Development 122, 473-480 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV9429

Interactions between rhombomeres modulate *Krox-20* and *follistatin* expression in the chick embryo hindbrain

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SUMMARY

The rhombomeres of the embryonic hindbrain display compartment properties, including cell lineage restriction, genetic definition and modular anatomical phenotype. Consistent with the idea that rhombomeres are autonomous developmental units, previous studies have shown that certain aspects of rhombomere phenotype are determined early, at the time of rhombomere formation. By contrast, the apoptotic depletion of neural crest from rhombomeres 3 and 5 is due to an interaction with their neighbouring rhombomeres, involving the signalling molecule Bmp4. In this paper, we have examined whether inter-rhombomere interactions control further aspects of rhombomere phenotype. We find that the expression of *Krox-20* and the repression of *follistatin* in r3 is dependent upon neighbour interaction, whereas these genes are expressed autonomously in r5. We further demonstrate that modulation of *Krox-20* and *follistatin* expression is not dependent on Bmp4, indicating the existence of multiple pathways of interaction between adjacent rhombomeres. We also show that, although some phenotypic aspects of r3 are controlled by neighbour interactions, the axial identity of the segment is intrinsically determined.

Key words: *Krox-20*, *follistatin*, rhombomere, chick, hindbrain

INTRODUCTION

The embryonic hindbrain is subdivided along the anteroposterior axis into eight metameric units, rhombomeres, that display characteristic properties of developmental compartments (Lumsden, 1990). Most significantly, cell mixing between adjacent rhombomeres is transiently restricted (Fraser et al., 1990; Birgbauer and Fraser, 1994), with the consequence that polyclonal cohorts of cells are segregated into discrete blocks during the early stages of cell-type specification, when the rhombomeres each acquire a unique molecular and anatomical identity (Lumsden and Keynes, 1989; McGinnis and Krumlauf, 1992; Prince and Lumsden, 1994). Rhombomere (r) 4, for example, is characterised by a population of contralaterally migrating vestibulo-acoustic (CVA) neurons and high level expression of *Hoxb-1* (Simon et al., 1995; Simon and Lumsden, 1993; Wilkinson et al., 1989; Sundin and Eichele, 1990). Transplantation experiments have demonstrated that the unique identity of a rhombomere may be determined from the stage at which it becomes defined by boundaries; thus, when a newly forming r4 is transplanted to either the r2 or the r6 position, it goes on to generate its characteristic CVA neurons (Simon et al., 1995) and becomes marked by high level *Hoxb-1* expression (Guthrie et al., 1992; Kuratani and Eichele, 1993; Simon et al., 1995). The picture that emerges from these studies is therefore one of developmental autonomy.

Our studies on the control of neural crest production in the hindbrain, however, have yielded a contrasting view; they suggest that rhombomeres do not develop in complete independence of each other, but can and do regulate certain aspects of the developmental programme of their neighbours (Graham et al., 1993). Fate-mapping studies have demonstrated that, while rhombomeres 1/2, 4 and 6 are foci of neural crest production, the intervening rhombomeres, r3 and r5, are extensively depleted of crest (Lumsden et al., 1991; Sechrist et al., 1993). The loss of neural crest precursors in r3 and r5 involves the induction of *Bmp-4* and *msx-2* expression in these cells and their subsequent apoptotic elimination (Graham et al., 1994). The absence of neural crest cells at these levels of the anteroposterior axis interrupts what is otherwise a continuous axial column of crest production from the dorsal neural tube. We have argued that this patterned elimination of crest may have particular morphogenetic significance in that it sculpts the remaining neural crest outflow from the hindbrain into discrete streams, each destined to enter a particular branchial arch and contribute to the corresponding cranial sensory ganglia (Lumsden et al., 1991). We have shown, furthermore, that programmed neural crest cell death is not autonomous to r3 and r5; rather, it is under the interactive control of the neighbouring even-numbered rhombomeres (Graham et al., 1993, 1994). When r3 and r5 are experimentally freed from the influence of their neighbours, either by heterotopic grafting or by isolating explants in culture, the expression of both *Bmp-4* and *msx-2* is down-regulated and the cell-death programme is commuted (Graham et al., 1994). If recombinant Bmp-4 protein is added to the explant cultures then both of these genes are re-expressed and the cell-death programme is reinstated. These observations suggest that a pivotal event in the control of crest elimination is the induction, by unknown factors emanating from the even-numbered rhombomeres, of *Bmp-4* expression in the odd-numbered rhombomeres (Graham et al., 1994).

Here we ask whether inter-rhombomere interaction is limited to the control of crest production or if it has other consequences. Towards that goal we have analysed a number of genes that are markers of rhombomere phenotype. Specifically, we analysed what effect rhombomere transposition has on the expression of *Krox-20*, *follistatin* and the *Hox* genes. *Krox-20* is expressed in r3 and r5 and has recently been shown via mutational analysis in mice to be required for the maintenance of these rhombomeres (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993, Nieto et al., 1995). *Follistatin* has been shown to be expressed in the even-numbered rhombomeres of mouse embryos (Albano et al., 1994, Feijen et al., 1994) and may, through binding activin or other TGFβs, play some role in regulating neurogenesis. We have isolated chick *follistatin* and find that in the chick embryo, as in the mouse, *follistatin* is expressed in even-numbered rhombomeres and not in r3. Unlike in the mouse, however, *follistatin* is expressed in the chick r5. The other genes that we employed in our analysis were *Hoxa-2* and *Hoxb-1*, which serve as markers of the axial identity of rhombomeres (Wilkinson et al., 1989; Sundin and Eichele, 1990; Prince and Lumsden, 1994).

The results of our experiments show that inter-rhombomere interactions do influence aspects of hindbrain development other than neural crest production. We found that the r3-specific expression of both *Krox-20* and *follistatin* can be modulated by neighbour interactions. Furthermore, we show that the controls of neural crest cell apoptosis and of *Krox-20*/*follistatin* expression in r3 are mediated by separate pathways of interaction with the even-numbered rhombomeres. We also further demonstrate that, although some aspects of r3 phenotype are affected by neighbour interactions, the axial identity of this segment is intrinsically programmed.

