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Relationship between spatially restricted *Krox-20* gene expression in branchial neural crest and segmentation in the chick embryo hindbrain

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Previous studies have suggested that the rostrocaudal patterning of branchial arches in the vertebrate embryo derives from a coordinate segmental specification of gene expression in rhombomeres (r) and neural crest. However, expression of the *Krox-20* gene is restricted to neural crest cells migrating to the third branchial arch, apparently from r5, whereas this rhombomere contributes cells to both the second and third arches. We examined in the chick embryo how this spatially restricted expression is established. Expression occurs in precursors in both r5 and r6, and we show by cell labelling that both rhombomeres contribute to *Krox-20*-expressing neural crest, emigration occurring first from r6 and later caudally from r5. *Krox-20* transcripts are not detected in some precursors in rostral r5, presaging the lack of expression in cells migrating rostrally from this rhombomere. After transposition of r6 to the position of r4 or r5, many *Krox-20*-expressing cells migrate rostral to the otic vesicle, whereas when r5 is transplanted to the position of r4, only a small number of migrating cells express *Krox-20*. These results indicate that, in the chick, *Krox-20* expression in branchial neural crest does not correlate with rhombomeric segmentation, and that there may be intrinsic differences in regulation between the r5 and r6 *Krox-20*-expressing populations.

Key words: branchial arches/hindbrain segmentation/*Hox* code/*Krox-20*/neural crest

Introduction

The genetic regulation of patterning in the branchial region of the vertebrate head may involve segmental relationships between the hindbrain, neural crest and branchial arches. Segmentation is apparent at the morphological level in the hindbrain as a series of bulges and constrictions, the rhombomeres, that form in the neural epithelium. With the formation of each rhombomere boundary, there is restriction of cell movement such that each rhombomere is a polyclonal compartment of cells which display limited intermixing with cells in neighbouring rhombomeres (Fraser *et al.*, 1990; Birgbauer and Fraser, 1994). This restriction is likely to be an important mechanism by which each rhombomere establishes and maintains a distinct identity, as later seen in the development of branchial motor nerves in precise segment-restricted patterns (Lumsden and Keynes, 1989).

In addition to underlying neuronal organization, hindbrain segmentation appears to be important for the patterning of neural crest cells that migrate from the neural tube to contribute to cranial ganglia and branchial arches (Hunt *et al.*, 1991; Lumsden *et al.*, 1991). At the gross morphological level in chick and rat embryos, neural crest cells are observed in three streams that are separated by neural crest-free zones adjacent to rhombomere (r) 3 and r5 (Anderson and Meier, 1981; Tan and Morriss-Kay, 1985; Serbedzija *et al.*, 1992). Cell-marking experiments indicate that neural crest cells originating from r2, r4 and r6 migrate to the first, second and third branchial arches, respectively (Lumsden *et al.*, 1991; Sechrist *et al.*, 1993). One study (Lumsden *et al.*, 1991) has suggested that r3 and r5 do not contribute neural crest to the branchial arches, perhaps because of localized cell death that leads to crest-free zones (Lumsden *et al.*, 1991; Jeffs *et al.*, 1992; Graham *et al.*, 1993). However, another study has shown that neural crest cells forming in r3 and r5 migrate rostrally or caudally from these rhombomeres to join the streams emanating from the flanking rhombomeres (Sechrist *et al.*, 1993). This suggests that factors other than the non-uniform generation of neural crest cells contribute to the patterning of neural crest migration in the hindbrain. For example, the otic vesicle appears to selectively attract some neural crest cells (Sechrist *et al.*, 1994).

Because neural crest cells in each arch form a characteristic pattern of bone and cartilage derivatives, either the site of origin or the destination of hindbrain neural crest cells could prescribe their arch-specific differentiation. Grafting experiments in the chick suggest that this spatial pattern is specified in the neural crest prior to its migration; when neural crest destined to form first arch structures is transplanted caudally so that it instead migrates into the second arch, first arch structures form in the second arch (Noden, 1983, 1988). These data suggest that the branchial arch neural crest is specified according to its rostrocaudal site of origin in the hindbrain.

Molecular correlates of the rostrocaudal specification of rhombomeres and neural crest are provided by the expression pattern of a number of genes, including members of the *Hox* family, *Krox-20* and retinoic acid receptors and binding proteins (reviewed by Wilkinson, 1993). In general, there is a correlated segmental expression of these genes in rhombomeres and in neural crest cells: e.g. the combination, or 'code', of *Hox* gene expression in r2, r4 and r6 is identical to that in neural crest

derivatives migrating to branchial arches 1, 2 and 3, respectively (Hunt *et al.*, 1991). This segmental *Hox* code was proposed to specify the rostrocaudal identity of branchial arch neural crest, a model supported by the phenotype of mice with null mutations in the *Hoxa-2* gene (Gendron-Maguire *et al.*, 1994; Rijli *et al.*, 1994). Exceptions to this correlated expression are that *Hoxa-2* is expressed in r2, but not r2-derived neural crest (Prince and Lumsden, 1994), and *Krox-20* in r3, but not r3-derived neural crest (Wilkinson *et al.*, 1989; Sham *et al.*, 1993). These latter data, nevertheless, are consistent with a segmental specification of rhombomeres and neural crest, with some aspects of gene expression independently regulated in these tissues. On the other hand, r3 and r5 each contribute neural crest cells to two branchial arches that have distinct patterns of gene expression; e.g. r5 contributes neural crest to the second and third arches, but only the latter has the same *Hox* code as r5. This discrepancy between branchial arch gene expression and a segmental specification of neural crest cells raises the question as to how the appropriate spatially restricted pattern of expression is regulated in r3- and r5-derived neural crest. Moreover, a limitation of published gene expression studies supporting a segmental specification of neural crest is that these assume, but do not test, that gene expression is a reliable marker of cell lineage. It is, therefore, essential to directly examine the relationship between gene expression and the origin of neural crest cells.

Krox-20 gene expression provides an amenable molecular marker for examining the relationship between hindbrain segmentation and the rostrocaudal specification of r5-derived neural crest. In all vertebrates examined, expression is restricted at early neurula stages to r3, r5 and neural crest migrating from the hindbrain towards the third branchial arch (Wilkinson *et al.*, 1989; Bradley *et al.*, 1992; Sham *et al.*, 1993). The expression in third arch neural crest is clearly related to hindbrain segmentation in *Xenopus* embryos where, before migration, *Krox-20*-expressing neural crest cells are precisely contiguous with r5 (Bradley *et al.*, 1993). However, in the chick, the relationship with segmentation is less clear, since neural crest cells migrate from r5 to both the second and third branchial arches, raising the question as to how *Krox-20* expression is restricted to the latter cells.

Here, we examine the relationship between *Krox-20* expression in neural crest cells and hindbrain segmentation in the chick embryo by combining cell marking, *in situ* hybridization and *in ovo* transplantation experiments. We find that pre-migratory neural crest cells in r6 and in caudal r5 express *Krox-20*, and expression is transiently maintained in these cells as they migrate caudal to the otic vesicle and towards the third arch. In contrast, many presumptive neural crest cells in rostral r5 do not express *Krox-20*, presaging the absence of *Krox-20* expression in cells migrating rostral to the otic vesicle. Transplantation experiments indicate that *Krox-20* expression appears to be autonomous in r6-derived neural crest, but that there are differences in the migration and/or regulation of *Krox-20* expression in r5-derived neural crest. We discuss the implications of these findings for the spatial restriction of gene expression in branchial neural crest.

Results

In the chick embryo hindbrain, *Krox-20* expression is upregulated first in the region of prospective r3 at the 3-somite stage and then in prospective r5 at the 7-somite stage prior to boundary formation for these rhombomeres (Nieto *et al.*, 1991). By the 19-somite stage in the chick (see Figure 2E), as in the mouse (Sham *et al.*, 1993), a narrow stream of *Krox-20*-expressing neural crest cells is observed immediately caudal to the otic vesicle, and then ventrally, with its dorsal extremity aligned with the r5/r6 boundary. In contrast, no *Krox-20*-expressing neural crest cells are observed adjacent to r3 or the rostral portion of r5. Although these data imply that *Krox-20*-expressing neural crest cells originate near the r5/r6 border, it is not evident whether these cells have arisen from r5 and/or r6.

