**INTRODUCTION**

During early vertebrate development the neuroepithelium of the hindbrain is transiently organised into a series of repeated units termed rhombomeres. Recent experiments in chick suggest that this rostrocaudal series of eight segments provides an early ground plan on which morphogenesis and neurogenesis are based. Single cell labelling experiments have demonstrated that each rhombomere represents a lineage restricted compartment (Fraser et al., 1990). The rhombomere pattern is related to an underlying cellular organisation (Lumsden and Keynes, 1989). For example, the cranial nerves are positioned with a two-segment periodicity; the cell bodies of branchiomotor nerves V, VII and IX being confined to rhombomeres 2 plus 3, 4 plus 5, and 6 plus 7 respectively, the nerve exit points lying within the even-numbered rhombomeres.

An increasing array of putative developmental control genes, encoding regulators of transcription, show rhombomere-specific domains of expression. The first such gene to be reported was Krox-20 (Wilkinson et al., 1989a; Nieto et al., 1992), which is expressed in mouse and chick hindbrain first in rhombomere 3 (r3) and slightly later in r5, before, or as, each rhombomere becomes delineated by boundaries. Many other genes have since been shown to have expression patterns which respect rhombomere boundaries. Perhaps most notable amongst these are the genes of the Hoxb cluster. Hoxb-1, the most 3′ gene in the complex, has an anterior limit of expression at the boundary between r3 and r4. By 8.5 days post coitum in the mouse, (Murphy et al., 1989; Wilkinson et al., 1989b) or stage 11 (Hamburger and Hamilton, 1951) in the chick, (Sundin and Eichele, 1990) the expression domain has receded caudally from r5 and r6 to leave a domain of up-regulated expression in r4. The genes of the Hoxb cluster have expression patterns reminiscent of the related Drosophila homeotic complex genes. In general, the more 3′ a gene lies in the Hoxb cluster the more anterior its expression domain, thus Hoxb-2 has a rostral limit of expression at the r2/3 boundary, Hoxb-3 at the r4/5 boundary and Hoxb-4 at the r6/7 boundary (Wilkinson et al., 1989b).

Neural crest production in the hindbrain also displays a two-segment periodicity. Three discontinuous streams of neural crest migrate away from the rhombencephalic dorsal neural tube, separated by two regions, r3 and r5 that are deficient in crest production (Lumsden et al., 1991; Sechrist et al., 1993). Neural crest derived from the midbrain, r1 and r2, contributes to the first branchial arch and the trigeminal ganglion, crest...
from r4 contributes to the second branchial arch and the facial and vestibuloacoustic ganglia, crest from r6 contributes to the third branchial arch and the superior ganglion of the IXth nerve.

The cranial neural crest differs from that of the trunk in several important aspects. Although both crest regions contribute to sensory and autonomic ganglia, the cranial neural crest additionally gives rise to a range of tissues that includes mesenchymal derivatives, such as connective tissue, cartilage and bone (Le Lievre and Le Douarin, 1975; Le Lievre, 1978; Noden, 1978, 1983). Cranial mesoderm is relatively sparse and, also unlike trunk mesoderm, is not clearly segregated into paraxial and lateral regions. The paraxial mesoderm of the trunk forms metameres (somites) with myogenic and skeletogenic potencies in addition to the ability to form dermis. The cranial mesoderm has retained myogenic and some skeletogenic capacity but the formation of skeleton and dermis has largely been taken over by the cranial neural crest; the neural crest-derived cephalic dermis is the source of most of the membranous bones of the skull (Coulby et al., 1992, 1993). Cranial neural crest also has the unique capacity to influence cephalic pattern formation (Noden, 1988). When neural tube containing presumptive first arch neural crest is transplanted into the position of that containing presumptive second arch neural crest, graft-derived ectomesenchyme migrates into the second arch but there develops first arch structures, including an ectopic beak (Noden, 1983). Furthermore, if cranial mesoderm is replaced by trunk paraxial mesoderm, the neural crest directs formation of somite-derived muscles appropriate to the new location (Noden, 1986). These results suggest that spatial programming of connective tissue-forming branchial arch neural crest cells occurs before the onset of migration, when the crest precursors are still within the neuroepithelium. Additionally, the neural crest-derived ectomesenchyme can impart this information to compliant myogenic cells (Noden, 1986).