MATERIALS AND METHODS

Cloning of chick *follistatin*

Fully degenerate oligonucleotide primers were designed against conserved amino acid sequences in the mammalian follistatins (Shimasaki et al., 1988; Albano et al., 1994). The 5′ primer - GGAATTCCARGCNGGNAAYTGYTGG - and the 3′ primer - GCTCTAGARCANGCNGCYTCYTTCAT - were derived from the amino acid sequences QAGNCW and MKEAAC, respectively. To aid subcloning, an *Eco*RI site was added to the 5′ primer and an *Xba*I site to the 3′ primer. RNA was prepared from a stage-12 chick hindbrain and oligo(dT)-primed cDNA was synthesised. This cDNA was used as the substrate in the PCR with the above primers. *Taq* polymerase was used in the manufacturers' buffers under the following conditions: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 2 minutes, 72°C for 2 minutes and a final step at 72°C for 10 minutes. The end result of this reaction was the amplification of an 800 bp fragment, which was then subcloned into Bluescript pSK. Sequencing demonstrated that this was a fragment of chick *follistatin.* To obtain a full-length clone, 2×10^6 pfu of a chick embryo cDNA library in λZAP (kindly provided by David Wilkinson) were plated and screened at high stringency. This resulted in the isolation of a full-length *follistatin* cDNA clone, which was mapped and sequenced. All sequencing was carried out using the Sequenase system (Amersham International, UK).

In situ hybridisation

Digoxygenin-labelled riboprobes (Boehringer, UK) were used to detect gene transcripts. The probes used in this study were as follows: the *Krox-20* probe was a 600 bp *Pst*I-*Apa*I fragment from the mouse gene (kindly provided by David Wilkinson), the *follistatin* probe was the original PCR fragment, the *Hoxa-2* probe was a 700 bp partial cDNA (Prince and Lumsden, 1994) and the *Hoxb-1* probe was a partial cDNA starting at approximately +500 and containing the entire 3′ untranslated region (kindly provided by Vicky Prince). For *Krox-20* in situ hybridizations, the protocol used was that of Wilkinson (1992). For *follistatin* and *Hoxb-1*, the protocol used was that of Domingos Henrique and David Ish-Horowicz (Henrique et al., 1995). This protocol is as follows: embryos were fixed in 4% paraformaldehyde and then washed twice in PBT (PBS with 0.1% Tween-20). Embryos were dehydrated by washing in 50% methanol/PBT and then twice in 100% methanol, rehydrated by passing them through a methanol/PBT series and then washed twice in PBT. The embryos were treated with proteinase K at 10 μg/ml in PBT for 10 minutes. They were then rinsed in PBT and fixed for 20 minutes in 4% paraformaldehyde containing 0.1% glutaraldehyde. The embryos were next washed in PBT and this was followed by a quick wash in hybridisation buffer/PBT (1:1). The embryos were preincubated in hybridisation buffer for 1 hour at 70°C and then the hybridisation buffer was changed and 1 μg of DIG-labelled riboprobe was added. The hybridisation buffer consisted of 50% formamide, 1.3× SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween-20, 0.5% CHAPS and 100 μg/ml heparin. After hybridisation the embryos were washed twice at 70°C in hybridisation buffer followed by one wash at the same temperature in MABT/hybridisation buffer (1:1). MABT is 100 mM maleic acid, 150 mM NaCl, 1% Tween-20, final pH 7.5. The embryos were then washed in MABT, followed by washing in MABT containing 2% Boehringer blocking reagent (Boehringer, UK) and 20% goat serum. The embryos were incubated overnight in the same solution containing alkaline phosphatase-coupled anti-DIG antibody at a 1:2000 dilution (Boehringer, UK). After incubation with the antibody, the embryos were washed in MABT over the next day. At the end of the day, the embryos were washed in NTMT (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1% Tween-20) twice for 10 minutes. The colour reaction was then developed in NTMT containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. For sectioning, embryos were embedded in gelatin-albumin, fixed with glutaraldehyde and cut at 50 μm on a vibratome.

Rhombomere transplantation

Rhombomeres were isolated at stage 9-10, as previously described (Graham et al., 1993). The hindbrain was dissected out and then treated with dispase (1 mg/ml in L15) containing DNase 1 for 15-20 minutes. In all of the experiments performed here the rhombomeres were isolated just after their formation. The rhombomeres were dissected free from adherent tissue using needles flame-sharpened from 100 μm diameter tungsten wire. Isolated rhombomeres were accumulated in L15 containing 5% FCS. For transplantation, eggs were windowed and the vitelline membrane was reflected. A gap in the neural tube corresponding to the desired site of insertion of the graft was created with the tungsten needles. The graft was moved into place and the egg resealed. The embryos were incubated for a further 6 hours before being harvested for in situ hybridisation.

Explant cultures of rhombomeres

Rhombomeres were isolated at stage 9-10, as described above. They were then cultured in F-12 medium in 4-well tissue culture dishes at 37°C/5% CO_2. In some cases, recombinant Bmp4 was added to the medium at a concentration of 10 ng/ml (Graham et al., 1994). After culturing for either 6 hours or overnight, the rhombomeres were transferred into 4% paraformaldehyde. To enable easy processing of these small pieces of tissue for in situ hybridisation, the embryos were immobilised in 8% gelatin (Graham et al., 1994). Specimens were then refixed in 4% paraformaldehyde and processed for in situ hybridizations as described above.

Neural crest cultures

Rhombomeres were isolated as above and then immediately incubated for 2 hours in F-12 media in a 4-well dish to ensure the down-regulation of *Krox-20* and the induction of *follistatin.* After this period, the embryos were then explanted onto a fibronectin-coated dish and

cultured in F-12 medium, either with or without Bmp4 at 10 ng/ml. After overnight incubation at 37°C/5% CO_2, the cultures were fixed by adding one tenth volume of 37% formaldehyde to each well. They were then processed for HNK-1 staining as previously described (Graham et al., 1994) and detected with a fluorescein-coupled anti-mouse Ig and viewed by confocal laser scanning microscopy (Bio Rad MRC 600).