Krox-20 expression in neural crest

As a first step in deducing the segmental origin of neural crest cells which express *Krox-20* as they migrate, we have analysed the time course of *Krox-20* expression. The domain of expression in prospective r5 is first detected at the 7- to 8-somite stage, at which time its rostral boundary lies approximately perpendicular to the rostrocaudal axis, with an uneven appearance at the r4/5 border. The caudal boundary of expression forms a wedge shape that extends backwards along the dorsal midline (Figure 1A). At the 9- to 11-somite stage, both the rostral and caudal boundaries become more distinct and the wedge shape of expression is still observed (Figure 1B and C). Flat mounting of the neural epithelium shows that this shape is due to the presence of *Krox-20*-expressing cells in both r5 and the dorsal part of r6 (Figure 1D), and transverse sections demonstrate that the *Krox-20* expression is confined to the midline region of r6, corresponding to the location of pre-migratory neural crest cells (Figure 1E). Neural crest cells expressing *Krox-20* emigrate starting at the 13- to 14-somite stage. There is considerable variability at this stage, with some embryos having only a few emigrating cells (Figure 1F), whereas others have a broad stream of *Krox-20* positive cells contiguous with r6 that courses caudally around the otic vesicle (Figure 2A). Within the dorsal midline of the r5 neuroepithelium, *Krox-20* transcripts are detected in a lower proportion of cells in the rostral compared with the caudal portion of r5 (Figure 2B). This pattern correlates with the absence of *Krox-20* expression in neural crest cells migrating rostral to the otic vesicle that have been shown to derive from rostral r5 (Sechrist *et al.*, 1993). By the 17-somite stage, little *Krox-20* expression is detected in the midline region of r6, although several small streams of cells migrating from rostral r6 appear to join the larger stream from r5 (Figure 2C and D). From the 17- to the 20-somite stage, the stream of *Krox-20*-expressing cells progressively narrows to a region adjoining the r5/6 border that appears to overlap caudal r5 (Figure 2E and F). By the 23-somite stage, no migrating *Krox-20*-expressing cells are detected (not shown). Overall, these data indicate that *Krox-20* expression occurs in presumptive neural crest cells in both r5 and r6. Is the source of migratory *Krox-20*-expressing neural crest cells r5 and/or r6?

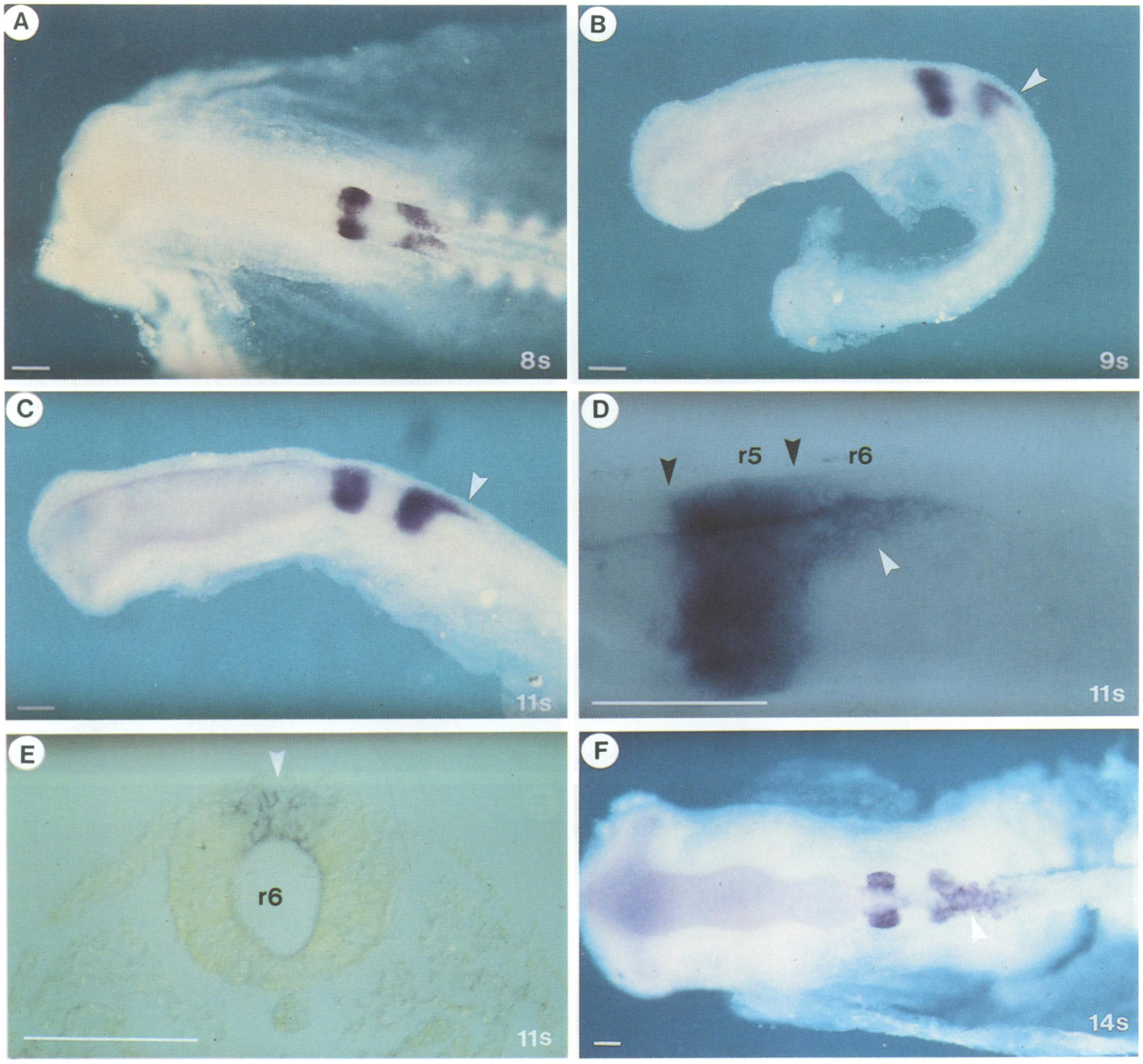

Fig. 1. Expression of *Krox-20* in pre-migratory neural crest visualized by whole-mount *in situ* hybridization analysis of the chick embryo. Chick embryos were photographed after whole-mount hybridization analysis, or after the flat mounting or sectioning of whole-mount hybridized embryos. The somite stages are indicated at the lower right of each photograph. (**A**) 8-Somite embryo. (**B**) 9-Somite embryo. (**C**) 11-Somite embryo. (**D**) Flat mount of the neural epithelium in 11-Somite embryo. (**E**) Transverse section through r6 of 11-somite embryo. (**F**) 14-Somite embryo. Emigration of neural crest is just starting in the 14-somite embryo shown in (F), but in other 14-somite embryos is more advanced (see Figure 2A). The white arrows indicate pre-migratory neural crest in the dorsal part of r6 and the black arrows the boundaries of r5. Rostral is to the left in all photographs. The bars indicate 100 μm.