The murine Hoxb genes are expressed not only in specific rhombomeres, but also in the neural crest cells that migrate out of the neural tube at that level (Hunt et al., 1991a,b). Perhaps due to the existence of crest-depleted areas at the level of r3 and r5, the expression of each gene in the neural crest is offset from that in the neural tube by a distance of one rhombomere’s width. This observation has led to the hypothesis that a ‘Hox code’, putatively involved in patterning the hindbrain, is passively carried into the branchial arches by the neural crest, to be further involved in patterning of craniofacial structures (Hunt et al., 1991b). The model implies that one function of hindbrain segmentation is to establish the prepattern of the neural crest that has been suggested by Noden’s experiments (1983, 1986), and that this patterning is directed by Hox gene expression. Vertebrate Hox genes have duplicate and diverged from an ancient ancestral gene complex to give rise to a total of 4 clusters (Graham et al., 1989). In many cases paralogous genes exist in different complexes; paralogues exhibit high levels of sequence identity, and occur in equivalent spatial locations within separate complexes. In the majority of cases investigated, the expression patterns of paralogous genes during early development share some or all features (Gaunt et al., 1989; Hunt et al., 1991a; Murphy and Hill, 1991). The original report that the mouse Hoxb-2 and Hoxa-2 genes share the same anterior expression limit at the r2/3 boundary, based on radioactive in situ hybridisation data (Hunt et al., 1991a), has recently been reinterpreted in the light of unpublished work and it is now accepted that there is expression of murine Hoxa-2 in r2, albeit in a subset of the cells (Krumlauf, 1993).

In this study we have cloned and sequenced the chick Hoxa-2 gene. We have used whole-mount in situ hybridisation to carefully analyse the early expression pattern of this gene particularly in the rhombencephalic neural tube and derived neural crest. Our experiments show that this gene is expressed in the hindbrain with an anterior limit precisely at the r1/2 boundary. The r2 expression is at a slightly reduced level with respect to more caudal expression domains but nevertheless includes the dorsal-most cells of the neural tube, which are precursors of neural crest and the neural crest primordium. The neural crest produced at the r2 level does not, however, express Hoxa-2. In contrast, the neural crest cells produced at the r4 level do express the gene. We have exploited the differential expression pattern of Hoxa-2 in neural tube and neural crest, plus the ease of manipulation of the chick embryo, to investigate prepattern-ning of the neural crest at a molecular level. Grafting experiments show that the neural crest is pre-programmed with respect to Hoxa-2 expression status prior to migration out of the neuroepithelium.

MATERIALS AND METHODS

Cloning of the Hoxa-2 homeobox using degenerate oligonucleotide primers

Primers

A:-5’ CAGACCTCCICGACITT/TCTCAGC/CTTGGATG 3’

B:-5’ CGGATCCTGGGCTICGCTTG/GTCCTG 3’

C:-5’ CCCAACATCCTATICGAT/CAGTTT/GATGCGC/CAAAC 3’

PCR amplification reactions

A 280 bp fragment encompassing the homeobox region of chick Hoxa-2 was generated by the polymerase chain reaction (PCR) with cDNA, synthesised from stage 15 chick embryo poly(A)+ RNA (Amersham cDNA synthesis system). Reactions were performed in a 100 μl volume at a magnesium chloride concentration of 2.5 mM using 1 unit of Taq polymerase (Promega). The reaction was heated to 94°C for 2 minutes then passed through thirty cycles of 94°C for 30 seconds, 55°C for 2 minutes then 72°C for 2.5 minutes, after completion of thirty cycles the reactions were incubated for a further 10 minutes at 72°C. The product of the first PCR reaction generated with primers A and B was used as a template for a second reaction with primers A and C. The final PCR product was gel purified and subcloned into a T-vector formed from pKS (Stratagene) as previously described (Marchuk et al., 1991).

Isolation and characterisation of cDNAs

A chick stage 12-16 cDNA library in lambda ZAPII (generously provided by Angela Nieto and David Wilkinson) was screened at high stringency (5× SSC, 5× Denhardt’s, 100 μg/ml salmon sperm DNA, 0.1% SDS at 65°C) with the 280 bp PCR-derived cDNA of Hoxa-2. pSK (Stratagene) plasmids harbouring the isolated clones were excised with exassist helper phage (Stratagene) according to the manufacturer’s instructions. Derived cDNA’s were restriction mapped and subcloned into pSK or M13 vectors. Double-stranded or single-stranded sequencing, as appropriate, was performed using Sequenase (United States Biochemical) according to manufacturer’s instructions.
Rhombomere transplantations