RESULTS

Isolation of chick follistatin

The predicted amino acid sequences of the mammalian follistatins were used to design degenerate primers for cloning chick *follistatin* through RT-PCR (Shimasaki et al., 1988; Albano et al., 1994). The PCR was carried out on cDNA from a stage-12 chick hindbrain. This procedure resulted in the cloning of an 800 bp fragment of chick *follistatin* and this fragment was then used to screen a chick embryo cDNA library to obtain full-length clones. The full-length coding sequence and the corresponding amino acid sequence are shown in Fig. 1.

The open reading frame of the chick cDNA that we have isolated encodes a 343-amino acid peptide, which shares many of the features that are found in follistatin clones from other species (Fig. 1) (Albano et al., 1994; Hemmati-Brivanlou et al., 1994). In all cases, there is a long signal sequence and, within the body of the protein, there are three copies of a module that has been termed the follistatin repeat (Fig. 1A) (Patthy and Nikolics, 1993). The follistatin repeat has been found in a number of other molecules, including agrin and sparc, and it has been suggested that it could act to facilitate the binding of growth factors. Two forms of follistatin have been identified in a number of species and these differ from each other in their possession of an acidic run in the carboxy terminus. The clone that we have isolated is the larger form, with the C-terminal acid-rich region. Fig. 1B also shows that the follistatins are remarkably well conserved, with the majority of the variation being found in the signal peptide. All of the cysteine residues, except the most C-terminal one in *Xenopus*, are conserved, as are the potential sites of N-linked glycosylation.

Expression of follistatin during the period of hindbrain segmentation

The reported expression of *follistatin* in the even-numbered rhombomeres of the mouse embryo (Albano et al., 1994; Feijen et al., 1994) led to our interest in analysing *follistatin* during hindbrain development in the chick. The expression of *follistatin* in the chick hindbrain begins to be evident at stage 9 (Hamburger and Hamilton, 1951) and, by stage 10, is at high levels in r2, r4, r5 and r6. The same pattern is observed at stage 12 (Fig. 2). This expression pattern differs from that reported for mouse, where *follistatin* was found to be expressed exclusively in even-numbered rhombomeres during comparable stages of development. In chick, we observed that *follistatin* also exhibits dorsoventral restrictions in its expression pattern within the hindbrain; expression is confined to the lateral regions of the neural tube and both the floor plate and the dorsum are markedly free of expression (Fig. 2D). The expression pattern described here for *follistatin* in the early segmenting hindbrain is still

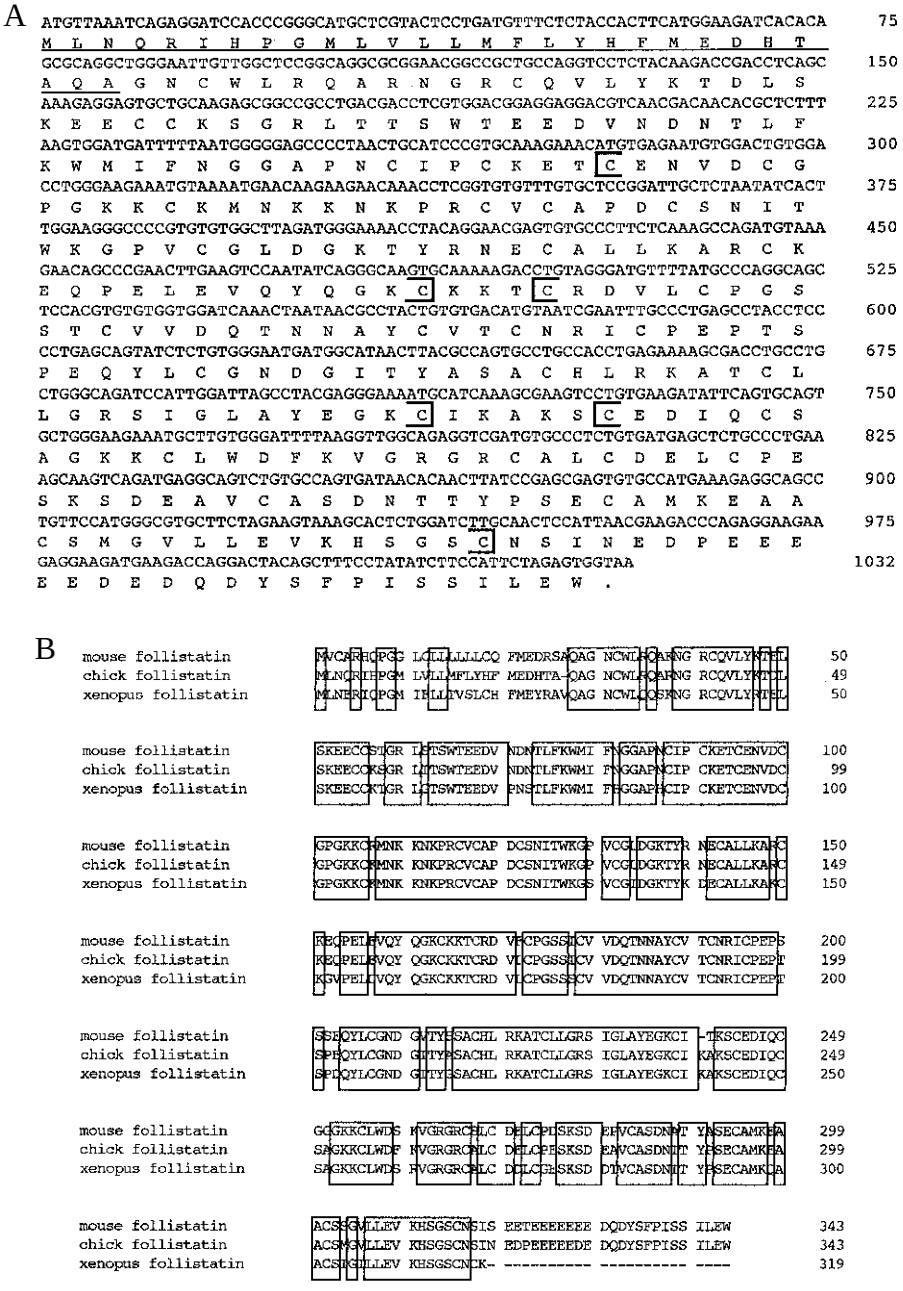
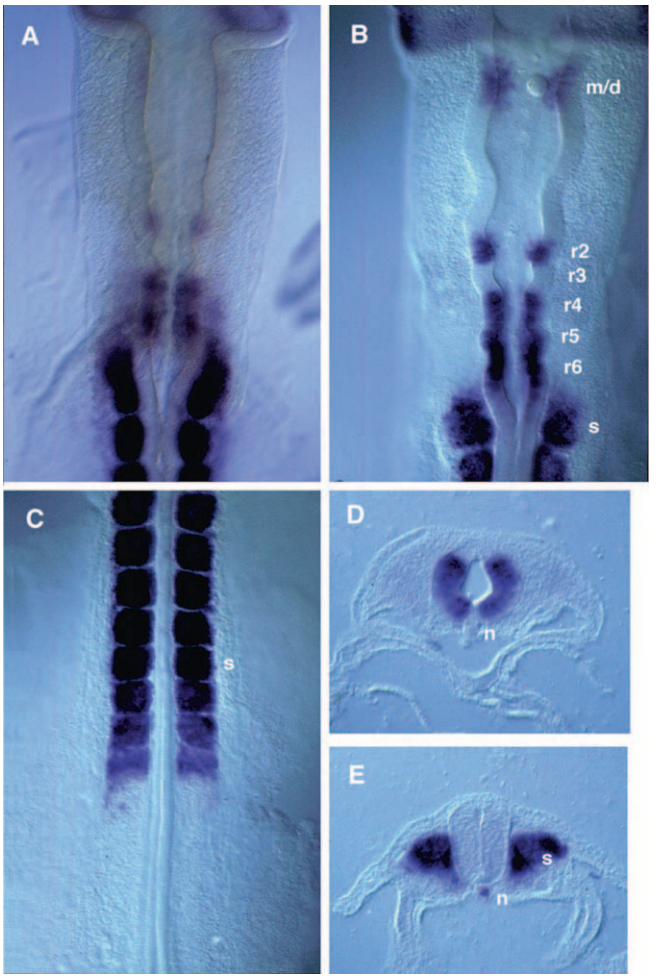