Cell-marking analysis of Krox-20-expressing neural crest

The site of origin of neural crest cells expressing *Krox-20* was determined by combining cell marking with *in situ* hybridization (Izpisúa-Belmonte *et al.*, 1993). 1,1-Dioctadecyl-3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate (DiI) was focally injected into dorsal r5 or r6 at the 8- to 13-somite stage, allowed to develop to the 13- to 20-somite stage, then fixed and the DiI photoconverted to an oxidized diaminobenzidine precipitate. This provides a permanent signal in the labelled neuroepithelium and neural crest cells that had migrated from it, and was followed by *in situ* hybridization to visualize the *Krox-20*-expressing cells. The results are summarized in Table I.

r5 injections. Eleven injections into r5 were confined within the rhombomere boundaries both at the time of injection and after fixation (Table I). All of these r5 injections labelled pre-migratory neural crest cells at the level of r5 (Figure 3A–D and G). In 5/11 embryos, the injection also labelled neural crest cells that had migrated rostral to the otic vesicle (Table I; Figure 3A and B). These rostrally directed groups of cells lacked *Krox-20* expression, consistent with the absence of *Krox-20*-expressing pre-migratory neural crest cells in rostral r5 (Figure 2B). In 4/11 embryos, labelled cells were also observed in the caudally directed stream at the level of r6 (Figure 3A–D). Many DiI-labelled cells were observed adjacent to dorsal r5 and r6, between the neural tube and

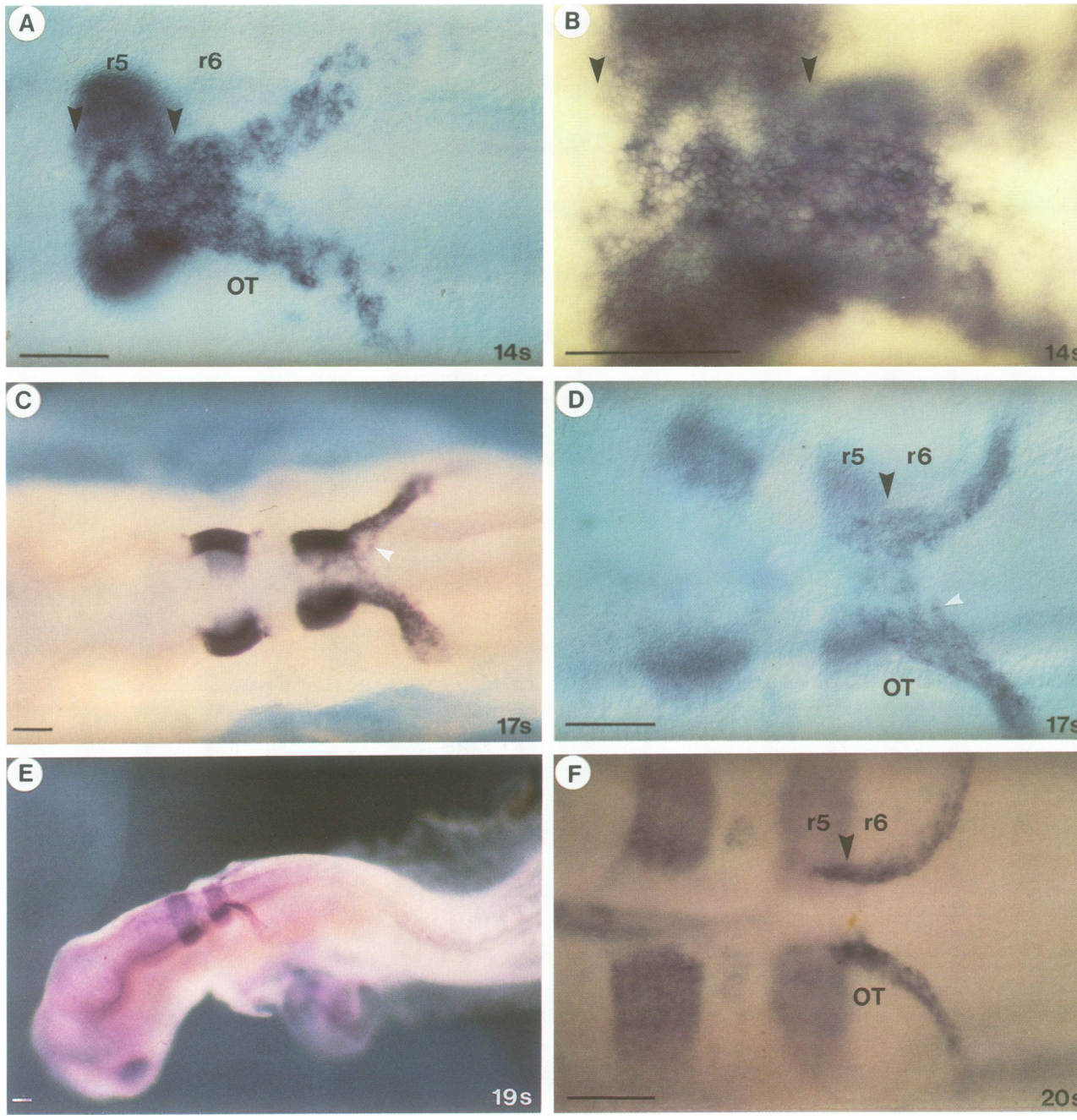

Fig. 2. Migration of *Krox-20*-expressing neural crest cells visualized by whole-mount hybridization. The somite stages are indicated on the lower right of each photograph. (**A**) Flat mount of the dorsal hindbrain in 14-somite embryo, focused on migrating crest. (**B**) Higher power view of flat mount shown in (A), focused on pre-migratory crest. (**C**) 17-Somite embryo. (**D**) Flat mount of dorsal hindbrain of 17-somite embryo. (**E**) 19-Somite embryo. (**F**) Flat mount of dorsal hindbrain of 20-somite embryo. In the flat-mount preparations, dorsal neural tube is medial and ventral tissue lateral. The stream of *Krox-20*-expressing crest is seen migrating adjacent and caudal to the otic vesicle (OT). The black arrows indicate boundaries of r5. The white arrows in (C) and (D) indicate a stream of crest cells apparently migrating from r6 to join the major stream contiguous with r5. Rostral is to the left in all photographs. The bars indicate 100 μm.

otic vesicle. However, only in one embryo fixed at the latest stages of detectable *Krox-20* expression (20-somite stage) were DiI-labelled cells observed in the most ventral portion of the *Krox-20*-expressing neural crest cells which extended around the otic vesicle and approached the third branchial arch (Table I; Figure 3C and D).

Injections at the r5/6 border. Four DiI injections spanned the r5/6 border. In 2/4 injections, the stream of *Krox-20*-expressing neural crest was more extensively DiI labelled than observed for injections into r5 alone analysed at the same stages (Figure 3E, F and H). This is consistent with the possibility that a significant proportion of the ventrally directed *Krox-20* stream arises from r6. Careful inspection of serial sections through DiI-labelled embryos suggests that labelled cells arising from caudal r5 and rostral r6 preferentially, but not exclusively, emigrate from the neural epithelium at the r5/6 border (not shown).

r6 injections. In 5/5 injections into r6, there was extensive labelling in the stream of *Krox-20*-expressing cells (Table I; Figure 4A–H). Significantly, DiI injections into mid to

Table I. Summary of DiI injections

Injection site	Somite stage of injection/analysis	Location of DiI-labelled neural crest			
		Ventral to r4	Dorsal r5	Dorsal r6	Ventral to r6
r5	8/16		⊕		
	8/16	+	⊕		
	8/16	+	⊕	⊕	
	9/13		⊕	⊕	
	9/16	+	⊕	⊕	
	10/14		⊕		
	10/16	+	⊕		
	10/19	+	⊕		
	10/19		⊕		
	12/20		⊕		
	12/20		⊕	⊕	⊕
caudal r5/	9/15		⊕	⊕	
rostral r6	9/16		⊕	⊕	
	9/17		⊕	⊕	⊕
	11/20		⊕	⊕	⊕
rostral r6	9/19			⊕	⊕
	10/17			⊕	⊕
mid-caudal	9/16				⊕
r6	11/19				⊕
	13/16				⊕

+ indicates DiI labelling detected in neural crest not expressing *Krox-20* (rostral to otic vesicle). ⊕ indicates DiI labelling detected in *Krox-20*-expressing neural crest. r5 injections with only rostrally directed streams of neural crest were not included in the analysis.

caudal r6 analysed at the 16- to 19-somite stage only labelled the ventral part of the stream of *Krox-20*-expressing cells, but failed to label neural crest cells above r5 or r6 (Figure 4A–F). This contrasts with injections into r5 or rostral r6, which labelled one or both of these populations (Figure 3). These observations are consistent with the *Krox-20*-expressing neural crest emigrating earlier from mid to caudal r6 than from r5 and rostral r6.