Rhode Island Red hens’ eggs from a local flock (Needle Farm, Enfield) were incubated at 37°C and 50% relative humidity to stages 9+ to 10+. Embryos were removed from eggs and pinned out on a Sylgard dish, small focal injections of a solution of Dil C18 (Molecular probes, D-282) at 5 mg/ml in dimethyl formamide were made close to the anterior boundary of r2 and r4, to mark the polarity of the regions to be grafted. Pairs of rhombomeres, r2+r3 or r4+r5 were then dissected out and treated with dispase 1 (Boehringer-Mannheim; 1 mg/ml in L-15 medium, Gibco) for 15-20 minutes to separate the neural tissue from the surrounding mesenchymal cells. The pairs of rhombomeres were transplanted to prepared stage-matched hosts. To prepare host embryos the egg was windowed, the embryo visualised by a sub-blastodermal injection of India ink, the vitelline membrane reflected and a gap in the neural tube corresponding to r2+r3 or r4+r5 made using needles of flame-sharpened 100 µm tungsten wire, leaving the notochord in place. The graft was manoeuvred into place using needles, the anteroposterior orientation of the graft being maintained by reference to the Dil label at its anterior end, the egg was then sealed with tape and incubation continued for a further 36 hours. Embryos were then harvested and analysed by whole-mount in situ hybridisation and vibratome sectioning. Grafts were performed of r2+r3 to the location of r4+r5, or r4+r5 to the location of r2+r3, as shown in Fig. 1. Control grafts were also performed in which each donor rhombomere pair was placed in its correct location in a host embryo. In a few cases the majority of the dorsal aspect of donor embryo r2 or r4 was labelled by injection of a solution of Dil C18 at 1 mg/ml in 0.3 M sucrose before dissecting out pairs of rhombomeres for transplantation into host embryos as described above. These specimens were also harvested after 36 hours incubation and fixed in 3.5% paraformaldehyde in phosphate-buffered saline, labelled cells were observed by fluorescence microscopy before processing for in situ hybridisation.

Whole-mount in situ hybridisation

Embryos were fixed overnight in 3.5% paraformaldehyde in phosphate-buffered saline (PBS), washed twice in PBT (PBS, 0.1% Tween-20) and then dehydrated by passing them successively through 25%, 50%, 75% and 100% methanol. The specimens were rehydrated by passing through the series in reverse and then washed twice in PBT and incubated for 1 hour in 6% hydrogen peroxide in PBT. After a PBT wash the embryos were treated with proteinase K (20 µg/ml) for 15 minutes and refixed in 0.2% glutaraldehyde/3.5% paraformaldehyde in PBS for 20 minutes at room temperature. The embryos were then transferred to hybridisation buffer (50% formamide, 5× SSC pH 4.5, 1% SDS, 50 µg/ml tRNA, 50 µg/ml heparin) and pre-hybridised for 1 hour at 70°C. Digoxigenin-labelled RNA probes were synthesised according to the manufacturers instructions (Boehringer-Mannheim) and added to hybridisation buffer at a final concentration of 1 µg/ml; hybridisation was carried out overnight at 70°C. The embryos were washed three times for thirty minutes in 50% formamide, 1% SDS, 5× SSC pH 4.5 at 70°C, then three times for 30 minutes in 50% formamide, 2× SSC pH 4.5 at 65°C. This was followed by washing in TBST (0.14 M NaCl, 10 mM KCl, 25 mM Tris-Cl pH 7.5, 1% Tween-20). The embryos were pre-blocked in 10% sheep serum in TBST before being incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim) that had been pre-absorbed with chick embryo powder. The embryos were then washed extensively in TBST and then in NTMT (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2, 1% Tween-20). The alkaline phosphatase was visualised by incubating embryos with 0.34 mg/ml nitroblue-tetrazolium chloride (BRL), 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BRL) in NTMT. The stained specimens were mounted in 10% PBS/50% glycerol and viewed under Nomarski optics. For sectioning, embryos were embedded in gelatin-albumin, fixed with glutaraldehyde and cut at 75 µm on a vibratome.

RESULTS

Isolation and characterisation of the chick Hoxa-2 gene

The polymerase chain reaction was used to isolate a 280 base-pair (bp) cDNA including the homeobox of the chick Hoxa-2 gene; this was used to screen an amplified stage 12-16 chick cDNA library at high stringency. Six independent cDNAs, ranging in length from 700 bp to 1650 bp, were isolated and the sequence of the gene from 100 bp 5′ of the presumed start of translation to 3′ of the polyadenylation signal was obtained.
Northern analysis using poly (A)+ RNA from E3 embryos (data not shown) revealed a single transcript of 1.6 kb, indicating that the largest cDNA obtained was approximately full length.

