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Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain

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SUMMARY

Neural crest cells originate at three discontinuous levels along the rostrocaudal axis of the chick rhombencephalon, centred on rhombomeres 1 and 2, 4 and 6, respectively. These are separated by the odd-numbered rhombomeres r3 and r5 which are depleted of migratory neural crest cells. Here we show elevated levels of apoptosis in the dorsal midline of r3 and r5, immediately following the formation of these rhombomeres at the developmental stage (10-12) when neural crest cells would be expected to emerge at these neuraxial levels. These regions are also marked by their expression of members of the *msx* family of homeobox genes with *msx-2* expression preceding apoptosis in a precisely co-localised pattern. In vitro and in ovo experiments have revealed that r3 and r5 are depleted of neural crest cells by an interaction within the neural epithelium: if isolated or distanced from their normal juxtaposition with even-numbered rhombomeres, both r3 and r5 produce migrating neural crest cells. When r3 or r5 are unconstrained in this way, allowing production of crest, *msx-2* expression is concomitantly down regulated. This suggests a correlation between *msx-2* and the programming of apoptosis in this system. The hindbrain neural crest is thus produced in discrete streams by mechanisms intrinsic to the neural epithelium. The crest cells that enter the underlying branchial region are organised into streams before they encounter the mesodermal environment lateral to the neural tube. This contrasts sharply with the situation in the trunk where neural crest production is uninterrupted along the neuraxis and the segmental accumulation of neurogenic crest cells is subsequently founded on an alternation of permissive and non-permissive qualities of the local mesodermal environment.

Key words: rhombomeres, neural crest, apoptosis, *msx* genes, chick nervous system

INTRODUCTION

The principal morphological differences between vertebrates and other chordates reside in the head and central to both development and evolution of the head is the deployment of neural crest cells and ectodermal placodes (Gans and Northcutt, 1983). There are also important differences between the head and the trunk with regards to the neural crest; whereas both regions produce sensory and autonomic ganglia, the cranial crest gives rise to a broader range of tissues that includes ectomesenchymal derivatives, such as connective tissue, cartilage and bone (Le Lievre and Le Douarin, 1975; Le Lievre, 1978; Noden, 1978, 1983). Cranial mesoderm, in contrast to its trunk counterpart, is not clearly segregated into paraxial and lateral regions and in the bird is largely myogenic in its developmental potential (Couly et al., 1992). The ectomesenchyme-derived cephalic dermis is the source of most of the membraneous bones of the skull (Couly et al., 1992, 1993). Thus many of the tissues produced by mesoderm in the trunk are derived from the neural crest in the head.

Possibly the most striking difference between cranial and trunk neural crest is the capacity of the cranial crest to control pattern in this region (Noden, 1988). When presumptive first arch neural crest is placed in the position of presumptive second arch neural crest, the graft-derived ectomesenchyme migrates into the second arch but subsequently develops first arch-specific structures (Noden, 1983). Moreover, when the cranial mesoderm is replaced by trunk paraxial mesoderm, it is directed by the neural crest to form muscles that are appropriate for its new location (Noden, 1986). Thus in the head region the neural crest may be morphogenetically specified before migration from the neural primordium and the emerging cells transpose their positional information to adjacent tissues with which they interact.

Much of the cranial neural crest derives from the rhombencephalon, a brain region that becomes overtly segmented shortly after neural tube closure. We have previously produced a fate map of rhombencephalic crest in the chick embryo using focal injections of the lipophilic dye DiI to map the sites of origin of the crest and to follow its migration, rhombomere by rhombomere (Lumsden et al., 1991). This study revealed a relationship between the sites

of emergence of neural crest cells and the segmented neural epithelium, such that neural crest from both r1 and r2 contributed to the trigeminal ganglion and the first branchial arch, that from r4 to the facial and vestibulo-acoustic ganglia and the second arch and that from r6 to the superior ganglion of the IXth nerve and the third branchial arch. Separating these three areas of crest production were two axial levels, r3 and r5, which did not contribute to the emergent neural crest once these rhombomeres become delineated by their boundaries. Thus the crest originates from three discontinuous levels and migrates ventrally in three distinct streams. This results in the specific filling of each of the corresponding underlying branchial arches and in the organisation of the cranial ganglia and the entry/exit points for the peripheral nerves at appropriate rostrocaudal levels of the rhombencephalon.

The discontinuous emigration of crest along the neuraxis may be achieved by mechanisms intrinsic to the neurectoderm. One possibility is that the specification of rhombomeres 3 and 5 includes the non-production of neural crest, another that cells are generated, perhaps continuously along the anteroposterior axis, but are selectively eliminated at these rhombomere levels by cell death. We have previously used the vital dye nile blue sulphate to visualise the contribution of programmed cell death in the embryos (Lumsden et al., 1991). The results demonstrated that the dorsal median aspects of r3 and r5 were associated with elevated levels of cell death, thus raising the possibility that neural crest cell production is continuous along the neuraxis but that most or all crest cells are eliminated from r3 and r5 before emigration. Our finding was subsequently confirmed by others, also using nile blue sulphate staining (Jeffs et al., 1992).

Interest in morphological and cellular aspects of hindbrain development has been complemented by burgeoning analyses of its molecular basis. Initially, a number of developmentally interesting genes were found to have expression patterns that respected rhombomere boundaries. The zinc-finger gene *Krox-20* was found to be expressed first in r3 and then also in r5 as these rhombomeres were being formed (Wilkinson et al., 1989), an expression pattern consistent with this gene being involved in the segmentation process. By contrast, the homologies and expression patterns of members of the Hox gene family suggest that they may be involved in the specification of rhombomere identity (Hunt et al., 1991). More recently, targeted mutation of two members of the *Hoxa (Hox 1)* cluster that are expressed in the rhombencephalon have resulted in phenotypes that support a role for these genes in rostrocaudal patterning of the hindbrain (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992).

While *Krox-20* and members of the Hox gene family would seem to be important in the patterning of the rhombencephalic region, the expression patterns and, in the case of the Hox genes, the mutational analysis would not obviously suggest an organising role for crest-free and crest-productive regions. We propose that better candidates for such a role might be the *msx* family of homeobox genes. This includes the *Drosophila msh* gene and the vertebrate *msx-1 (Hox-7)* and *msx-2 (Hox-8)* genes amongst others (Hill et al., 1989; Robert et al., 1989; Coelho et al., 1991; Monaghan et al., 1991; Robert et al., 1991; Suzuki et al., 1991; Yokouchi et al., 1991). The *msx* genes are expressed in the branchial arches (Robert et al., 1989; Takahashi et al., 1990) and the gene family is thought to have expanded in number at the origin of vertebrates and the first appearance of the neural crest (Holland, 1991).

The aim of the present work was to investigate further the discontinuous production of crest cells in the avian hindbrain at both the cellular and molecular level; on the one hand, we sought to understand how crest free areas become established while, on the other, we set out to analyse what role the members of the *msx* gene family might play in the process and how the expression of these genes may relate to the cellular events.

MATERIALS AND METHODS

Visualisation of programmed cell death

Rhode Island Red hens eggs from a local flock (Needle Farm, Enfield) were incubated at 37°C and 50% RH to stages 9-12. Embryos were removed from eggs and the whole rhombencephalic region was dissected out. These tissues were incubated for 30 minutes at 37°C in Howards Ringer containing 5 μg/ml acridine orange. The specimens were then washed two times 1 minute in Ringer. Fluorescence was observed immediately using a rhodamine filter set (Zeiss set 15) producing a signal specific for apoptotic cell death.

Electron microscopy

Embryos were dissected rapidly and fixed for 4 hours at 4°C in 2.5% glutaraldeyde in 0.1 M phosphate buffer (pH 7.3). Tissues were washed overnight in phosphate buffer, osmicated for 1 hour in 1% aqueous osmium tetroxide and dehydrated in an ascending methanol series. After embedding in Epon (TAAB), thin and semithin sections were cut. Semithin sections (1 μm) were stained with toluidine blue, and ultrathin sections with uranyl acetate and lead citrate. Ultrathin sections were viewed at 75 kV in a Hitachi H700 transmission electron microscope.