Destinations of r5 and r6 neural crest cells

Our results indicate that r5 contributes to the dorsal part and r6 contributes to the ventral part of the *Krox-20*-expressing caudal stream at the 16- to 19-somite stage. These conclusions are based on fixing embryos at early stages of migration, but the ultimate fate of these cells has not been tested. It is possible that r5-derived cells follow the earlier migrating r6 neural crest, or that they die and never reach the third branchial arch. To distinguish between these possibilities, embryos were injected with DiI as described above, but were allowed to develop to later stages. In 21- to 28-somite embryos, in which *Krox-20* expression is no longer detected in neural crest cells, viable DiI-labelled migratory neural crest cells were visible from both r5 and r6 injections. r5-derived neural crest cells were observed in the superior ganglion of the IXth nerve adjacent to the neural tube (Figure 5A and B). In addition, they often approached the third arch, where they contributed to the IXth nerve and associated petrosal ganglion or appeared as mesenchymal cells in association with the third aortic arch. These data suggest that caudally migrating r5 cells do not die. Injections into r6-labelled cells in the third and fourth branchial arches (Figure 5C). Labelled cells were observed as mesenchymal cells deep within the arch (near the fourth and fifth aortic arches), as well as in the IXth and Xth nerve and associated ganglia (petrosal and nodosal) at the dorsal portion of the arches. The DiI-labelled cells contributed to the non-neurogenic portion of the distal ganglia, as evidenced by their lack of neurofilament immunoreactivity (not shown), consistent with the epibranchial placode origin of neurons in the distal ganglia of cranial nerves VII, IX and X (Ayer-LeLievre and Le Douarin, 1982; D'Amico-Martel and Noden, 1983). Together with the above results, these data suggest that both r5 and r6 contribute neural crest cells that migrate into the third arch. In addition, r5 contributes labelled cells to the second branchial arch, whereas r6 contributes cells to the fourth branchial arch (Figure 5A and C).

Krox-20 expression in neural crest after rhombomere rotation

Our studies reveal that whereas r5 contributes neural crest to the second and third branchial arches, *Krox-20* expression occurs only in cells migrating to the latter and thus does not correlate with segmentation. This restriction of *Krox-20* expression in r5 neural crest appears to be initiated prior to emigration of these cells (Figure 2B). Two possible mechanisms can be envisaged to underlie the regulation of *Krox-20* expression in neural crest precursors. One possibility is that neural crest expression is specified autonomously in the neural epithelium through a rostrocaudal polarity or subdivision that is established within presumptive r5. The neural crest cells that express *Krox-20* then migrate caudal to the otic vesicle, whereas non-expressing cells migrate rostrally. Alternatively, extrinsic signals that exist rostral, but not caudal, to the otic vesicle could downregulate *Krox-20* expression in presumptive neural crest. To distinguish these possibilities, we analysed the effect of transplanting r5 and r6 to new locations by carrying out rostrocaudal rotations prior to neural crest emigration.

We first examined the expression of *Krox-20* after the rostrocaudal rotation of r5–r6 or r4–r6 at the 8- to 12-somite stage, followed by fixation at the 15- to 22-somite

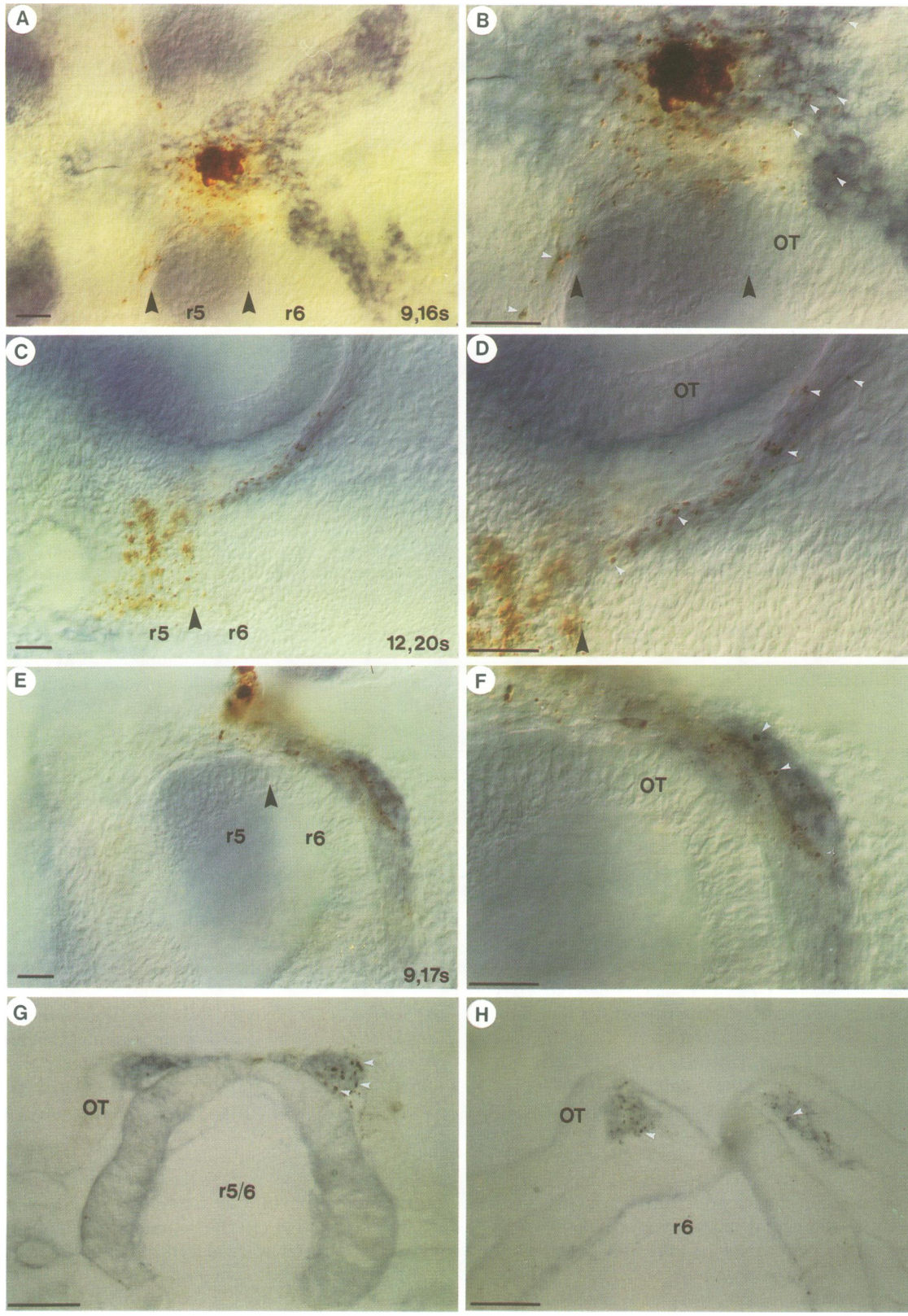

Fig. 3. Cell fate analysis of neural crest arising from r5 or r5/rostral r6. Embryos were injected into r5 or the r5/6 border with DiI and, after further development, fixed and the DiI signal photoconverted. After *in situ* hybridization to detect *Krox-20* transcripts, embryos were flat mounted to view the dorsal hindbrain and neural crest, and then sectioned. The somite stages of injection and fixation are indicated on the lower right. (**A**) Flat mount of embryo injected into r5 at 9 somites and fixed at 16 somites. (**B**) High-power view of (A). (**C**) Flat mount of embryo injected into r5 at 12 somites and fixed at 20 somites. (**D**) High-power view of (C). (**E**) Flat mount of embryo injected into the r5/6 border at 9 somites and fixed at 17 somites. (**F**) High-power view of (E). (**G**) Transverse section at level of r5/6 border of embryo shown in (A, B). (**H**) Transverse section at level of r6 of embryo shown in (E, F). The black arrows indicate boundaries of r5. The white arrows indicate examples of DiI-labelled neural crest cells seen either migrating rostral to the otic vesicle (OT) in (B) or above r5/r6 and caudal to the otic vesicle in (B, D, F–H). The bars indicate 50 μm.