The amino acid sequence of the cloned homeobox is 100% identical to the homeodomain sequences of murine Hoxa-2 (Tan et al., 1992), human HOXB-2 (Acampora et al., 1989), and murine Hoxb-2 (Rubock et al., 1990). However, comparison with the full length sequence of murine Hoxa-2 (Tan et al., 1992) revealed extensive homology beyond the homeodomain, confirming the identity of the cloned gene as chick Hoxa-2 (Fig. 2B). The overall level of amino acid identity between chick and mouse Hoxa-2 is 85% (Fig. 2B), yet between chick Hoxa-2 and human HOXB-2 there is only 49% identity (full amino acid sequence is unavailable for murine Hoxb-2).

The ATG designated as the translational start lies within a divergent Kozak initiation consensus sequence (6/10 nucleotides conserved; Kozak, 1987). This divergent Kozak sequence is identical to that reported for murine Hoxa-2 (Tan et al., 1992); in addition the 100 bp of sequence 5′ of the putative translational start of chick Hoxa-2 has 75% nucleotide identity with the murine Hoxa-2 upstream sequence. The chick Hoxa-2 sequence also includes a conserved hexapeptide, EYPWMK (amino acids 94-99), characteristic of many Hox genes.

**Expression of Hoxa-2 during early development**

The expression pattern of Hoxa-2 in chick embryos from Hamburger-Hamilton stage 4 to 24 was analysed by whole-mount in situ hybridisation (Figs 3, 4A,B). Expression is first observed at Hamburger-Hamilton stage 4, in the caudal most part of the primitive streak (Fig. 3A), by stage 5 the level of expression has increased and the domain of expression extends rostrally towards Hensen’s node (Fig. 3B). Between stages 7 and 8, as somites begin to condense, expression becomes localised to the somites and is also retained in the regressing Hensen’s node (Fig. 3C-F). Expression within the neuroepithelium commences at stage 8 (Fig. 3E,F), reaching a diffuse anterior limit at approximately the presumptive anterior boundary of r2 by stage 9 (Fig. 3F,G). Throughout these stages expression is maintained in the regressing Hensen’s node and in the developing somites. The anterior limit of Hoxa-2 expression becomes discrete by stage 11 (Fig. 3I), close to the time at which the rhombomere 1/2 boundary becomes morphologically distinct (stage 11+; Vaage, 1969). The r1/2 boundary continues to be the anterior limit of expression (see Fig. 4B) until at least stage 24, at which time rhombomere boundaries become less distinct morphologically (Vaage, 1969).

The level of expression of Hoxa-2, as assessed by whole-mount in situ analysis, is slightly lower in r2 than in more caudal rhombomeres up until stage 17 of embryonic development when the expression level becomes similar in all the rhombomeres (compare Figs 3K and 4A,B). More caudally, there is no obvious variation in Hoxa-2 expression level between rhombomeres at the stages analysed (Fig. 3H-L). At stage 10+ (Fig. 3I) Hoxa-2-expressing neural crest cells can be seen emerging from the dorsal aspect of r4 (the crest migrates from the otic region between stages 9+ and 11; Tosney, 1982; Lumsden et al., 1991). The emerging neural crest cells continue to express Hoxa-2 as they migrate into, and fill, the second branchial arch (Fig. 3I-L). Similarly, neural crest cells migrating from the level of r6 into the third branchial arch also continue to express the gene, as do crest cells migrating more caudally. Unexpectedly, despite the presence of Hoxa-2 expression throughout the
neural tube at the r2 level, Hoxa-2 expression cannot be detected in neural crest derived from r2 (Fig. 3H-L; trigeminal level crest migrates between stages 9 and 11; Tosney, 1982, Lumsden et al., 1991). A lateral view of a stage 17 embryo shows expression of Hoxa-2 in the entire second branchial arch, the facial (VII) nerve and VII/VIII ganglia (Fig. 4A), but no expression outside the neural tube more rostrally, in the first branchial arch or trigeminal (V) ganglion.

Expression in the somites continues throughout the stages analysed, but from stage 9 onwards is at a higher level in the more rostral somites. By stage 24, somite expression is markedly reduced caudal to the wing bud. No expression was detected in the otic vesicle or in the developing limb buds at the stages analysed (see Fig. 3L).

As the level of chick Hoxa-2 expression is slightly lower in r2 than in more caudal rhombomeres we considered that expression might be limited to the ventral half of the neural tube at this axial level, as has been reported for the murine Hoxa-2 gene (Krumlauf, 1993). Such an expression pattern could imply that Hoxa-2 is not expressed in the dorsal population of cells that gives rise to neural crest, and could thus explain the absence of expression in r2-derived neural crest and the first branchial arch. To address this possibility vibratome sections were cut transversely through the hindbrain of embryos after performing whole-mount in situ hybridisation.