Neural crest prelabelling and explant culture

Small focal injections of a solution of DiI C_{18} (Molecular probes, D-282) at 1 mg/ml in 0.3 M sucrose were used to label the dorsal aspect of individual rhombomeres during the period of neural crest production i.e. from stage 9 to 11. For this procedure, the embryos were pinned out on a Sylgard dish and injected as described previously (Lumsden et al., 1991). The rhombencephalon was then dissected out and treated with dispase (1 mg/ml in L-15 media) for 15-20 minutes to separate the neural tissue from the surrounding mesenchymal cells. Individual rhombomeres or pairs of rhombomeres were dissected out using needles flame-sharpened from 100 μm diameter pure tungsten wire. The isolated segments were incubated on fibronectin-coated glass coverslips; these had first been treated with poly-L-lysine (4 μg/ml in water) for 30 minutes at room temperature and then air dried before being coated with fibronectin at 10 μg/ml in F-12 media (Gibco-BRL) for 3 hours. The explants were incubated on the treated coverslips in F-12/10% FBS media (Gibco-BRL) at 37°C in 8% CO_2 for 24 hours. The cultures were then washed with PBS and fixed for 1 hour at room temperature in 4% paraformaldehyde containing 2.5% DABCO. The coverslips were then mounted under 10% PBS/90% glycerol again containing 2.5% DABCO and viewed by laser scanning confocal microscopy (BioRad MRC 500).

Immunohistochemistry

Explants of individual segments that had been dissected out and incubated as above were stained with the Leu-7 antibody (Becton-Dickinson), which is directed against the HNK-1 epitope, at a

1:100 dilution in PBS/5% NGS/1% triton for 1 hour at room temperature. This was then followed by a 1 hour incubation with an FITC-conjugated anti-mouse Ig antibody. The specimens were observed under blue excitation (Zeiss filter set 09).

Isolation of the *msx-2* probe

A chick stage 10 cDNA library (generously provided by Martyn Goulding) was screened at low stringency (5× SSPE, 5× Denhardts, 100 μg/ml salmon sperm DNA, 150 μg/ml yeast tRNA, 1% SDS at 56°C) with a mix of the mouse *msx* homeoboxes (Holland et al., 1991). Restriction fragments of positive clones were isolated and subcloned into the pBluescript plasmid (Stratagene). The DNA sequence of the subclone was determined via the dideoxy chain termination method using Sequenase (United States Biochemical) according to manufacturers instructions.

Probes used for in situ hybridisation

The *msx-2* probe extends 350 bp 5′ of the *Eco*RI site in the homeobox (Yokouchi et al., 1991). The *msx-1* probe was a kind gift of Dr Benoit Robert. The region used for the in situ hybridisation studies extended from the *Bgl*II site in the homeobox 594 bp to the 3′ end of the cDNA (Robert et al., 1991).

Whole-mount in situ hybridisation

Embryos were fixed overnight in 4% paraformaldehyde, 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/4% 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 prehybridised for 1 hour at 70°C. Digoxigenin-labelled RNA probes were synthesised according to the manufacturers instructions (Boehringer) and added to hybridisation buffer at a final concentration of 1 μg/ml; hybridisation was overnight at 70°C. The embryos were washed two times 30 minutes in 50% formamide, 1% SDS, 5× SSC pH 4.5 at 70°C. This was followed by treatment with RNase A (100 μg/ml in TE containing 0.5 M NaCl) twice for 30 minutes at 37°C. After RNase treatment, embryos were washed in 50% formamide, 2× SSC pH 4.5 at 65°C twice for 30 minutes. This was followed by washing in TBST (0.14 M NaCl, 10 mM KCl, 25 mM Tris-HCl pH 7.5, 1% Tween-20). The embryos were preblocked in 10% sheep serum in TBST before being incubated overnight with alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer-Mannheim) that had been preabsorbed 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 $MgCl_2$, 1% Tween-20). The alkaline phosphatase was visualised by incubating embryos with nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate in NTMT. The stained specimens were mounted in 10% PBS/90% glycerol and viewed under Nomarski optics. For sectioning, embryos were embedded in gelatin-albumin, fixed with glutaraldehyde and cut at 50 μm on a vibratome.

RESULTS

High levels of apoptosis are associated with the dorsal midline of r3 and r5

We have previously demonstrated with nile blue sulphate that the dorsal midlines of r3 and r5 are associated with elevated levels of cell death (Lumsden et al., 1991). To extend this observation and to investigate further the nature of this cell death, we have used acridine orange and transmission electron microscopy. Vital staining with acridine orange has been used previously to examine sites of apoptosis (Spreij, 1971; Wolf and Ready, 1991).

The first group of cells staining strongly with acridine orange in the rhombencephalon were evident at stage 10 over the dorsal midline of r3, just as this rhombomere becomes distinct and during the early phase of neural crest production in the rostral rhombencephalon (Fig. 1A). By stage 11 the staining over r3 was greatly enhanced both in intensity and in extent with the median band of strongly staining cells now reaching into the r2 territory (Fig. 1B). By this stage there was also staining over the dorsal midline of r5. R4 was markedly free of acridine orange staining. These results exactly match those obtained previously with nile blue sulphate (Lumsden et al., 1991). There is a rostral-to-caudal maturation in the staining pattern, with r3 being labelled before r5 and only the dorsal midline region that would be associated with neural crest production is stained (Fig. 1). It is also apparent that both dyes reveal more intense staining in r3 than in r5 and that the r3 staining encroaches into r2 territory.

That the acridine orange staining marked apoptotic cells was confirmed by transmission EM analysis. Fig. 1 shows transverse sections through r3 and r4 at stage 11. It is apparent from the semi-thin sections that, while neural crest cells can be seen migrating from r4 (Fig. 1E), r3 is free of neural crest (Fig. 1C). Our EM analysis of the dorsal midline region of each rhombomere reveals that while the cells of in this region of r4 are healthy those in r3 are not and exhibit a large number of pycnotic nuclei (Fig. 1F,D). Nuclei in r3 (and r5, data not shown) are seen in various characteristic stages of apoptosis (Wylie et al., 1980), particularly prominent are the crescentic caps of condensed chromatin in some of the cells (Fig. 1D). These results are suggestive of and consistent with neural crest being produced by the rhombomeres 3 and 5 but then being subsequently eliminated by apoptosis; these levels of the rhombencephalon are thereafter rendered crest free.

Tissue culture of rhombomere explants

In an attempt to rescue r3 and r5 crest from cell death, we turned to tissue culture. Individual stage 10 rhombomeres, completely cleaned of contaminating mesenchyme, were prepared as described above and were plated onto fibronectin/poly-L-lysine substrata to allow neural crest cells to crawl out. Surprisingly, we found that both even- and odd-numbered rhombomeres produced cells that migrated away from the neural tube and that these cells had a typical neural crest cell morphology (Fig. 2) (Rogers et al., 1990). Explants were stained for the neural crest marker HNK-1 to verify that the cells were neural crest and to check for variation between the even-numbered and the odd-numbered explants. A comparison between Fig. 2A and B shows that there was no obvious difference between the two categories of explant. Thus, in sharp contrast to the in vivo situation, the single r3 or r5 can produce healthy neural crest cells when removed from the animal and cultured in isolation (Fig. 2C).