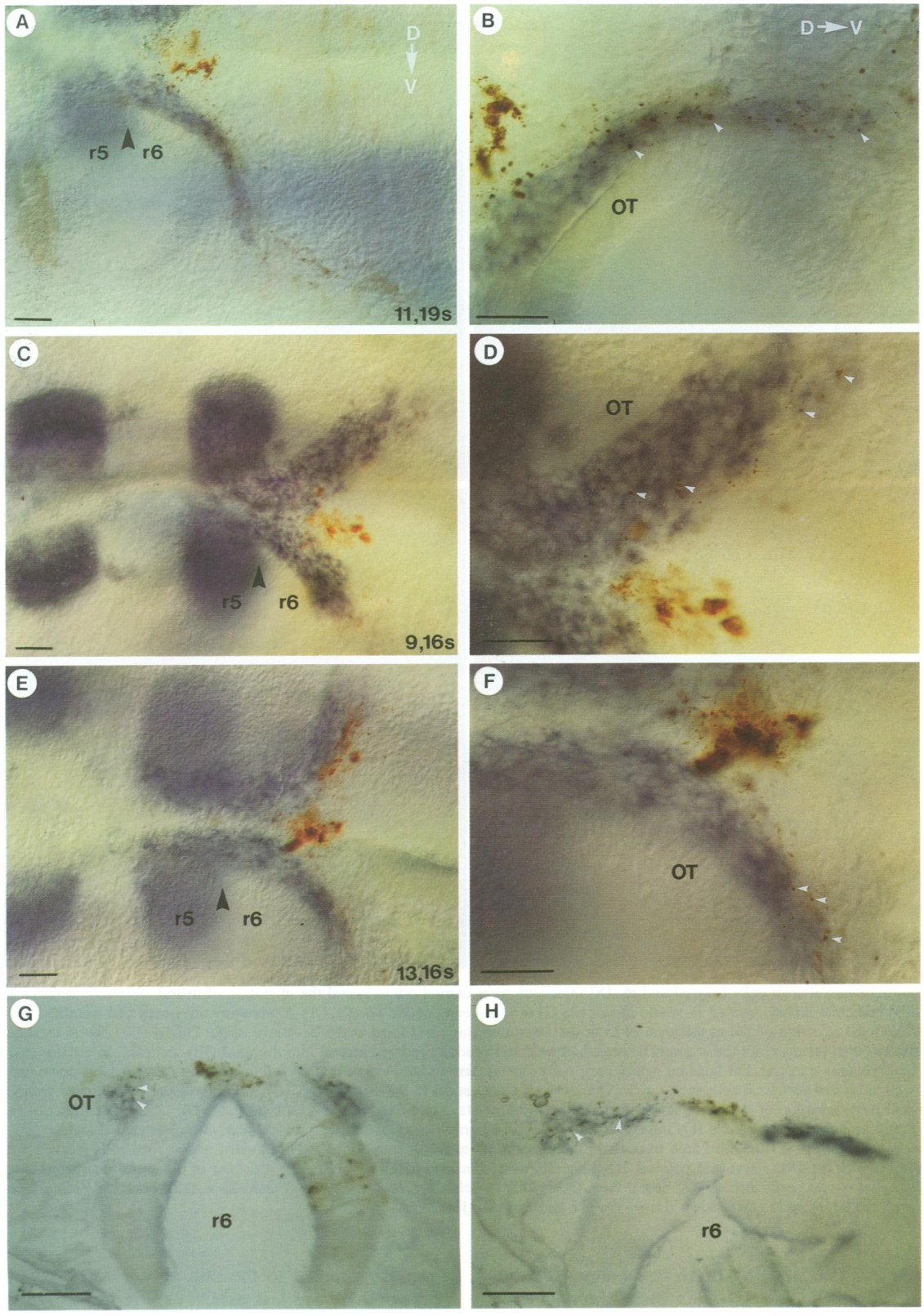

Fig. 4. Cell fate analysis of neural crest arising from r6. DiI injection into r6 and fixation followed by photoconversion were carried out at the indicated somite stages, and then *in situ* hybridization was performed with *Krox-20* probe. (**A**) Embryo injected into mid-caudal r6 at 11 somites and fixed at 19 somites. (**B**) Higher power photograph of (A), rotated by 90°, as indicated by D→V (dorsal→ventral). (**C**) Embryo injected into mid-caudal r6 at 9 somites and fixed at 16 somites. (**D**) High-power view of (C). (**E**) Embryo injected into mid-caudal r6 at 13 somites and fixed at 16 somites. (**F**) High-power view of (E). (**G**) Transverse section at level of r6 of embryo shown in (A, B). (**H**) Transverse section at level of r6 of embryo shown in (C, D). Black arrows indicate boundaries of r5. White arrows indicate examples of DiI-labelled cells. OT, otic vesicle. The bars indicate 50 μm.