At stage 10, when neural crest migration is occurring from the r2 level, sections through the neural tube at the level of presumptive r2 shows no dorsoventral restriction of Hoxa-2 expression (Fig. 4C). Indeed, expressing cells can be seen in the most dorsal aspect of the neural tube, apparently about to migrate out into the periphery. Thinner frozen sections (10 µm) of stage 10 embryos were also cut, these verified that Hoxa-2 expression extended throughout the neural crest precursor region of the neural tube (data not shown). However, by stage 11, and later, the majority of neural crest cells have migrated away from the neural tube, laterally and ventrally, and do not express the gene (Fig. 4D,E). In contrast, at the r4 level ectomesenchymal cells expressing the gene can be seen in transverse sections (Fig. 4F,G). Rather than revealing low levels of Hoxa-2 expression dorsally at the level of r2, vibratome sectioning showed that there is a somewhat higher level of expression in the dorsal part of the neural tube and in the neural crest primordium than more ventrally. Transverse sections also revealed that r2 and r3 floor plate cells do not express Hoxa-2, but r4 and more caudal floor plate cells do express the gene (compare Fig. 4D,E with 4F,G). There is no expression detected in the notochord at these levels.

**Isochronic rhombomere grafting experiments**

The unexpected finding that Hoxa-2 is not expressed in r2-derived neural crest, despite being expressed in the crest progenitor region at this axial level at the time of crest production, implies that transcription of the gene is rapidly down-regulated at the onset of neural crest migration from r2. This down-regulation might be intrinsic to r2 neuroepithelial cells, expression being pre-programmed to shut down as migration commences. Alternatively, the down-regulation might be a function of the environment into which the cells are migrating; cells migrating out of r2 towards the first branchial arch may receive a signal from surrounding cells (i.e. epidermal ectoderm or paraxial mesoderm) that causes a rapid reduction in the rate of Hoxa-2 transcription, whilst cells migrating out of r4 towards the second branchial arch do not receive such a signal.

To distinguish between these hypotheses we made use of the ease of manipulation of the early chick embryo; a series of isochronic rhombomere grafting experiments was carried out to test directly the effect on Hoxa-2 expression of the environment into which neural crest cells migrate (Fig. 1). We grafted pairs of rhombomeres, either r2+r3 or r4+r5, rather than individual rhombomeres to provide larger pieces of tissue that were simpler to manoeuvre. Because neural crest production by rhombomeres 3 and 5 is depleted compared with their even-numbered neighbours (Lumsden et al., 1991; Graham et al., 1993), the experiment addresses the effect of allowing r2- and r4-derived neural crest to migrate through each other’s normal environment. Embryos of stages 9–10+, during the initial early phase of cranial neural crest production, were used for grafting. These experiments are similar in design to those carried out by Noden (1983), who showed that first branchial arch neural crest can migrate into and populate the second branchial arch if the first arch crest primordium (including the r2 level) is transplanted in place of the second arch crest primordium (including the r4 level) at stages preceding migration.

Rhombomere pairs from donor embryos, either r2+r3 or r4+r5, were marked at their anterior end with a small focal injection of DiI then dissected out and surrounding mesenchymal tissue removed enzymatically to produce clean cylinders of intact neural tube. The rhombomere pairs were inserted into prepared stage-matched donors, the anteroposterior orientation of the grafts being maintained by reference to the labelled anterior edge. Grafts of r2+r3 were made into the position of r4+r5 and vice-versa. Control experiments, in which rhombomere pairs were placed into host embryos at their normal locations, were performed in order to assess the effect of the manipulations.

Those manipulated embryos that survived the operation and in which the grafted region appeared to have healed adequately into the host neural tube (approximately 65% of experimental embryos) were analysed at stage 16-18 for Hoxa-2 expression. The results of all the grafting experiments performed are collated in Table 1. The control embryos in which a donor r2+r3 was placed was into the position of the host r2+r3 (Fig. 5A), or a donor r4+r5 was placed into the position of the host r4+r5 (Fig. 5B), were indistinguishable from unmanipulated embryos. This result showed that the transient presence of cut edges in the neural tube, from which ectopic neural crest cells might possibly be produced, does not alter Hoxa-2 expression. In contrast, experiments in which r2+r3 was transplanted into the

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Fig. 3. Whole-mount in situ analysis of Hoxa-2 expression in chick embryos from Hamburger-Hamilton stage 4 to 16. All views are from the dorsal aspect and with rostral to the right. (A) Stage 4, arrowhead indicates Hensen’s node. (B) Stage 5; (C) stage 7 (1 somite), arrowhead indicates first somite. (D) Stage 8– (3 somite). (E) Stage 8 (4 somite). (F) Stage 9– (6 somite). (G) Stage 10– (9 somite). (H) Stage 10 (10 somite), arrowhead indicates presumptive r1/r2 boundary. (I) Stage 10+ (11 somite), arrowhead indicates r4-derived migrating neural crest. (J) Stage 11+ (14 somite), arrowhead indicates r1/r2 boundary. (K) Stage 12+ (17 somite). (L) Stage 16, arrowhead indicates otic vesicle.