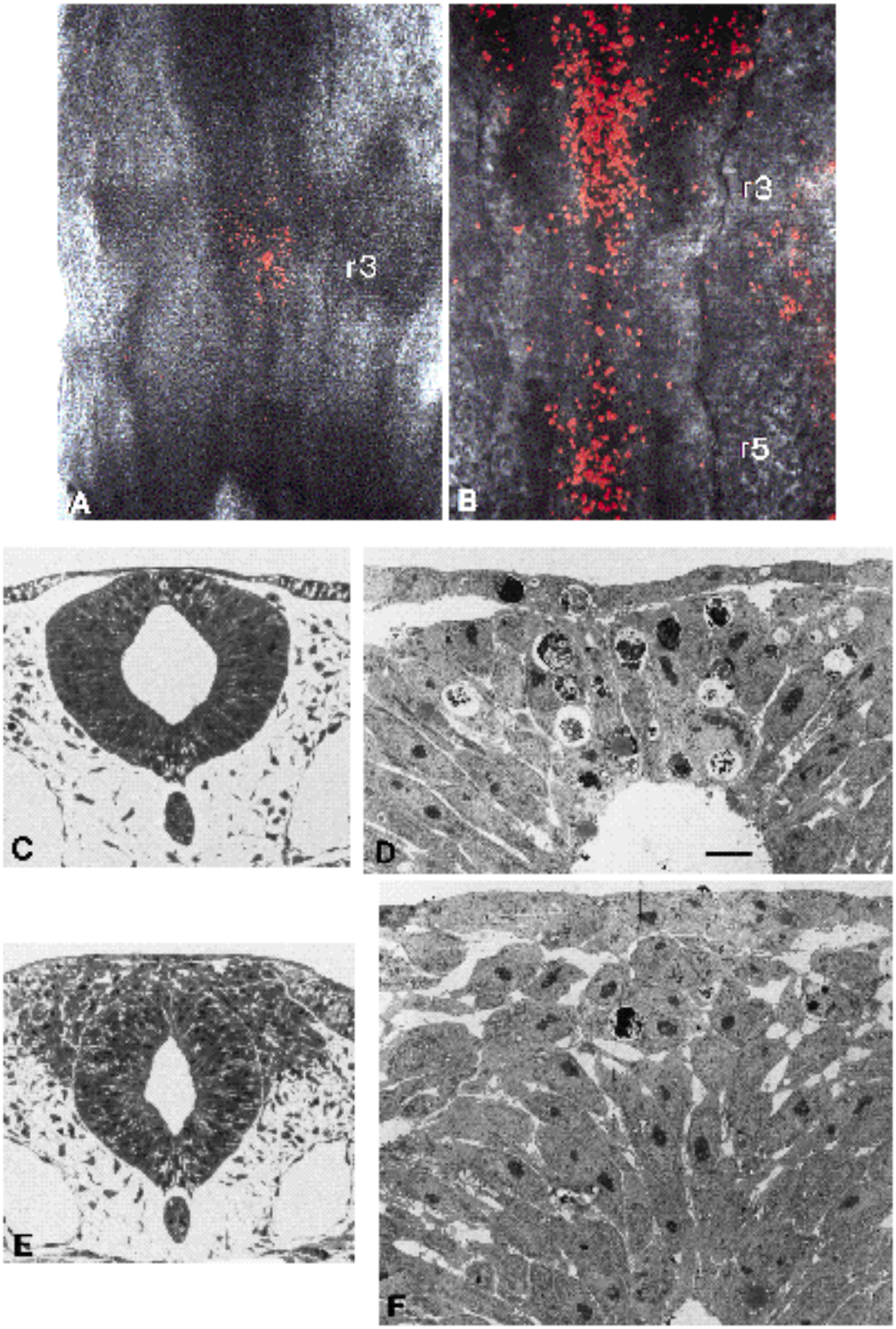

Fig. 1. Apoptosis in the hindbrain during the period of neural crest production. (A) Visualisation of the sites of apoptosis at stage 10 using acridine orange. Staining in the dorsal midline of r3. (B) At stage 11 acridine orange staining is now also found to extend from r3 into r2 and is present in r5. (C) Transverse semithin section through the dorsal midline of r3 at stage 11; the space between the neural tube and overlying ectoderm is empty of crest cells. (D) Transmission electron micrograph of the dorsal midline region in C. (E) Transverse semithin section through the dorsal midline of r4 at stage 11; abundant crest cells occupy the region above and beside the neural tube. (F) Transmission electron micrograph of the dorsal midline region in E. Scale bar (D,F), 5 μm.

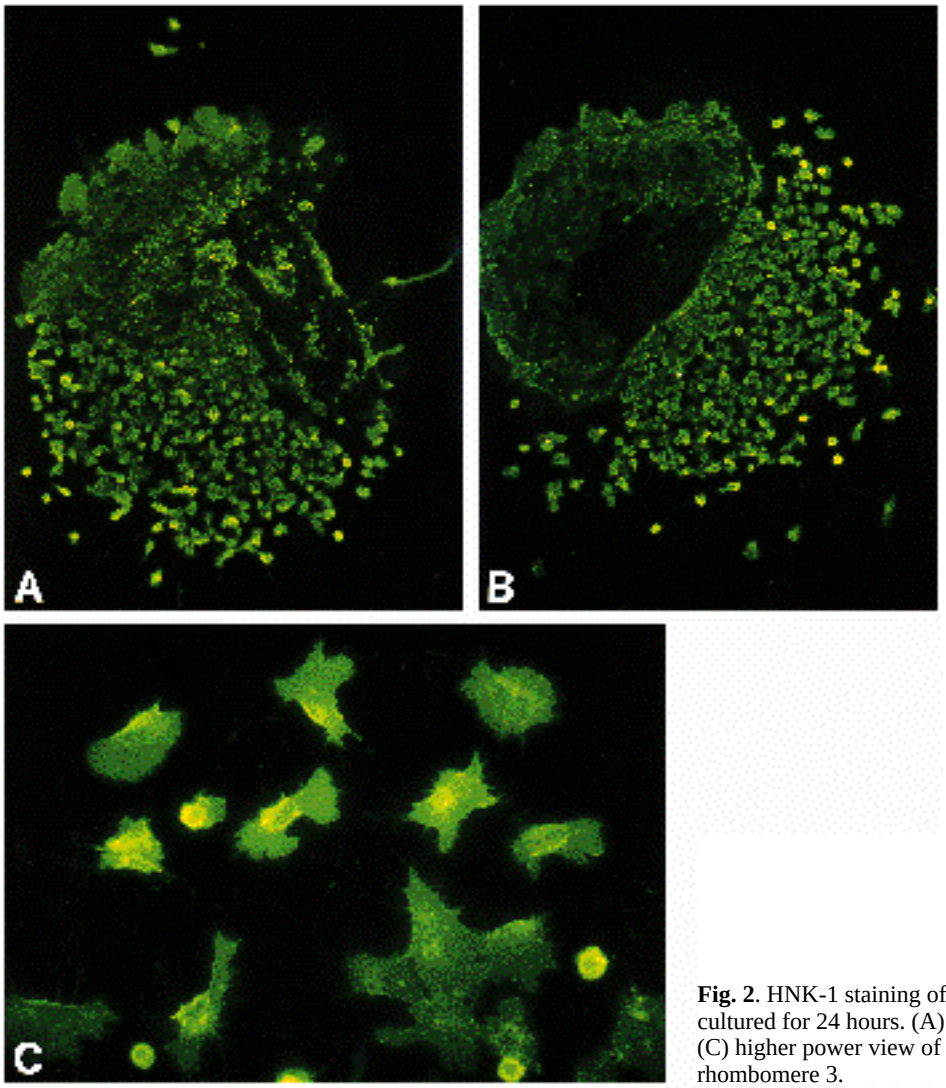

Fig. 2. HNK-1 staining of isolated stage 10 rhombomere explants cultured for 24 hours. (A) Rhombomere 4; (B) rhombomere 3 and (C) higher power view of HNK-1-positive cells emerged from rhombomere 3.