Fig. 1. (A) Complete coding sequence of chick *follistatin*. The probable signal sequence is underlined and each of the three follistatin repeats are bracketed. (B) Comparison of the predicted protein sequences of mouse, chick and *Xenopus* follistatin. This comparison demonstrates that, within vertebrates, follistatin is highly conserved. Sequence submitted to EMBL; accession number X87609.



evident up to stage 15 but, at later stages, it alters and *follistatin* is expressed in all rhombomeres, particularly in rhombomere boundaries and in stripes running along the rostrocaudal axis of the hindbrain (A. G. and A. L., unpublished).

During the early period of rhombencephalic segmentation *follistatin* is also expressed at other sites in the embryo. Within the nervous system, expression was noted at the mesencephalic-diencephalic junction and in the forming optic territories (Fig. 2B), both of which were described as sites of *follistatin* expression in the mouse, although not at a comparably early stage (Feijen et al., 1994). In contrast, in the chick as in the mouse, *follistatin* is strongly expressed in the somites but not in the segmental plate (Fig. 2C). This gene has been described as being expressed in the notochord of *Xenopus* embryos (Hemmati-Brivanlou et al., 1994), and we also find expression in the chick notochord (Fig. 2E). Interestingly, the notochordal expression in chick does not extend the full length of the axis, suggesting that the notochord is regionalised (Fig. 2D,E).

Alteration of *Krox-20* and *follistatin* expression after tissue transplantation

Our previous work has demonstrated that neural crest depletion in r3 and r5 is controlled by an interaction with their neighbouring rhombomeres (Graham et al., 1993, 1994). We wished to see whether any other aspects of the phenotypes of these rhombomeres could be affected by inter-rhombomere interactions. Here we have employed similar manipulations to analyse what effect is produced upon the expression of two markers,

Fig. 2. Expression of *follistatin* in the hindbrain region at (A) stage 10 and (B) at stage 12. (C) *follistatin* expression in newly formed somites at stage 12. Expression of *follistatin* seen in transverse section through (D) r4 and (E) r8. r, rhombomere; s, somite; n, notochord; m/d, mesencephalic/diencephalic junction.