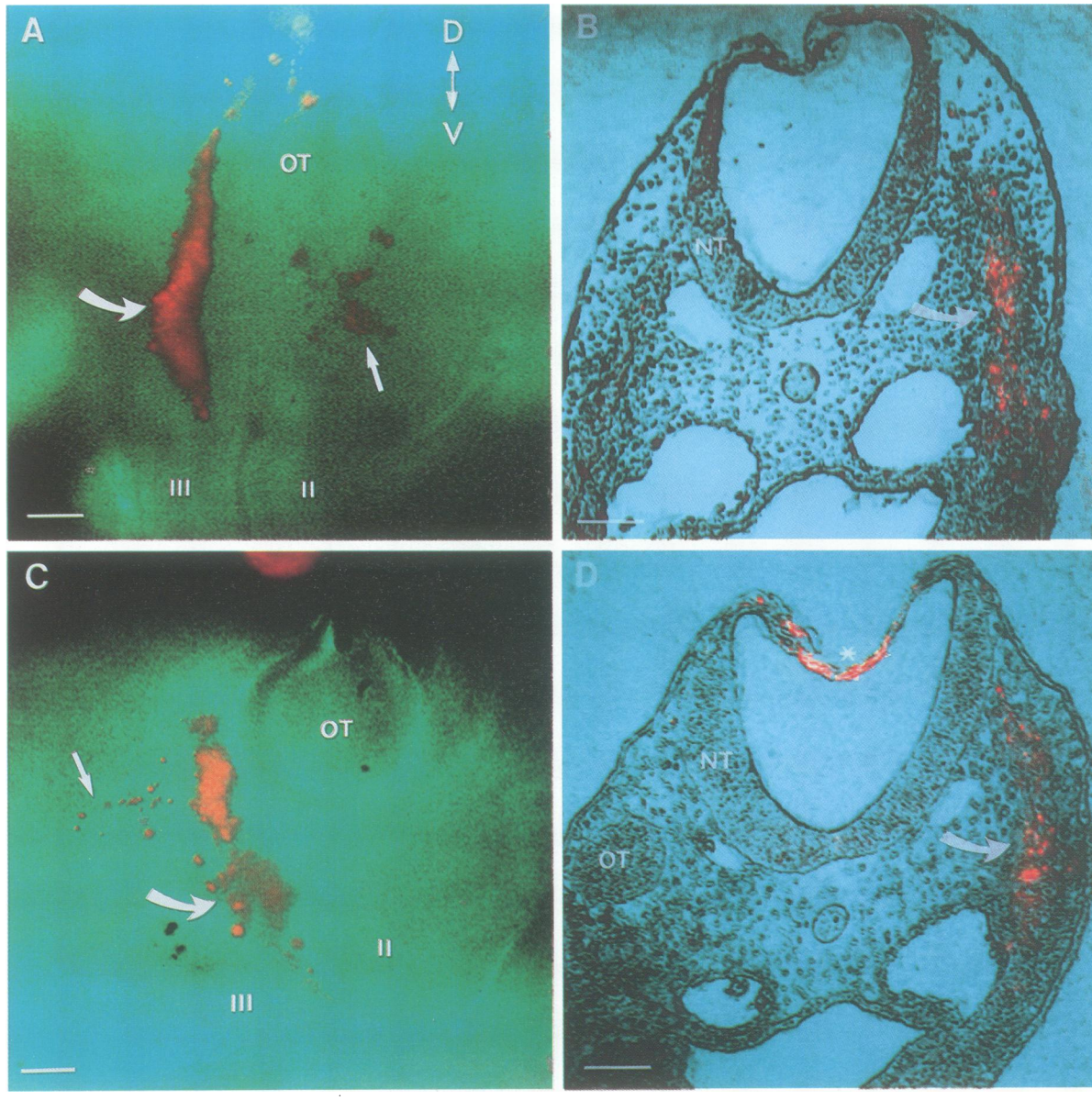

Fig. 5. Migration of neural crest cells from r5 and r6. DiI injections were carried out to reveal the migration of neural crest cells from r5 (A, B) or r6 (C, D) to the branchial arches, viewed in whole mount (A, C) or transverse section (B, D). (**A**) A whole-mount image of an embryo which received a small focal injection of DiI at the level of r5 at the 10-somite stage and fixed at the 25-somite stage. DiI-labelled neural crest cells migrated rostrally (small arrow) into the second (II) branchial arch and caudally (curved arrow) into the third (III) arch. (**B**) In a transverse section through the embryo shown in (A), DiI-labelled neural crest cells (curved arrow) are evident in association with the forming IXth nerve and petrosal ganglion as well as in mesenchymal cells in the arch. The section is just caudal to the otic vesicle. (**C**) A whole-mount image of an embryo which received a small focal injection of DiI at the level of r6 at the 14-somite stage and fixed at the 30-somite stage. DiI-labelled neural crest cells migrated caudally around the otic vesicle (OT) into the third (III; curved arrow) and toward the fourth (small arrow) branchial arches. (**D**) A transverse section at the level of the third arch through an embryo similar to that pictured in (C). DiI-labelled neural crest cells (curved arrow) are evident associated with the IXth nerve and petrosal ganglion. DiI-labelled neural tube cells were also observed in the dorsal neuroepithelium (*) of r6, where the injection was performed. NT = neural tube; OT = otic vesicle; II = second branchial arch; III = third branchial arch; all photographs are oriented with dorsal up and ventral down (D⟷V). The bars indicate 100 μm.

stage (Table II). DiI labelling of r6 or r4 indicates that neural crest migrates ventrally from these rhombomeres in accordance with their new location after rotation: from r6 rostral to the otic vesicle (Figure 6A and B) or from r4 caudal to the otic vesicle (Figure 6C and D). In 12/12 embryos, *Krox-20*-expressing neural crest cells were observed adjacent to the transplanted r6, rostral to the otic vesicle. The ventral extent of *Krox-20* expression in these cells varied from embryo to embryo. In three embryos (Figure 7A and B), the ventral extent was similar to that occurring caudal to the otic vesicle in normal embryos, but in others was less extensive (Figure 7C and D). These results indicate that neural crest cells from r6 can maintain *Krox-20* expression and migrate rostral to the otic vesicle. However, it is possible that there is a more rapid down-regulation of expression in this ectopic location.

It was not possible to determine from these experiments whether *Krox-20*-expressing cells were migrating from r5 and rostral to the otic vesicle, since these cannot be distinguished from those arising from r6. However, in

Table II. Summary of *Krox-20* expression in neural crest after r5/r6 rotation

Somite stage of rotation/analysis	Location of *Krox-20*-expressing neural crest			
	Rostral to OT		Caudal to OT	
	Dorsal to r6	Ventral migration	Contiguous with r5	Not contiguous with r5
8/21	+	++	+	
9/15	+	+		
9/16	+	+++		
9/16	+	+++		
9/16	+	++	+	
9/17	+	+		
9/18	+	+		
9/18	+	++	+	
9/19	+	+	+	+
11/17	+	++		+
11/20	+	+++		
12/17	+	++		+

+ indicates the presence of *Krox-20*-expressing neural crest cells in the indicated location. For the column labelled 'ventral migration' the number of + symbols indicates the extent of the ventral migration; +++ is similar to that occurring caudal to the otic vesicle in normal embryos.

4/12 embryos, *Krox-20*-expressing neural crest cells were observed contiguous with rotated r5 and migrating caudal to the otic vesicle (Figure 7C and D), suggesting that there was some regulation to achieve the correct post-otic expression or direction of migration after rotation. In 3/12 embryos, *Krox-20*-expressing neural crest cells were found more caudally and not contiguous with r5 (Figure 7D); it is possible that these cells have arisen from residual caudal r6 not included in the rotation, rather than from r5.

To examine whether *Krox-20*-expressing cells arising from r5 can migrate rostral to the otic vesicle, we performed rostrocaudal rotations of r4 and much of r5. These rotations were performed at the 8- to 12-somite stage and fixed for analysis at the 19- to 21-somite stage (Table III). In 9/9 embryos, *Krox-20*-expressing neural crest was observed migrating caudal to the otic vesicle, since they largely derive from r6 and residual caudal r5 which were left intact. Although in these rotations a portion (25–50%) of r5 was left adjacent to r6, in 7/9 embryos this stream of neural crest was not contiguous with the residual r5 in its normal orientation (Figure 8A and C). This is consistent with *Krox-20*-expressing neural crest cells emigrating earlier from r6 than from r5, such that r5 contributes the dorsal part of the stream; these cells emerging from mid-r5 have been translocated rostrally by tissue rotation. What is the fate of these transplanted cells? Although DiI labelling indicates that extensive neural crest cell migration from r5 occurs after r4/r5 rotation (Figure 6E and F), in 5/9 embryos *Krox-20* expression was detected in a very small number of neural crest cells immediately adjacent to either the endogenous (Figure 8B; same embryo as Figure 6E and F) or an ectopic (Figure 8C and D) otic vesicle. In 4/9 embryos, no *Krox-20* expression was detected in these cells (Figure 8A).

Discussion

Segmental origin and migration of neural crest expressing Krox-20 in the chick embryo

Our studies have revealed an unexpectedly complex picture of the relationship between hindbrain segmentation and the spatial restriction of *Krox-20* expression in neural crest. In whole-mount preparations, *Krox-20*-expressing neural crest cells are seen in a stream that courses caudally around the otic vesicle and towards the third branchial arch. The proportion of third arch neural crest derived from *Krox-20*-expressing precursors is not clear, since expression is downregulated during migration, but several lines of evidence suggest a major contribution. First, in transgenic mice in which lacZ expression is regulated by Krox-20 protein, the stable β-galactosidase reporter is observed in many neural crest cells in the third arch (Sham *et al.*, 1993). Second, the *Sek-1* gene is co-expressed with *Krox-20* in r3, r5 and post-otic neural crest (Nieto *et al.*, 1992), but expression is less transient and persists in neural crest cells that comprise a major portion of third arch mesenchyme (M.A.Nieto and D.G.Wilkinson, unpublished observations). We find that in the chick, *Krox-20* expression occurs in neural crest precursors not only in r5, but also in r6. By combining DiI labelling with *in situ* hybridization, we show that both of these precursor populations contribute to the stream of *Krox-20*-expressing neural crest and these cells appear to emigrate in a defined order, initially from r6 and shortly thereafter from r5 (summarized in Figure 9). In contrast, *Krox-20* expression is not detected in r5-derived neural crest cells migrating rostral to the otic vesicle, and this appears to be presaged by an absence of expression in many pre-migratory neural crest cells in rostral r5.

How is Krox-20 expression restricted to post-otic neural crest?

We find that neural crest from r6 continues to express *Krox-20* as it migrates ventrally after transplantation adjacent to the rostral side of the otic vesicle. In some transplantations, *Krox-20*-expressing neural crest cells migrating rostral to the otic vesicle were detected less ventrally than normally occurs caudal to the otic vesicle. This could be due to a delay in migration, but we cannot rule out that the environment causes a premature downregulation of expression. Nevertheless, our findings indicate that *Krox-20* expression in neural crest arising from r6 is autonomous at the time of transplantation. Similarly, expression of the *Hoxa-2* gene in neural crest arising from r2 and r4 has been found to be independent of the local environment (Prince and Lumsden, 1994). However, unlike r5, neural crest cells arising from r6 are not, during normal development, confronted by the need to express distinct phenotypes in accordance with pre- or post-otic migration. It may, therefore, be significant that our transplantation experiments reveal a difference in *Krox-20* expression in neural crest arising from r5. Although a large number of neural crest cells migrate ventrally from rostrally transplanted r5, *Krox-20* expression is detected in, at most, a minor population of cells immediately adjacent to the otic vesicle. One interpretation is that the phenotype of r5-derived neural crest cells may be influenced by extrinsic signals that lead to a