Analysis of neural crest production in single and double rhombomere explants

To establish that the crest cells produced by rhombomeres in culture were derived from the normal location (i.e. the dorsal midline) and did not arise de novo as a result of creating new cut surfaces during their dissection, we applied small focal injections of DiI to the dorsal aspect of the neuroepithelium of rhombomeres before explanting them. Since the rhombomeres are plated as naked rings of neural tube, stripped of mesenchymal investments, any labelled cells that migrated away from the explant and possessed typical neural crest morphology could be reliably assumed to be neural crest cells derived from the dorsal midline of that rhombomere. The results of these experiments were scored both visually and by counting the number of labelled migrating cells in each preparation. The numbers derived from this sort of analysis should be viewed only as an index, however, and not as a rigorous measure of the number of cells produced in each situation.

We found that for individual rhombomeres, either even- or odd-numbered, a relatively large number of labelled neural crest cells migrated away from the explant in all cases analysed (Fig. 3, Table 1). Fig. 3 shows labelled cells with a typical neural crest morphology emigrating from explants of r3 (A) and r5 (B). Labelling of even-numbered rhombomeres when still joined with their normally adjacent odd-numbered rhombomere also resulted invariably in labelled cells being present in the emigrating crest cell population (Table 1). In all cases, the even-numbered rhombomere contributed a large number of labelled cells to the crest population. The figures in Table 1 suggest no major difference between the number of labelled cells produced by even-numbered rhombomeres when they are explanted alone or when explanted as part of a doublet.

In sharp distinction, dorsal prelabelling of r3 or r5 in a double rhombomere explant did not produce labelled cells in the emigrating crest cell population. An example of r3 labelled and then explanted in conjunction with r2 is shown in Fig. 3C while Fig. 3D and 3E show examples of labelled r5 explanted with r4 and r6, respectively. In these cases there are healthy neural crest cells present around each of the explants but no label is seen in these cells. Rather, the label remains in the explant or in small round cells closely associated with the explant. These display a morphology typical

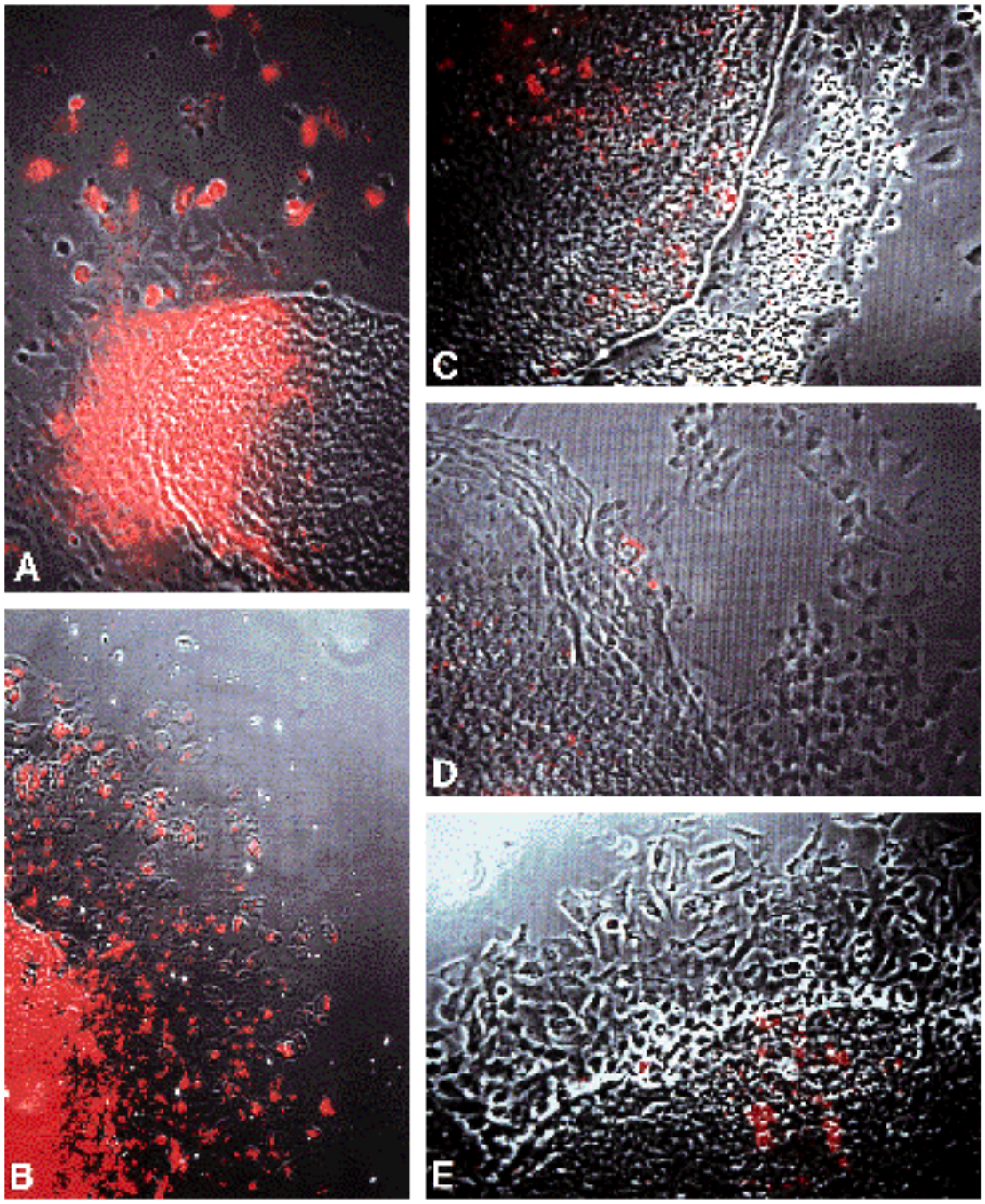

Fig. 3. Explants of DiI-prelabelled odd-numbered rhombomeres cultured in isolation (A,B) or in conjunction with an even-numbered neighbour (C-E). DiI was injected into the dorsal midline before explantation from the embryo. Dye-labelled cells can be seen migrating from odd-numbered rhombomeres cultured in isolation; (A) r3 (B) r5. Prelabelled odd-numbered rhombomeres cultured in conjunction with an even-numbered neighbour do not contribute to the crest cell population. (C) Prelabelled r3 co-cultured with r2; (D) prelabelled r5 co-cultured with r4 and (E) prelabelled r5 co-cultured with r6.

of dying cells in tissue culture. The same was seen for r5 (r4/5 Fig. 3D, r5/6 Fig. 3E). Since none of the label is found in the crest population of these explants but in dying cells, it can be concluded that the crest cells must have arisen from the even rhombomere of the doublet. It should be noted that in a very few cases a small number of labelled cells were observed which most likely derive from some of the dye reaching the edge of the even-numbered segment (Table 1). The fact that in explants of double rhombomeres one can observe crest-productive and crest-free areas that map to even and odd rhombomeres, respectively, implies that the tissue culture experiments mirror what is observed in the embryo.

The data in Table 1 demonstrate that there is a significant difference in the production of crest between the odd-numbered rhombomeres explanted on their own and when

Table 1. Number of labelled cells migrating from prelabelled even- or odd-numbered rhombomeres when cultured either in isolation or in rhombomere pairs

2*	93 (*n*=8)	2*3	99 (*n*=9)	3*	40 (*n*=22)	23*	4 (*n*=7)
4*	118 (*n*=7)	34*	69 (*n*=7)	5*	44 (*n*=7)	3*4	2 (*n*=12)
6*	62 (*n*=8)	4*5	59 (*n*=12)			45*	4 (*n*=15)
		56*	72 (*n*=6)			5*6	4 (*n*=10)

An asterisk to the right of the number indicates which rhombomere was prelabelled.

they are explanted with an even-numbered neighbour. Analysis using the Students *t*-test gave a probability that these results could occur by chance of less than 0.005% in all cases. These results therefore suggest that the odd-numbered rhombomeres are capable of producing crest and will do so if they are isolated from their neighbours. Since these experiments used only neural tissue it is probable that the even-numbered rhombomeres themselves are the source of a negative influence upon the odd-numbered rhombomeres that renders them effectively crest free, most likely through the induction of cell death.