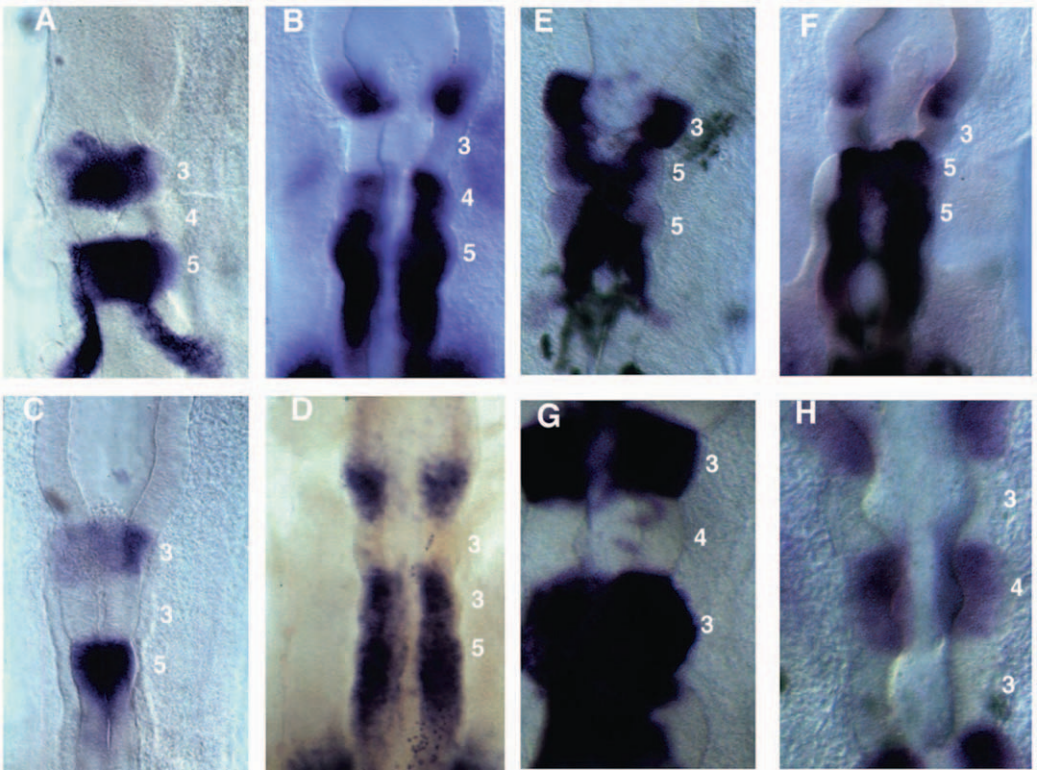


Fig. 3 Effect of rhombomere transposition upon the expression of (A,C,E,G) *Krox-20* and (B,D,F,H) *follistatin*. (A,B) Control manipulation of placing r3 into r3; (C,D) r3 into the place of r4; (E,F) r5 into the position of r4; (G,H) r3 into the r5 position.

Krox-20 and *follistatin*. The embryos were harvested 6 hours after surgery and then fixed for whole-mount in situ hybridisation. The embryos were incubated for 6 hours and not overnight because the rhombomere-restricted expression pattern of *follistatin* is transient and does not persist beyond stage 14.

Krox-20 is normally expressed at high levels in both r3 and r5 (Fig. 3, Nieto et al., 1995). To test whether or not *Krox-20* expression is dependent upon neighbour interaction, we transplanted newly formed r3 or r5 into the position of r4 at stage 9-10. We found that, when r3 is grafted into the position of r4, *Krox-20* expression is down-regulated in all cases (Fig. 3C; Table 1), suggesting that tissue interactions are important in maintaining *Krox-20* expression. Yet, by contrast, when r5 is grafted into the place of r4 then the expression of *Krox-20* is unaffected and high levels of transcripts are still evident (Fig. 3E; Table 1). We also noted that the expression of *Krox-20* in the host r3, flanking the transplanted r3, was also down-regulated, although to a variable extent (data not shown). As a control for the effect of surgical manipulation in these experiments, we grafted a donor r3 into the r3 position of a host embryo. In all cases analysed, this procedure did not alter the normal expression of *Krox-20* (Fig. 3A; Table 1).

During early hindbrain development *follistatin* is strongly expressed in r2, r4, r5 and r6 but not in r3 (Fig. 2). Again we tested the role of tissue interactions in the control of *follistatin* expression by moving newly formed r3 or r5 into the position

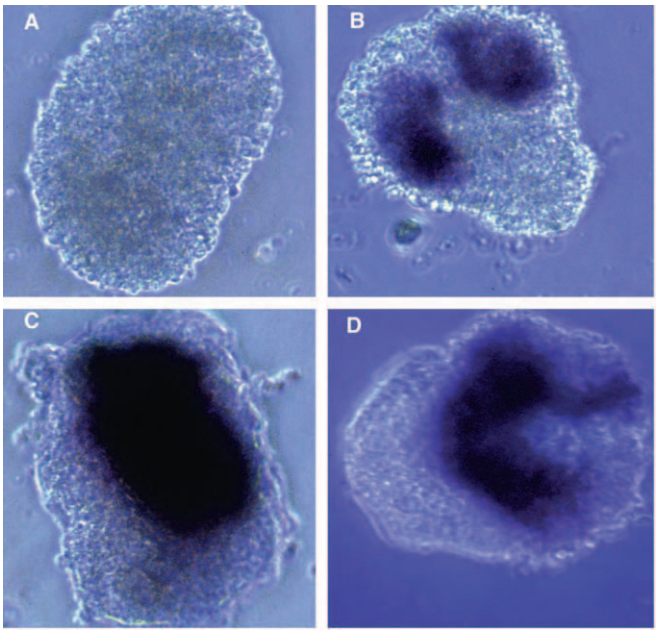


Fig. 4. Explant cultures of isolated rhombomeres stained either for (A,C) *Krox-20* or (B,D) *follistatin*. (A,B). Isolated r3; (C,D) r5.

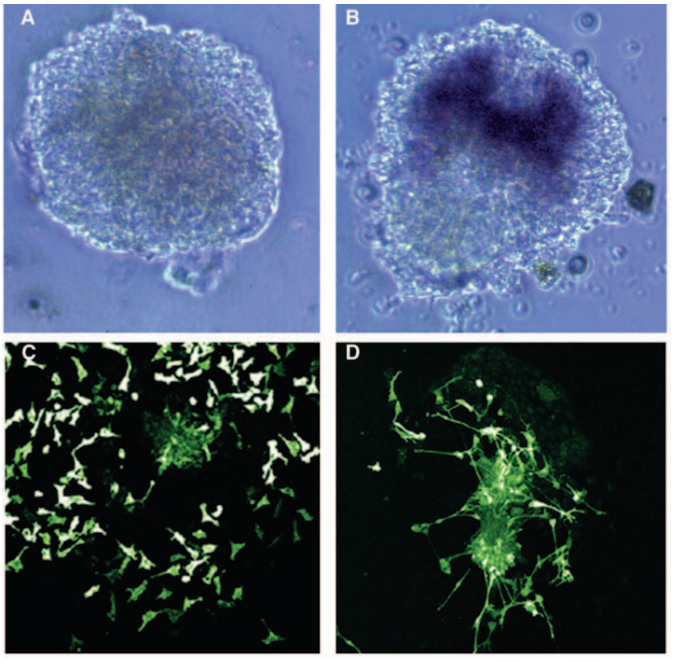


Fig. 5. *Krox-20* expression is not maintained nor is *follistatin* suppressed if isolated explants of r3 are treated with (A,B) 10 ng/ml recombinant Bmp4. Neural crest explants cultures of r3 after prior incubation for 2 hours in F-12 either in (C) control media or (D) in the same with 10/ng/ml Bmp4.