Fig. 4. Whole-mount in situ analysis of Hoxa-2 expression. (A) Lateral view of stage 17 embryo rostral to the right. (B) Flat-mounted hindbrain of stage 17 embryo showing the precise correlation between the rostral limit of expression and the r1/2 boundary. (C-G) Vibratome sections of whole-mount in situ analysed embryos were cut at 75 µm. (C) Transverse section through r2 of a stage 10 embryo, arrowheads indicate lateral limit of expression in the dorsal neural tube and neural crest primordium. (D) Transverse section through r2 of stage 11− embryo. (E) Transverse section through r2 of stage 13+ embryo. (F) Transverse section through r4 of stage 11− embryo; arrowheads mark the lateral margin of Hoxa-2-expressing crest. (G) Transverse section through r4 of stage 13+ embryo.
position of r4+r5 produced distinct changes in the Hoxa-2 expression pattern in the periphery (Fig. 5C-E). In some embryos no Hoxa-2 expression at all was found in the second branchial arch, which in these experimental embryos is being populated by neural crest cells derived from the grafted r2 (Fig. 5C). In others, only a subset of the second arch cells expressed Hoxa-2 (Fig. 5D,E). The number of Hoxa-2-expressing cells in the second arch increased with the stage of the donor and host embryos, presumably reflecting the number of Hoxa-2-positive crest cells that had migrated out of the host r4 before grafting. In support of this idea we found that when r2+r3 was grafted into the position of r4+r5 at stage 12, close to the end of the period of neural crest migration from r4, the resulting embryos were indistinguishable from unmanipulated or control embryos.
Fig. 6. Isochronic grafting experiments with Dil-prelabelled rhombomere pairs. Embryos were analysed at stage 16-18; labelled neural crest cells were located by fluorescence microscopy before whole-mount in situ analysis. (A) Graft of r2+r3 to r4+r5 (stage 9); bright-field image, arrowhead indicates second branchial arch. (B) Fluorescence image; Dil-labelled cells have migrated throughout the proximodistal extent of the second branchial arch. (C) Whole-mount in situ analysis; loss of Hoxa-2 expression accompanies the presence of r2-derived neural crest cells in the second branchial arch. (D) Graft of r4+r5 to r2+r3 (stage 9); bright-field and fluorescence image showing Dil-labelled cells migrating into the first branchial arch (arrowhead). (E) High magnification of first branchial arch; fluorescence image. (F) Whole-mount in situ analysis; Hoxa-2 expression accompanies presence of r4-derived neural crest cells in the first branchial arch.
(data not shown). The results of these grafting experiments showed that removal of r2 from its normal position into the environment normally occupied by r4 does not prevent down-regulation of Hoxa-2 expression in r2-derived neural crest cells.

In the complementary experiment in which r4+r5 was grafted into the position of r2+r3 Hoxa-2-expressing cells were found in the first branchial arch (Fig. 5F-H), although no such expression was ever observed in unmanipulated or control embryos. In the majority of cases a trail of Hoxa-2-expressing neural crest cells, derived from the grafted r4, was seen migrating into the first branchial arch. The number of Hoxa-2-expressing crest cells in the first branchial arch varied between experimental embryos (Fig. 5F-H) and once again correlated with the stage at which grafting was performed, i.e. at later stages fewer Hoxa-2-expressing cells were present in the first branchial arch. Despite this variation in the number of Hoxa-2-positive cells they were generally found at all proximal-distal levels along the first branchial arch. This result suggests that crest cells do not simply fill the arch in a strict distal to proximal order according to time of migration but that they intermix, possibly migrating to defined spatial locations. We never observed Hoxa-2 expression in 100% of the cells of the first branchial arch, a result that is predicted by previous observations that a considerable proportion of the neural crest component of the first arch is derived from the midbrain (Le Douarin, 1983; Lumsden et al., 1991). The results of experiments in which r4+r5 was grafted to the r2+r3 position demonstrated that Hoxa-2 expression of r4-derived neural crest is not down-regulated by the r2 environment, r4-derived crest cells continued to express the gene just as when migrating into their normal second arch environment.