Experimental manipulation of rhombomere environment

To test whether this inhibitory effect of even-numbered rhombomeres on the crest production of odd-numbered rhombomeres occurs in vivo, we used microsurgical techniques to create an experimental situation where an odd-numbered rhombomere was not flanked by an even rhombomere. Thus we removed the r4 from a host embryo and replaced it with a DiI prelabelled r3, removed from a donor embryo of the same stage such that the donor r3 was abutted by the host r3 and r5 (Fig. 4). In 16 experiments, all carried out at stage 10 and analysed 24 hours later, we found that the labelled donor r3 produced a stream of neural crest cells that consistently migrated towards and into the second branchial arch, although in some instances labelled cells were found in the first and third arches (Fig. 5A). By contrast, in all 8 control experiments, also at stage 10, where the labelled r3 was placed into the r3 position of a host embryo no neural crest cells were produced by the prelabelled r3 (Fig. 5B).

While the tissue culture experiments point towards the even-numbered rhombomeres as the source of the negative effect on crest production, it is possible that the mesoderm adjacent to r3 and r5 could also play a role in depleting these segments of crest. If the mesoderm has no role to play then the distancing of the caudal end of host r3 and the rostral end of the host r5 from any even rhombomeres, through the removal of r4, may result in crest production from these sites in the host (Fig. 4). We tested this possibility in 11 experiments by labelling the host r3 and r5 immediately after removing r4 and before insertion of an unlabelled donor r3. The cut ends were labelled as these were furthest from any even-numbered rhombomere influence. This experimental situation usually resulted in some labelled neural crest being produced from the host r3 and r5 even though they are set in their normal mesodermal environment (Fig. 5). The amount of crest produced in this circumstance, however, never matched that of the transposed r3 or that of the normally flanking rhombomeres, r2, r4 and r6. In certain instances, the labelled crest abnormally emanating from r5 circumnavigated the otocyst suggesting that this structure does not normally present a barrier to neural crest production and emigration.

These experiments taken together would suggest that it is the inhibitory effect of the even-numbered rhombomere alone that is responsible for suppressing crest production in r3 and r5 through the induction or enhancement of apoptosis in the cells of the dorsal neural tube.

Isolation of *msx-2* and analysis of its expression pattern and that of *msx-1* during the period of rhombencephalic neural crest production

We wished to analyse genes of the *msx* family during the period of neural crest production in the hindbrain for the reasons outlined above. To isolate chicken *msx-1* and *msx-2*, we screened a stage 10 cDNA library, covering the early period of hindbrain crest production, at low stringency with a mix of the three known murine *msx* homeoboxes (Holland, 1991). The screen resulted in the isolation of a number of phage. Twelve of these were sequenced and proved to be clones of *msx-2* (Coelho et al., 1991; Yokouchi et al., 1991)

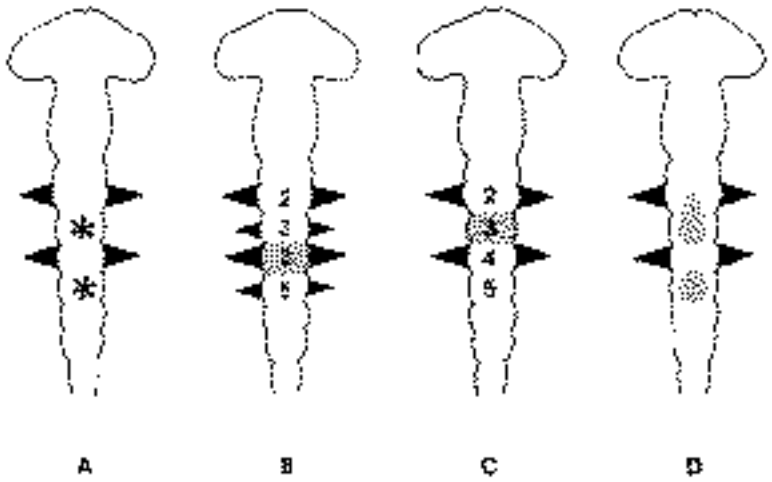

Fig. 4. Diagrammatic representation of the stage 10/11 chick embryo brain showing the results obtained in this study and the microsurgical manipulations employed. (A) Neural crest cells migrate from r2 and r4 (arrowheads), whereas those in r3 and r5 are eliminated by apoptosis (asterisks). (B) Replacement of r4 by r3 results in crest emeregnce both from the donor r3 (large arrowhead) and, to a lesser extent, from the flanking host rhombomeres (small arrowheads). (C) Control orthotopic replacement of r3 results in the absence of crest from the donor rhombomere. (D) The patterns of apoptosis and *msx-2* expression (stippled) coincide.

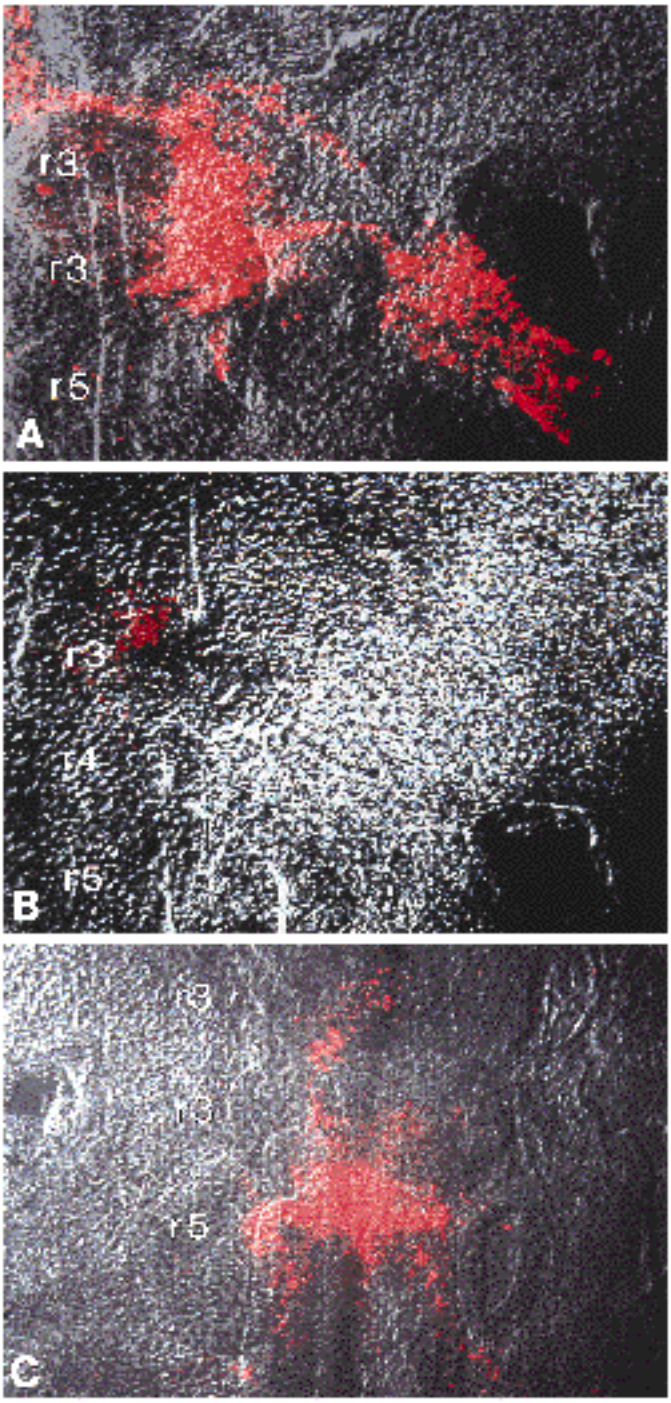

Fig. 5. Release of odd-numbered rhombomeres from even-numbered rhombomere influence by manipulation of the rhombomere environment. (A) 24 hours after removal of the stage 10 host r4 and its replacement with a DiI-prelabelled donor r3, labelled neural crest cells were found to have migrated from the donor odd-numbered rhombomere. (B) Control manipulation replacing the host r3 with a DiI-prelabelled donor r3 does not result in labelled crest cells migrating from the neural tube. (C) DiI labelling the host r3 and r5 prior to the replacement of the host r4 with an unlabelled donor r3 results in labelled neural crest cells emanating from the host odd-numbered rhombomeres.

and not of other members of this gene family. We have been generously provided with the *msx-1* clone from Dr Benoit Robert and as yet there have been no reports of any other chick members of this family.

