rhombomeric 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. 1991b), 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. 1991c), and this is supported by patterns of gene expression, which show identical limits in both tissues as indicated in Fig. 3C.

The expression pattern 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. 1991a). 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
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. 1991a). 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 12 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 9 1/2 d.p.c., and this domain is restricted to a single
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 (x100), Section D (x200), Section E (x240), Section F (x200).

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
ESTABLISHMENT OF THE BRANCHIAL HOX CODE

Presomitic to 1 Somite (7.75–8.0 dpc) 8 to 12 Somites (8.5 dpc)

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.
The conservation of the vertebrate Hox complexes and 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 Vaughan, 1979; Altman and Bayer, 1984; Wentworth, 1984a,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 level.

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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.

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**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/r7 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 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.

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. (1991a).

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 10 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 Capeschi, 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, X, 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 argue 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 rhomboeres 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 Capeschi, 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, 1983a,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, rhomboeres 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 one 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 characteristically vertebrate skeleton, either dural 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 Drosophilia, 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 Drosophilia 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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