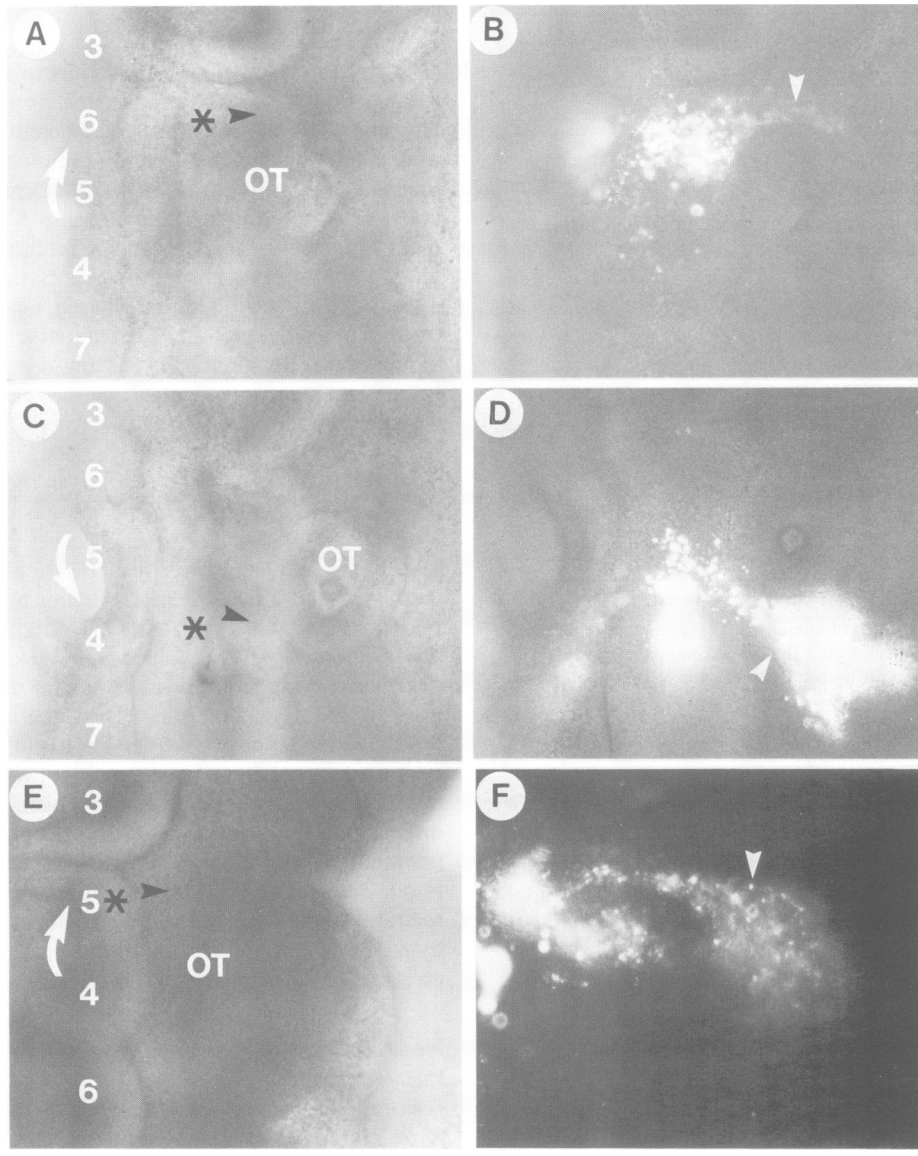

Fig. 6. Migration of neural crest cells from r4, r5 and r6 after rotation to new location. DiI was injected into r4, r5 or r6 in order to reveal neural crest cell migration from these rhombomeres after the rotation of the r4−r6 region. On the left are shown bright-field images, and on the right the corresponding fluorescence image of the DiI label. Left-hand panels: the rotation is indicated by the curved white arrow, the site of DiI labelling by *, and the direction of labelled neural crest migration by a black arrow. Right-hand panels: migrating neural crest is indicated by the white arrow. (**A**, **B**) r6 labelled at the 9/10-somite stage, followed by r4−r6 rotation and analysis at the 19-somite stage. Labelled neural crest cells are observed migrating rostral to the otic vesicle (OT). (**C**, **D**) r4 labelled at the 9/10-somite stage, followed by r4−r6 rotation and analysis at the 20-somite stage. Labelled neural crest cells are detected migrating caudal to the otic vesicle. (**E**, **F**) r5 labelled at the 8-somite stage, followed by r4−r5 rotation and analysis at the 19-somite stage. Labelled neural crest cells have migrated extensively rostral to the otic vesicle. This embryo corresponds to that of Figure 8B which, in contrast, indicates *Krox-20* expression only in a few neural crest cells adjacent to r5.

downregulation of *Krox-20* expression in pre-otic cells, which based on the pattern of expression in normal embryos appears to be initiated prior to emigration. Another possibility is that the cells detected migrating to the second arch derive from non-expressing precursors, while *Krox-20*-expressing cells fail to migrate. It is intriguing that in some embryos *Krox-20*-expressing cells migrated caudally from r5 after r5/r6 rotation, since this is suggestive of regulation towards the normal expression in post-otic neural crest. These could be cells derived from rostral r5 that, in the post-otic environment, have not downregulated expression. Alternatively, it is possible that there is a preferential caudal migration of r5-derived *Krox-20*-expressing cells.

Implications for models of branchial neural crest patterning

Current models propose that there is a coordinate segmental specification of *Hox* gene expression in rhombomeres and neural crest that underlies branchial arch patterning (Hunt *et al.*, 1991). However, this proposal is inconsistent with the finding that r3 and r5 each contribute neural crest cells to two arches (Sechrist *et al.*, 1993): the *Hox* code of r5 correlates with the third, but not the second arch, yet contributes neural crest to both.

The relationship between the function of the *Krox-20* and *Hox* genes is at present unclear, but it seems likely that their expression may be coupled to similar or identical upstream mechanisms that establish the rostrocaudal

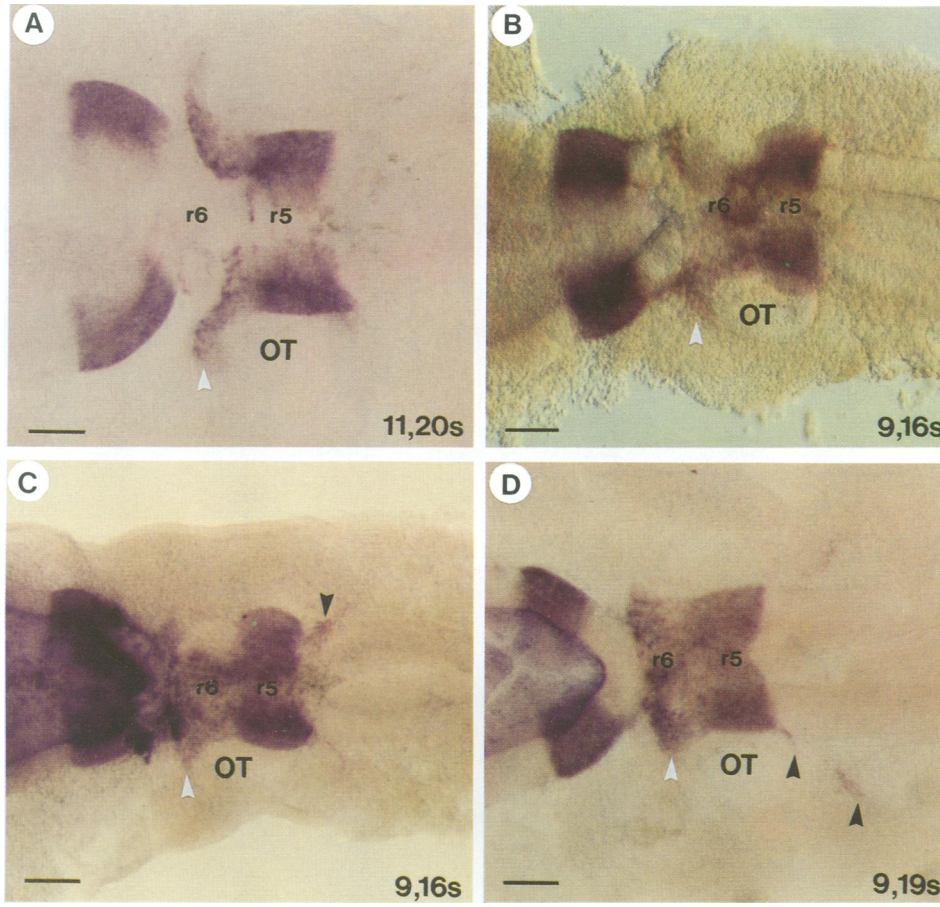

Fig. 7. Expression of *Krox-20* in neural crest after r5/r6 rotation. r5 and r6 were rotated at the 8- to 12-somite stage, prior to migration of neural crest from these rhombomeres, then fixed at the 15- to 21-somite stage for *in situ* hybridization with *Krox-20* probe. The somite stage of rotation and fixation are indicated at the bottom right of each photograph (**A**–**D**). The white arrows indicate *Krox-20*-expressing neural crest cells rostral to the otic vesicle (OT). The black arrows indicate *Krox-20*-expressing neural crest cells caudal to the otic vesicle; in (C) and (D) these are contiguous with rotated r5, and in (D) a more caudal, non-contiguous population is also observed. The bars indicate 100 μm.