Early in the period of neural crest production, at stage 10, both of these genes are expressed in the rhombencephalon with *msx-1* expression being more abundant and more widespread than that of *msx-2*. Expression of *msx-1* extends either side of the dorsal midline up to the level of r3 (Fig. 6A). Surprisingly, in r3 but at no other point along the rostrocaudal axis, the expression of this gene crosses the dorsal midline (Fig. 6A). At this same stage, *msx-2* is exclusively expressed over the dorsal aspect of r3 and extending slightly into r2 but at no other place in the CNS (Fig. 6B). Fig. 6C illustrates that the expression of *msx-1* on either side of the midline has extended rostrally in a stage 11 embryo and that it now also crosses the dorsal midline in r5. In comparison, Fig. 6D shows *msx-2* expressing strongly in r3 and in a band of cells extending into r2 and also now in r5 at stage 11. Noticeably, both genes exhibit stronger expression in r3 than r5. After stage 12, following the peak period of neural crest production, both of these genes exhibit uninterrupted expression along the dorsal aspect of the neural tube, as has been described by other workers (data not shown, Suzuki et al., 1991; Yokouchi et al., 1991). Vibratome sectioning of stage 11 whole mounts in the transverse plane reveals that in r3 and r5 both genes are expressed exclusively in the dorsal neuroectoderm and are not expressed in the overlying ectoderm or in the adjacent mesoderm (Fig. 6E,F). The expression pattern of *msx-1* encompasses more of the dorsal region of r3 and r5 than *msx-2*, whose domain of expression is wholly contained within that of *msx-1*.

Although these genes exhibit later expression in the branchial arches, there is never expression of either gene in the neural crest cells that are migrating away from the neural tube. Instead these genes have been found to be expressed in the dorsal regions of r3 and r5, those rhombomeres from which migrating neural crest is depleted but which are associated with elevated levels of programmed cell death.

Altered expression of *msx-2* following rhombomere transposition

The in situ hybridisation analysis of *msx-2* points towards a close spatial and temporal association between the expression of this gene and apoptosis during rhombencephalic crest production. We have shown that the replacement of r4 with a donor r3 results in crest production from that rhombomere and also from the flanking host r3 and r5. One test of any association between apoptosis and *msx-2* expression is to analyse the expression of this gene following this rhombomere manipulation.

The manipulations were carried out as described above, at stage 10, and are illustrated in Fig. 4. In four control experiments where a host r3 was excised and replaced with a donor r3, and neural crest production remains inhibited, we found that *msx-2* expression was unchanged (Fig. 7A,B). By contrast, in all of the 8 cases where the host r4 was replaced with a donor r3 the expression of *msx-2* was severely affected. As is shown in Fig. 7C,D, the transplanted r3 is devoid of *msx-2* message. In a number of instances, the flanking host r3 and r5 also exhibited reduced levels of

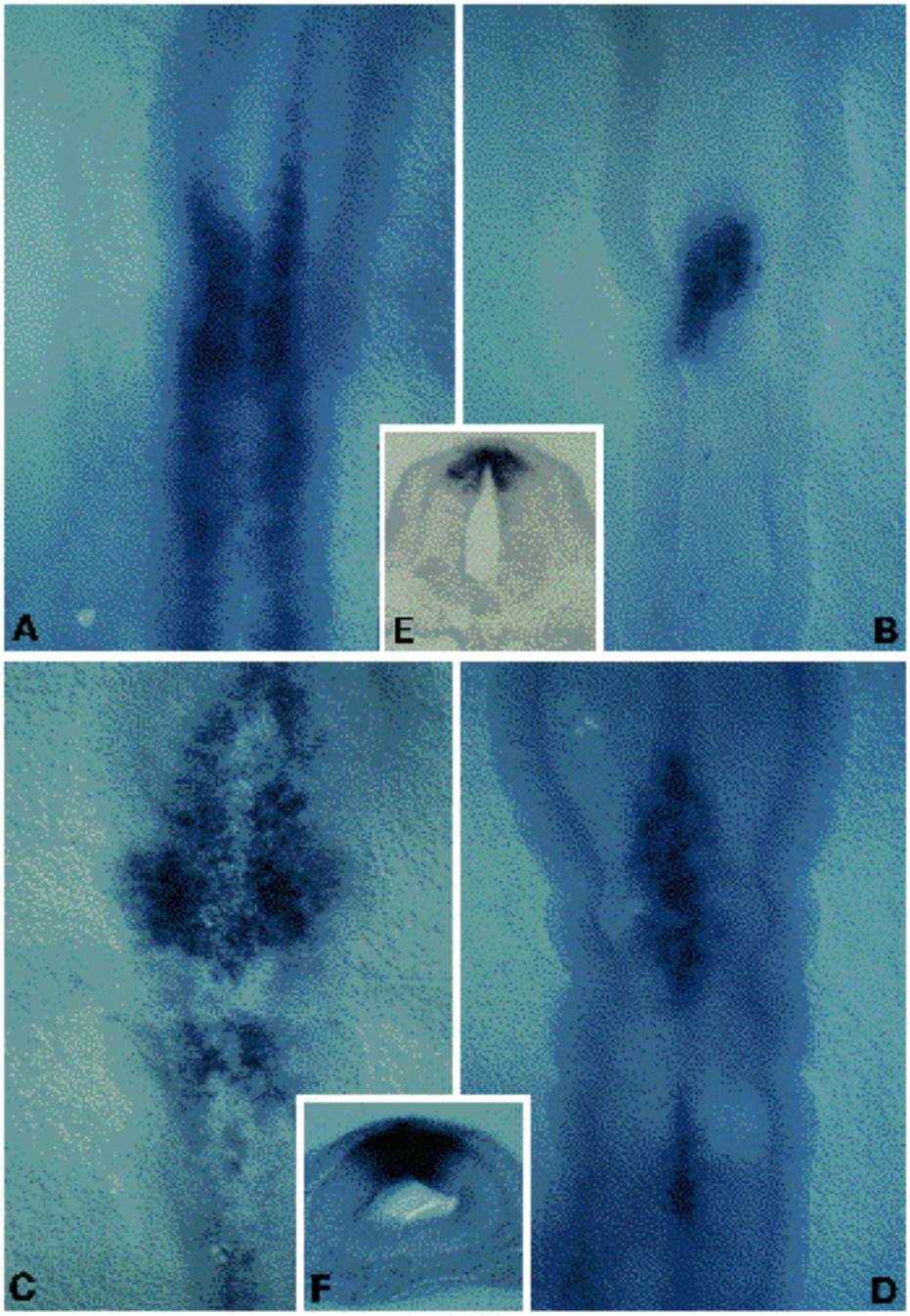

Fig. 6. Patterns of expression of *msx-1* and *msx-2* in the rhombencephalon during the period of neural crest production as shown by digoxigenin whole-mount in situ hybridisation. (A) *msx-1* expression at stage 10 extends in two stripes parallel to the midline and crosses the midline only at r3. (B) *msx-2* expression at stage 10 is only in r3. (C) *msx-1* expression at stage 11 has widened so that it now crosses the midline also at r5. (D) *msx-2* expression has now extended from r3 into r2 and is also found over r5. (E) A transverse section through r5 at stage 11 revealing *msx-2* expression to be concentrated in the dorsal aspect of the neural tube. (F) Expression of *msx-1* in the dorsal midline of r5 at stage 11.

expression of this gene (Fig. 7C), correlated with the increased flow of crest from these levels of the axis. Thus where an odd-numbered rhombomere does not normally produce neural crest but is associated with apoptosis, *msx-2* expression is associated with these regions, yet if odd-numbered rhombomeres are freed from the influence of even rhombomeres and allowed to produce crest then the expression of *msx-2* is downregulated. These experiments would suggest an association between the expression of *msx-2* and apoptosis. If a causal relationship exists then it is not all-or-nothing; emergence of crest cells seems to correlate with a reduction of *msx-2* expression and does not require that it be shut off entirely.