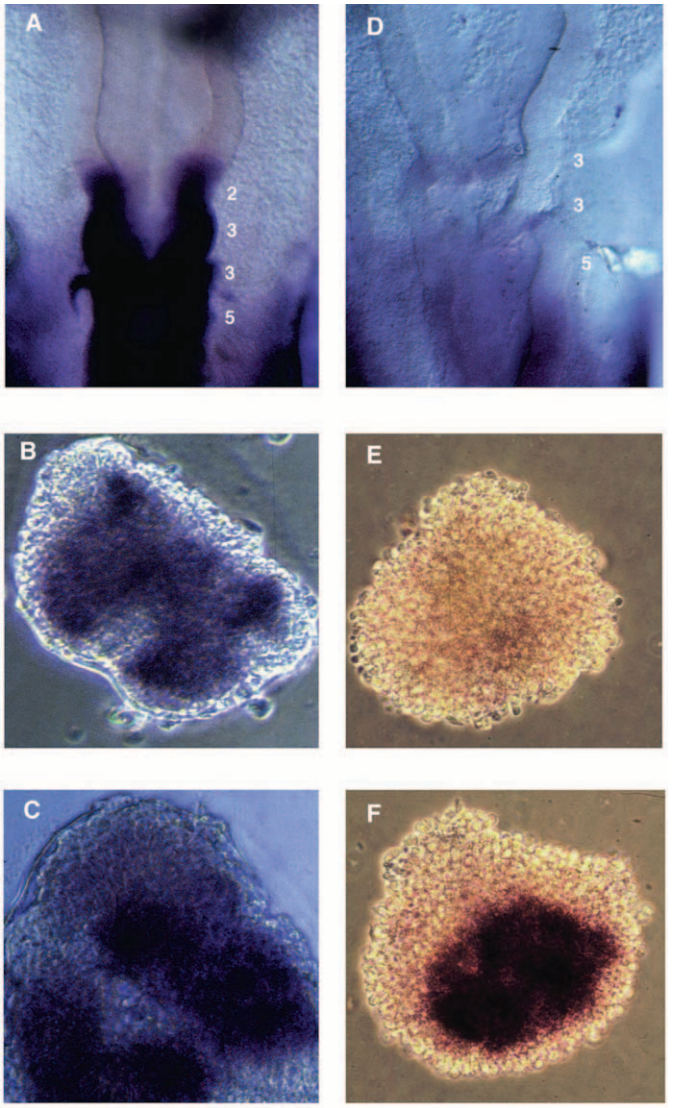


Fig. 6. *Hox* gene expression in transposed r3 (A,D) and in isolated explants of r3 (B,E) and r4 (C,F). Probed for (A-C) *Hoxa-2*; (D-F) *Hoxb-1*.

Table 1. Number of grafted embryos of each class

	Tissue transplantations (*n*)			
	r3-r3	r3-r4	r5-r4	r3-r5
Krox-20	7	13	7	7
follistatin	7	14	11	10
Hoxa-2	-	7	-	
Hoxb-1	-	11	-	-

of r4. As with *Krox-20*, *follistatin* expression is modulated when r3 is freed from the influence of its even-neighbours. In all cases analysed, the transposition of r3 into the r4 position resulted in this gene being up-regulated (Fig. 3D; Table 1). As with *Krox-20*, we noted some expression in the host r3 after this manipulation (data not shown). We have also grafted r5, which normally expresses *follistatin*, and have found that the expression of this gene is unaffected by this manipulation. Again, to control for any effects that the surgical procedure may have we have grafted r3 into the r3 position of a host embryo. In all cases *follistatin* expression was unaffected (Fig. 3B; Table 1).

These results suggest that the expression of *Krox-20* and *follistatin* in r3 is dependent upon neighbour interactions, presumably from the adjacent segments r2 and r4. The experiments also show that the r4-mesenchymal environment did not seem to affect the expression of these genes since, although they were modified when r3 was placed into the r4 position, no difference was found when r5 was moved to this site. To test this further, we transposed r3 into the r5 position. The grafted segment is still flanked by one of its normal neighbours, r4, but it is now in a new mesenchymal environment. When such manipulated embryos were probed for either *Krox-20* or *follistatin* expression, we found that in both the normal pattern of r3 expression prevailed (Fig. 3G,H; Table 1). That is, *Krox-20* expression was maintained and *follistatin* expression was not induced.

Gene expression in rhombomere explants

To further analyse the nature of the interactions that control *Krox-20* and *follistatin* expression we have also examined the effect of isolating rhombomeres in culture. The rhombomeres were dissected out of the neural tube just after their formation and cleaned of all adherent tissues before culturing. The results obtained were consistent with those from tissue transposition in vivo: *Krox-20* and *follistatin* expression in r3 was affected by isolation of this rhombomere in culture, with *Krox-20* expression being turned off while *follistatin* is turned on (Fig. 4A,B). We have also found that the alterations in the expression of these genes after r3 isolation was evident even after 1 hour (data not shown). In control explants of r3 conjoined with r4, the expression patterns of these genes were as in the embryo (data not shown). When r5 was explanted into culture we found that there was no effect upon the expression of either gene: both *Krox-20* and *follistatin* were strongly expressed in isolated r5, as in vivo (Fig. 4C,D). We have also placed r4 and r6 in culture and found no effect upon the expression of *follistatin* (data not shown).