Experiments were performed to confirm that neural crest cells migrate as predicted from a transplanted rhombomere into the position appropriate to that rhombomere’s ectopic location. Neural crest primordia at the dorsal aspect of r2 or r4 of donor embryos were labelled extensively by injection of a solution of DiI in 0.3 M sucrose directly into the dorsal neuroepithelium. Rhombomere pairs were then dissected out as described above and inserted into prepared stage-matched donors, the antero-posterior orientation of the grafts being maintained by reference to the labelled (anterior) rhombomere. The location of the labelled neural crest cells was analysed at stage 16-18 by fluorescence microscopy. In embryos in which r2+r3 was transplanted to the position of r4+r5, labelled cells were observed throughout the proximal-distal extent of the second branchial arch (Fig. 6A,B). Whole-mount in situ analysis of such embryos confirmed that the labelled region corresponded to a domain of reduced Hoxa-2 expression (Fig. 6C). In the converse experiment in which r4+r5 was transplanted to the position of r2+r3 labelled cells were observed throughout the proximal-distal extent of the first branchial arch (Fig. 6D,E); the labelled cells correspond to the region of Hoxa-2 expression (Fig. 6F). These observations confirmed that neural crest migrates according to the location of the ectopic rhombomere in our grafting experiments.

It has been shown in a previous study that the unique disposition of the trigeminal and facial branchiomotor neurons is maintained by donor rhombomeres 2 and 4 respectively when grafted to each others normal locations (Guthrie et al., 1992). However, the peripheral nervous systems of the grafted embryos used in this study did not appear to be grossly different to those of unmanipulated embryos. To investigate this phenomenon, peripheral nervous systems of both experimental and unmanipulated embryos were revealed using an anti-68K neurofilament antibody (Lumsden and Keynes, 1989). At stage 17 no significant changes could be seen in location or morphology of the cranial ganglia and nerves following grafting experiments (data not shown). This observation suggests that the nature of each cranial ganglion may be determined primarily by its placodal component, which is not affected in these grafting experiments.

**DISCUSSION**

In this study we have shown that the anterior limits of Hoxa-2 expression are non-equivalent in different tissues; there are separate rostral expression limits in the neural tube, neural crest and floor plate. The expression domain of Hoxa-2 in the chick neural tube extends further rostral than that of any of the clustered Hox genes reported to date; Hoxa-2 is the only developmental control gene known to map to the r1/r2 boundary. Interestingly, the presence of a rostral limit of Hoxa-2 expression precedes formation of the boundary between rhombomeres 1 and 2. The limit is somewhat diffuse from stages 9 to 11, suggesting that prospective rhombomere 1 and 2 territories are in the process of being specified at this time, but becomes sharp immediately before the appearance of a morphological boundary. The clarification of the edge of the Hoxa-2 expression domain may reflect either a sorting out behaviour of expressing and non-expressing cells, or alternatively ongoing transcriptional regulation. Transverse sections revealed that Hoxa-2 is expressed in the floor plate of r4 and more caudally, but not in the floor plate of r2 and r3, the gene product may therefore be involved in setting up putative differences in floor plate identity at these axial levels.

In the case of the murine Hoxb cluster genes, each is expressed within a specific domain of the hindbrain, and also in neural crest cells derived from levels of the neural tube within that domain (Hunt et al., 1991b). In contrast, chick Hoxa-2 is not expressed with such a pattern; despite expression in r2 the gene is not expressed in r2-derived neural crest. This exceptional expression behaviour with respect to the other Hox genes may reflect divergence of the group II Hox cluster genes (Hoxa-2 and b-2). The group II and III (Hoxa-3, b-3, d-3) Hox cluster genes are most closely related to the Drosophila proboscipedia (pb) gene and all have been proposed to derive from a common ancestral gene (reviewed by Gaunt, 1991). If the group III genes are considered to have retained pb-like behaviour, allowing group II genes to take on a new more anterior role, then all Hox genes other than those of group II fall into the expected pattern of colinearity i.e. the more 3′ a gene lies within a cluster the more anterior its expression domain.