DISCUSSION

We have shown that isolated odd-numbered rhombomeres, r3 and r5, that are normally depleted of neural crest, will produce crest cells when isolated in culture. When these rhombomeres are cultured in conjunction with an even-numbered neighbour, however, they do not produce crest cells. Through surgical manipulation of hindbrain segments in vivo a situation was created where an odd-numbered rhombomere is flanked by two host odd-numbered rhombomeres. Here both the donor and host odd rhombomeres produce neural crest. The in vitro and in vivo experiments demonstrate that odd-numbered rhombomeres will produce

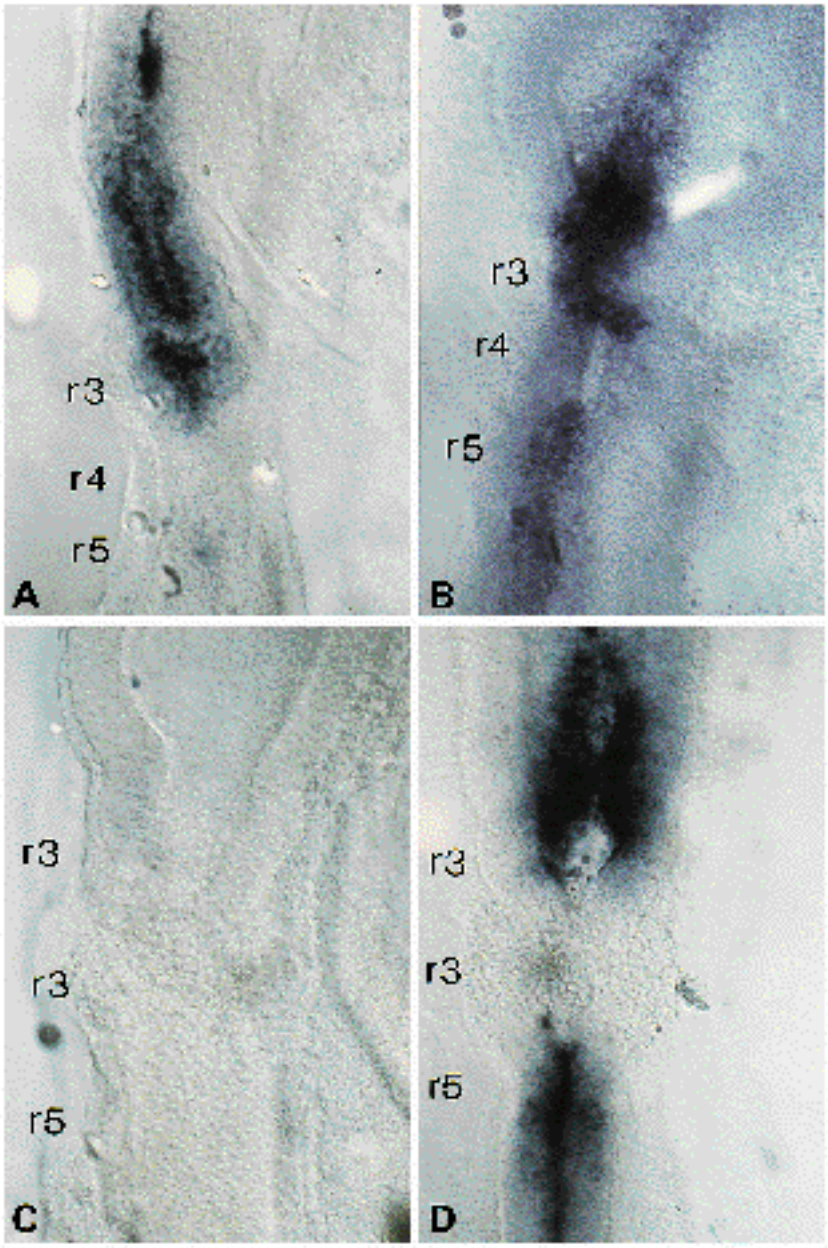

Fig. 7. Expression of *msx-2* in manipulated embryos, analysed 6 hours after grafting at stage 10. (A,B) Expression is unaltered in control embryos where a host r3 is replaced by a donor r3. (C) Expression is lost from the donor r3 when it replaces the host r4 and is down regulated in the host r3 and r5. (D) Expression is absent from the donor r3 when it replaces the host r4.

crest if they are freed from the influence of even-numbered rhombomeres. The tissue culture experiments and the results from the surgically manipulated embryos suggest that the even-numbered rhombomeres exert a dominant negative effect upon the odd-numbered rhombomeres that suppresses neural crest production, most likely through the induction of cell death. We have also analysed the expression of members of the *msx* family of homeobox genes during the period of neural crest production. We do not find these genes expressed in either premigratory or migrating crest but rather their expression patterns co-localise with patterns of programmed cell death. This is a particularly close association for *msx-2*; transplantation experiments that allow the transposed r3 to contribute crest cells resulted in the abolition of *msx-2* expression in the graft and downregulated expression in the host r3 and r5. This suggests a link between the expression of *msx-2* and apoptosis in the rhombencephalon during neural crest production.

We have previously constructed a fate map of the chick rhombencephalic crest with sufficient spatial resolution to reveal a segmental origin for neural crest in the hindbrain (Lumsden et al., 1991). This study found that there were three streams of neural crest in the hindbrain separated by two regions, r3 and r5, where crest production is dramatically reduced and which were associated with elevated levels of apoptosis in the dorsal midline of the neural tube. One of the most significant conclusions that can be drawn from the present study is that spatial segregation of the avian rhombencephalic neural crest can be achieved solely by mechanisms that are intrinsic to the neurectoderm. Through both in vitro and in vivo experiments, we can conclude that the crest in odd rhombomeres is depleted after stage 10

through an interaction with even rhombomeres. Before stage 10, it is possible that early crest individuation extends continuously along the neuraxis. As soon as the rhombomeres form, however, the even-numbered rhombomeres exert a dominant negative effect on the odd-numbered rhombomeres that induces programmed cell death in their presumptive crest population. If r3 and r5 are freed from this influence either in vitro or in vivo then they continue to produce healthy, migratory neural crest cells.

The in vitro and in vivo experiments do not reveal any obligate requirement for mesoderm in the segmental outflow of the rhombencephalic crest. First, crest-free and crest-productive regions exist in neurectodermal explants in tissue culture that are free of any mesodermal influence. Secondly, in transplantation experiments, neural crest can be produced by odd-numbered rhombomeres that are distanced from even-numbered rhombomeres (i.e. by replacing r4 with r3) but which are situated in their appropriate mesodermal environment.