Bmp4 does not modulate *Krox-20* or *follistatin* gene expression

It can be concluded from both the grafting and the *in vitro* experiments that, as with the control of crest apoptosis in the hindbrain, the expression of *Krox-20* and *follistatin* is modulated by neighbour interactions within the hindbrain itself. The cell-death programme of the premigratory neural crest in r3 and r5, which is abrogated when these rhombomeres are cultured in isolation, is reinstated by the addition of recombinant Bmp4 to these cultures (Graham et al., 1994). Here we have assessed whether the addition of recombinant *Bmp-4* to r3 cultures could also reinstate expression of *Krox-20* and repress *follistatin*. The addition of Bmp4, even at high concentrations (10 ng/ml; Fig. 5A,B), does not cause *Krox-20* expression to be maintained nor does it sponsor the repression of *follistatin* in r3. This result suggests that, although both the crest cell-death programme and the expression of *Krox-20* and *follistatin* in r3 are controlled by the even-numbered rhombomeres, their control is independent of each other. Yet there is the possibility that these events are linked and that the Bmp4-sponsored depletion of crest may be dependent upon the initial expression of *Krox-20* and the lack of *follistatin*. To analyse this possibility, we first cultured r3 explants for 2 hours to allow *Krox-20* to turn off and *follistatin* to be induced. The isolated r3s were then explanted onto a fibronectin matrix and cultured overnight, either in control media or in the same but with 10 ng/ml recombinant Bmp4. We found that, under these conditions, the addition of Bmp4 to the isolated explants of r3 still resulted in the depletion of crest cells (Fig. 5C,D). Thus the Bmp4-mediated response of the crest cells is not dependent upon the expression of *Krox-20* or the absence of *follistatin*. This does therefore suggest that neural crest apoptosis and the expression of *Krox-20* and *follistatin* in r3 are controlled independently by neighbour interactions.

Hox expression in transposed and cultured rhombomeres

The experiments described here and previously have demonstrated that certain phenotypic aspects of r3 are dependent upon interactions with the adjacent rhombomeres. This leaves open the question of whether or not r3 maintains its axial identity when it is transposed in the embryo or explanted into culture. To address this question we have used two *Hox* gene probes, *Hoxa-2* and *Hoxb-1*. The *Hoxa-2* gene is normally expressed from a rostral limit at the r1/2 boundary extending caudad from the early stages of hindbrain segmentation (Prince and Lumsden, 1994). *Hoxb-1* expression in the hindbrain initially extends from r4 caudally until about stage 10+, when it becomes progressively restricted to r4 (and r7 caudad) following the down-regulation of this gene in r5 and r6 (Sundin and Eichele, 1990; Guthrie et al., 1992). Thus r3 identity is marked by the expression of *Hoxa-2* and the absence of *Hoxb-1* and r4 identity by the expression of both genes.

Although the transposition of r3 into the r4 position affects crest production and *Krox-20* and *follistatin* expression, we do not find any effect upon the *Hox* genes (Fig. 6A,D; Table 1). *Hoxa-2* expression is maintained when r3 is grafted into r4 and *Hoxb-1* expression is not induced. This result is also observed in vitro. When r3 is isolated in culture it maintains *Hoxa-2* expression but does not acquire *Hoxb-1* (Fig. 6B,E). In contrast, r4 explants in culture strongly express both *Hoxa-2* and *Hoxb-1* (Fig. 6C,F). Thus, in agreement with other studies (Guthrie et al., 1992; Simon et al., 1995), the repertoire of *Hox* gene expression appropriate to each formed rhombomere is intrinsic to that segment. This is also consistent with the fact that we found that the responsiveness of r3 and r5 to Bmp4 was intrinsic to these rhombomeres and that it was not dependent upon tissue interactions (Fig. 5, Graham et al., 1994). Taken together these results support the conclusion that,

although aspects of r3 phenotype are modulated by neighbour interactions, its axial identity is intrinsically determined.

DISCUSSION

The results presented here show that inter-rhombomere interactions control more than just neural crest production. We have used *Krox-20* and *follistatin* as markers of r3 phenotype, with *Krox-20* providing a positive marker and *follistatin* a negative marker, and have shown that the expression of *Krox-20* and the repression of *follistatin* in r3 are dependent upon interactions with the neighbouring rhombomeres. Furthermore, we have shown that, although the apoptotic elimination of crest cells from r3 is also dependent upon interactions with the neighbouring segments, these two interactions are not linked and appear to depend on parallel pathways. Our results also demonstrate that, although certain aspects of r3 phenotype are modulated by segment interactions, its axial identity is intrinsically determined.

Follistatin was first isolated from follicular fluid as a factor that could bind to, and inhibit, activin; the strong sequence conservation amongst the vertebrate genes suggests that this property would also be shared by the chick protein (Fig. 1B). Whether *follistatin* plays such a role during early chick hindbrain development is not clear since the expression patterns of activins have not yet been described in this system. The results from other species, moreover, do not give a consistent picture: in mouse, there would appear to be no activin expression during hindbrain development (Albano et al., 1994; Feijen et al., 1994) while, in *Xenopus, inhibin* β_B is expressed in all the rhombomeres (Dohrmann et al., 1993). The lack of correlation between the expression of *follistatin* and *activin* in both the hindbrain and somites in the mouse embryo suggests that *follistatin* could also modulate the effects of other members of the TGF-β superfamily. This possibility has received support from the recent mutational analysis of *activins* and *follistatin* in mice, which have shown that the phenotypes associated with the *follistatin* knock-out are more extensive than those found with the *activin* knock-outs (Matzuk et al., 1995a, 1995b). It should also be noted that *follistatin* has been shown to act as a paracrine modulator of progesterone production in bovine thecal cells, suggesting that *follistatin* could act directly to modulate developmental processes (Shukovski et al., 1993).