It has been assumed that in general the paralogous groups of Hox cluster genes share anterior limits of expression (Hunt et al., 1991a). In this study we have shown that Hoxa-2 does not share an anterior limit with its parologue Hoxb-2 (Wilkinson, 1989b), but instead ceases expression at one rhombomere boundary more rostrally. Similarly, Geada et al. (1992) have demonstrated that the Hoxc-4 gene has an anterior expression limit one rhombomere more caudal than its paralogues Hoxb-4
and d-4. The group I genes also show distinct expression domains: Hoxd-3 shows no expression at all in the neural tube at the stages analysed, whilst a-1 and b-1 expression differ significantly at later stages when expression of both genes recedes caudally but only Hoxb-1 retains a high level expression domain in r4 (Hunt et al., 1991a; Sundin and Eichele, 1990). To add yet a further level of complexity, there are also differing rhombomere-specific domains of elevated and reduced expression of paralogous genes within their overall expression domains (Krumlauf, 1993). The existence of a simple ‘Hox code’ for the head (Hunt et al., 1991a,b) was originally proposed based on two main assumptions - that paralogous genes are expressed with equivalent anterior limits, and that Hox gene expression patterns are passively carried into the branchial arches by the neural crest, to be further involved in specification of craniofacial structures. The results of this and other recent studies continue to support the idea that some or all spatial information is present in the premigratory crest, and that one role of hindbrain segmentation is to help set up such a prepattern. However, it is becoming increasingly clear that the ‘Hox code’ is more complex than originally suggested. In particular the results of this study argue against a passive transfer of information from neural tube to neural crest. A summary of Hox gene expression patterns in the hindbrain and branchial arch region, taking into account recent results, is shown in Fig. 7. The expression of murine Hoxa-2 has recently been reported to be confined to a ventral subset of r2 cells (Krumlauf, 1993), in contrast we find that chick Hoxa-2 expression occurs at all doroventral levels of r2, with a somewhat higher expression level dorsally. This difference between Hoxa-2 expression patterns in rodent and chick hindbrain may reflect differences in the timing and organisation of hindbrain and cranial neural crest development in the two species (Tosney, 1982; Tan and Morriss-Kay, 1985). However, conservation of the basic ‘code’, with Hoxa-2 expression present in r2, yet absent in r2-derived neural crest in both species, lends weight to the idea that this gene plays an important evolutionarily conserved role in patterning of this region.

Our principal and unexpected observation, that Hoxa-2 is not expressed in the r2-derived neural crest, despite being expressed in the premigratory crest cell population of r2, led us to investigate whether the rapid down-regulation of Hoxa-2 transcription is intrinsic to these cells or mediated by extrinsic factors. The results of our isochronic rhombomere grafting experiments strongly support our first hypothesis, that the down-regulation of Hoxa-2 transcription in neural crest from the r2 level, and the maintenance of Hoxa-2 expression in neural crest from the r4 level, is intrinsic to the neuroepithelium. The neural crest is thus pre-programmed to cease or maintain Hoxa-2 expression, in r2 and r4 respectively, before migration from the neural tube. The rapid down-regulation of transcription in r2 crest could be mediated either by loss of a positively acting transcription factor or, alternatively, by gain of a negatively acting factor at the time of commitment of r2-derived neural crest cells. The results of our isochronic rhombomere grafting experiments both confirm and extend those of Guthrie et al. (1992) and Kuratani and Eichele (1993). In both these studies expression of Hoxb-1 was analysed following grafting of r4 to the site of r2 (Guthrie et al., 1992) or other rhombomeres (Kuratani and Eichele, 1993). Hoxb-1 expression revealed that the molecular identity of r4 is set up prior to boundary formation and maintained following transposition of r4 to an ectopic site. However, as Hoxb-1 expression is down-regulated in the neural crest soon after the onset of crest migration, analysis of the expression of this particular gene following rhombomere grafting, was uninformative regarding molecular identity of the neural crest.

Pre-programming of Hoxa-2 expression status in the premigratory neural crest, as revealed by our rhombomere grafting experiments, is consistent with the conclusions of earlier studies showing that presumptive first arch neural crest is prepatterned with respect to branchial arch patterning and skeletogenesis (Noden, 1983). The neural crest plays a vital role in patterning the craniofacial region, demonstrating a unique instructive capacity over the mesodermal contribution (Noden, 1986). However, not all non-crest cells are naive or require instruction from the crest, in particular the neural placodes show autonomy with regard to their patterning (Noden, 1988). We found no changes to the peripheral nervous system following rhombomere grafting experiments, which do not change the positions of the placodes. Similar results were noted by Kuratani and Eichele (1993), who found that only on grafting of an even-numbered rhombomere into the place of an odd-numbered rhombomere, thus producing neural crest in a normally crest-free area, was the peripheral nervous system affected. These results suggest that the placodes may play a dominant role in organisation of the peripheral nervous system. What role the Hoxa-2 gene product plays in patterning the vertebrate head remains to be determined. Transgenic mice

Fig. 7. Summary of Hox gene expression in the hindbrain. Data are compiled from the following sources: Gaunt et al. (1989); Geada et al. (1992); Hunt et al. (1991a,b); Krumlauf (1993); Murphy and Hill (1991); Wilkinson et al. (1989b) and this study. Filled area denotes region of up-regulated Hoxb-1 expression. Dense hatching denotes areas of high level expression and sparse hatching denotes areas of lower level expression. Dotted lines show transient expression of group I genes.


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