Our results argue that the streaming of cranial neural crest is organised according to developmental mechanisms that differ from those operating in the trunk. Segmentation of the trunk neural crest is imposed by the serially reiterated mesodermal sclerotome and establishes a precise correspondence between the peripheral nervous system and the somitic mesodermal derivatives (Keynes and Stern, 1984; Rickman et al., 1985). There is no evidence for morphogenetic prespecification at different rostrocaudal levels of the trunk neural crest. The head, by contrast, has been shown to contain morphogenetically specified premigratory crest (Noden, 1983) and the segregation of cranial neural crest into three discrete streams may ensure the specific filling of each of the branchial arches and the correct development of each of the individual cranial ganglia. Given that cranial crest acquires its positional information prior to emigration from the neuroepithelium (Noden, 1983) and that rhombomeres are primary units of regional specification (Guthrie et al., 1992; Kuratani and Eichele, 1993) then, as we have previously argued (Lumsden and Keynes, 1989), it is implicit that the segmentation of the cranial crest should relate to the segmentation of the rhombencephalon. It should also be apparent that the primary mechanism that segments the hindbrain crest (apoptosis) does not act within the migration environment but acts in the neuroepithelium itself. A number of aspects of avian head development would also seem to preclude the segmentation of crest by the mesoderm. These include the lack of segmented sclerotome (Couly et al., 1992), the fact that the majority of cranial crest does not enter the mesoderm but moves between the mesoderm and the overlying ectoderm (Noden, 1975; Tosney, 1982) and that none of the molecules that are differentially expressed in anterior and posterior sclerotome and thus implicated in trunk crest guidance (Erickson, 1986; Stern et al., 1986; Mackie, 1988) are distributed in a manner that would suggest they are performing the same role in the head. All of the available data are consistent with segmentation of the neural crest in the rhombencephalic region by mechanisms intrinsic to the neurectoderm.

The association of elevated levels of programmed cell death in r3 and r5 suggests that neural crest production is uninterrupted along the rostrocaudal axis but that r3 and r5 are rendered crest free by specific elimination of that cell population, following immediately after this region becomes segmented. This would appear to be an example of morphogenetic cell death, analogous to that observed in the limb, whereby populations of cells are separated from each other by local areas of apoptosis (Glucksmann, 1951; Saunders, 1966). Analysis with both nile blue sulphate and acridine orange staining and with transmission electron microscopy reveal that the cell death that characterises the dorsal midline in r3 and r5 has features typical of apoptosis (Wylie et al., 1980).

Limb tissue exhibits apoptosis in situ and will execute this cell death programme on schedule when explanted into culture (Fallon and Saunders, 1968). Apoptosis in the rhombencephalon displays novel characteristics in that its execution in the odd rhombomeres requires signals from the adjacent even rhombomeres and it will not occur if they are freed from even rhombomere influence. Thus programmed cell death in the hindbrain would not seem to be a case of cell suicide acting autonomously within the neural crest of the odd-numbered rhombomeres. Moreover, in our experiments crest-free and crest-productive areas can be found in double rhombomere explants cultured in rich media. Apoptosis in r3 and r5 is therefore unlikely to be due to the activation of an intrinsic programme, which results from the lack of growth factors, as has been suggested recently for other systems (Raff, 1992). Rather, what we see is a dominant negative effect of the adjacent tissue upon the crest of the odd-numbered rhombomeres.

Cell death, rather than the establishment of regions that never produce crest, is used to segregate outflow of the chick rhombencephalic crest. This may suggest that segmentation of the neural crest has been superimposed upon an unsegmented ancestral condition. Segmentation of the hindbrain crest could have evolved with changes in its interaction with, and its patterning of, the branchial arches. Interestingly, a scenario has been suggested for the transition between primitive agnathan vertebrates and jaw-bearing fish that involves the re-organisation of the three most rostral branchial arches. These three arches would have been modified to give rise to the structures of the jaw region of extant jawed vertebrates: arch 0 was modified to form the trabeculae cranii, arch 1 became the mandibular arch (i.e. palatoquadrate cartilage and maxilla of the upper jaw and Meckel's cartilage and mandible of the lower jaw), while arch 2 (hyomandibular) became the stapes and part of the hyoid (Reichert's cartilage). It has been suggested that the three most rostral arches of agnathan stage would have had the same quality as the third branchial arch of extant gnathostomes, with typical branchial nerves comparable to the glossopharyngeal (Romer, 1971). Noticeably the segregation of the neural crest into streams in higher vertebrates only relates to arches 1 and 2 and is not found to be associated with the third and fourth arches. Thus, segmentation of hindbrain crest may have evolved with the allocation of crest to different states of morphogenetic specification that would be necessary for the elaboration of the jaw region and therefore with alterations in the patterning of the most rostral arches.

It was also from taking an evolutionary perspective that we were prompted to analyse the expression patterns of

members of the *msx* gene family, together with the fact that they exhibit expression in the branchial arches. Our analysis of the expression of these genes in the rhombencephalon during the period of neural crest production has revealed that there is a close relationship between the patterns of *msx-1* and *msx-2* expression, as has been seen in other locations (Davidson and Hill, 1991). More specifically, we have found that these genes are not expressed in migrating neural crest, rather they are strongly expressed in the dorsal aspect of r3 and r5, those rhombomeres depleted in migratory neural crest. This would suggest that the later expression of these genes in neural crest derivatives such as the branchial arches is separately established and not related by lineage to the expression in the rhombencephalon.

These *msx* genes may play a role in neural crest production but, given the striking correspondence between their expression and the patterns of programmed cell death in the rhombencephalon, this may be in a patterning role that involves the selective elimination of specific populations of cells. Indeed, the expression pattern of *msx-2* in the rhombencephalon is spatially and temporally very similar to the pattern of apoptosis. The only difference is that the expression of this gene precedes the patterns of apoptosis which is what one would expect of a transcription factor possibly involved in this cellular phenomenon. In both the vital dye staining and the in situ hybridisation expression studies, staining is evident in r3 before r5 and expression in the r3 area is found to increase in intensity and to encroach into the r2 territory. The correlation between programmed cell death and *msx-1* expression is not so pronounced but it is intriguing that this gene only crosses the dorsal midline in r3 and r5. An analogous situation is also observed in the limb where the patterns of expression of these two genes, particularly that of *msx-2*, correlate with regions of programmed cell death, although such a correlation cannot be drawn for all sites of *msx-2* expression (Coelho et al., 1991; Yokouchi et al., 1991). Interestingly, the expression of both these genes in each of these embryonic fields again precedes programmed cell death.

The relationship between the expression of *msx-2* and patterns of cell death in two separate embryonic fields suggests an association between the two that is reinforced by the rhombomere transposition experiments. We have shown that when a host r4 is replaced with a donor r3 this rhombomere produces neural crest, as do the host r3 and r5. When the expression of *msx-2* is analysed in such embryos, it is found that the gene is no longer expressed in the transplanted r3 and is also downregulated in the host r3 and r5. This would suggest an intimate link between the expression of *msx-2* and apoptosis. This experiment also highlights the fact that the expression of this homeobox gene, *msx-2*, in contrast to that of another, *Hoxb-1* (Guthrie et al. 1992, Kuratani and Eichele, 1993), is not autonomous but is regulated by tissue interaction.

The negative factor(s) produced by the even rhombomeres elicits an effect only in the prospective crest population of r3 and r5. This factor would presumably interact with some receptor, possibly at the cell surface if it is diffusible, and then initiate the cascade of events that leads to the death of these cells. Since the receptor(s) must only be present in a specific population in r3 and r5, it could not be regulated exclusively by *Krox-20*, a transcription factor which is restricted to these rhombomeres (Wilkinson et al., 1989), but would also have to have some component in its regulation that was crest cell specific. Our experiments suggest that *msx-1* and *msx-2* are good candidates. Given that the expression of *msx-1* relates more closely than *msx-2* to the segment boundaries, it is possible that this gene responds to cues from the odd-numbered rhombomere, possibly involving *Krox-20*, and that *msx-2* is then expressed in the presumptive apoptotic population. The resolution of any such interactions obviously awaits further genetic analysis but it is interesting to note that the close relationship between the expression patterns of *msx-1* and *msx-2*, in a number of instances, suggests that they may be subject to common control and that they may regulate each other (Robert et al., 1991; Davidson and Hill, 1991).

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