The expression pattern of *follistatin* in the mouse, where it is expressed in the even but not the odd rhombomeres, is consistent with it either modulating the signal (or being the signal itself) that passes between neighbouring rhombomeres. The expression pattern that we have described in the chick, however, with high expression also in r5, is not consistent with this role. The pattern of expression of this gene in the developing rhombencephalon suggests that it may ensure that the effect of other TGF-β family members is restricted to those territories that do not express follistatin. Thus, the absence of *follistatin* expression in r3 may ensure that this rhombomere can respond to TGF-βs that promote its specific development. The lack of expression in the dorsal and ventral aspects of all rhombomeres may also expose these domains to the influence of such molecules emanating from flanking tissues such as the dorsal surface ectoderm and the underlying notochord. It is also of interest that *follistatin* expression is detected in more caudal regions of the notochord where it could negate the effect of any such notochordal signal in the trunk (Fig. 2).

Although alternate rhombomeres do share a number of common properties, such as *Krox-20* expression for r3 and r5, even here there are underlying differences. In the case of r3, we have seen that high level expression of *Krox-20* is dependent upon neighbour interactions while in r5, *Krox-20* expression is controlled by an intrinsic mechanism (Figs 4, 5). While we do not know the mechanism that bolsters r3 expression of *Krox-20*, an attractive candidate for such a regulatory role in r5 would be the *kreisler* gene. *kreisler*, encoding a member of the *maf* family of transcription factors, has recently been positionally cloned and found to be expressed in r5 and r6 (Cordes and Barsh, 1994). More significantly, analysis of the *kreisler* mutant phenotype has shown that this gene lies upstream of *Krox-20* (Frohman et al., 1993; McKay et al., 1994). If *kreisler* is an activator of *Krox-20* then its expression should also be unaffected by removing r5 from its neighbouring segments. We are currently examining the expression of *ck-kreisler* in rhombomere transposition experiments.

The existence of different control mechanisms in r3 and r5 is also evident from our *follistatin* results. As for *Krox-20*, neighbour interactions are important for controlling the expression of *follistatin* in r3, while in r5 the expression of this gene is controlled by an autonomous mechanism (Figs 4, 5). Given these parallels, there is the possibility that *Krox-20* could control *follistatin* expression. It clearly must act within the context of other factors, since it would have to repress *follistatin* in r3 but support it in r5. Consistent with the suggestion that *Krox-20* could regulate *follistatin*, the sequence of the porcine *follistatin* promoter (Miyanaga and Shimasaki et al., 1993) contains two potential *Krox-20* binding sites which differ from the published consensus by only one base (Chavrier et al., 1990).

The isolation of r3 from its adjacent segments relieves it from their constraining influence, enabling it to exhibit traits that are not normally associated with it. Despite this phenotypic plasticity, a number of lines of evidence suggest that when r3 is isolated from its neighbours it still possesses an intrinsic identity. It would not seem to adopt an r5 fate because r5 is marked by the expression of both *Krox-20* and *follistatin*. Our analyses with *Hoxa-2* and *Hoxb-1* demonstrate that r3 maintains its normal *Hox* gene expression patterns, i.e. positive for *Hoxa-2* but negative for *Hoxb-1*. That r3 retains its identity in these manipulations is also demonstrated by the retention of Bmp-responsiveness of this rhombomere, even after it has been cultured for 2 hours and *Krox-20* has become down- regulated and *follistatin* induced. This is in contrast to r4, which never exhibits Bmp4 responsiveness. It would also seem unlikely that by removing r3 from the influence of it neighbours it is taking on a more mature state. Although r3 does express *follistatin* at later stages, this is not until 20 hours after the stage at which the rhombomere is isolated in these experiments, and the induction of *follistatin* expression in r3 explants occurs within 1 hour. Furthermore, the isolated and/or ectopic r3s generate neural crest cells irrespective of their expression of *follistatin*; in vivo, *follistatin* expression in r3 follows some considerable time after the cessation of neural crest production. These results are consistent with previous studies that have also demonstrated that rhombomere identity is intrinsically determined (Guthrie et al., 1992; Kuratani and Eichele, 1993; Prince and Lumsden, 1994; Simon et al., 1995).

Although rhombomeres possess a number of attributes consistent with their development as autonomous units (Guthrie et al., 1992; Simon et al., 1995), the results presented here show that

rhombomeres do interact with each other to control other aspects of phenotype. Both the transposition experiments and the in vitro studies indicate that, as with the control of neural crest production (Graham et al., 1993), the regulation of *Krox-20* and *follistatin* expression in r3 is achieved by an interactive mechanism within the hindbrain itself. The significance of this interactive mechanism is as yet unclear. It also appears that the interaction between rhombomeres is unidirectional, in that it is the development of r3 and r5 that is affected by the even-numbered rhombomeres, whereas we have found no evidence for a reciprocal modulation of the even-numbered rhombomeres. One other important finding from these studies is that the interaction between r3 and its even-numbered neighbours involves the activation of at least two separate pathways. One culminates in neural crest apoptosis and involves Bmp4, while the other is concerned with the regulation of *Krox-20* and *follistatin* expression and is independent of Bmp4.

It is often said that segmentation confers a degree of independence upon each segment that could subsequently result in the generation of local anatomical variation - a prerequisite for evolutionary change. Yet the hindbrain is a remarkably well-conserved structure across the vertebrate groups, both in terms of the molecular basis of its development and of its neuroanatomical architecture. This may reflect the fact that rhombomeres are not fully autonomous developmental units, in that interactions between the rhombomeres are necessary to ensure normal development.

We would like to thank David Wilkinson for his gift of a chick cDNA library and the *Krox-20* probe and Vicky Prince for the *Hoxb-1* probe. Thanks also to Ian McKay and Gord Fishell for reading an early draft of the manuscript. This work was supported by the Medical Research Council and the Howard Hughes Medical Institute. AL is an International Research Scholar of the HHMI.

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(Accepted 15 November 1995)