identity of neural crest. *Hoxb-3* gene expression occurs in r5, where it is expressed at high relative levels, and in neural crest migrating to the third, but not the second, branchial arch. It is attractive to propose that, as for *Krox-20*, this pattern is achieved by a specification of expression only in neural crest precursors in r5 that selectively migrate caudal to the otic vesicle. Indeed, a direct coupling may occur between *Krox-20* and *Hox* gene expression. The transcription factor encoded by the *Krox-20* gene binds to an enhancer element of the *Hoxb-2* gene that upregulates expression in r3, r5 and post-otic neural crest (Sham *et al.*, 1993). For this *Hox* gene, expression also occurs in r4 and neural crest migrating from r4 to the second arch (regulated by Krox-20-independent elements), and thus a restriction to post-otic r5 neural crest is not required to establish the appropriate branchial *Hox* code. However, it will be very interesting to ascertain whether the *Hoxb-3* gene is also regulated by Krox-20 since this could underlie the restriction of expression to post-otic neural crest.

A potential role of the conserved expression of *Krox-20* in neural crest destined for the third arch is revealed by studies of mouse embryos with null mutations in the *Krox-20* gene (Schneider-Maunoury *et al.*, 1993). In heterozygous mutant embryos, lacZ expression from the disrupted *Krox-20* locus occurs in a pattern identical to *Krox-20* transcripts and only in post-otic crest. However, in homozygous mutants, lacZ expression is detected in neural crest cells both rostral and caudal to the otic vesicle,

Table III. Summary of *Krox-20* expression in neural crest after r4/r5 rotation

Somite stage of rotation/analysis	Location of *Krox-20*-expressing neural crest		
	Rostral to OT	Caudal to OT	
		Contiguous with r5	Not contiguous with r5
8/19	+	+	
8/19	+	+	
9/20	+		+
11/20			+
11/20			+
11/21			+
11/21	+		+
12/20			+
12/21	+		+

+ indicates the presence of *Kros-20*-expressing neural crest cells in the indicated location. Only a small number of cells are observed rostral to the otic vesicle.

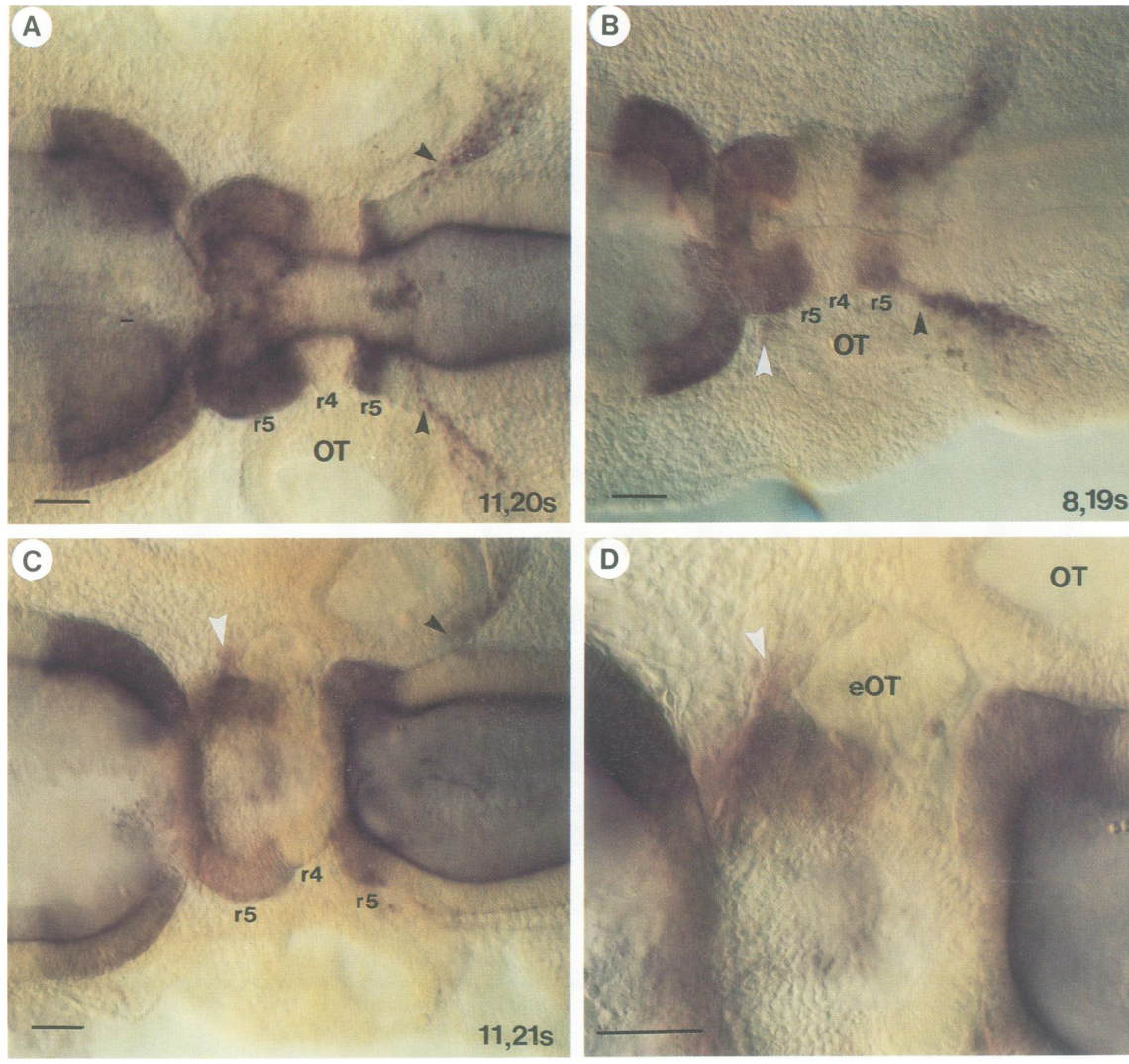

Fig. 8. Expression of *Krox-20* in neural crest after r4/r5 rotation. r4 and part of r5 were rotated at the 8- to 12-somite stage, then fixed at the 19- to 21-somite stage for *in situ* hybridization with *Krox-20* probe. The somite stage of rotation and fixation are indicated at the bottom right of each photograph (**A**–**C**); (**D**) is a higher magnification view of (C). The white arrows indicate the small number of *Krox-20*-expressing neural crest cells rostral to the endogenous (OT) or ectopic (eOT) otic vesicle. The black arrows indicate *Krox-20*-expressing neural crest cells caudal to the otic vesicle; in (A) and (D) these are not contiguous with the rotated r5, but are contiguous in (B). The bars indicate 50 μm.

with no alteration in the location of the otic vesicle that might explain this apparent difference in migration. Although defects in branchial arch patterning have not been reported, this raises the intriguing possibility that *Krox-20* function is required for the appropriate rostrocaudal migration of cells from r5.

Is the regulation of Krox-20 expression in neural crest conserved in other vertebrates?

Studies in the mouse embryo have detected *Krox-20* expression in neural crest cells contiguous with r5 that migrate caudal to the otic vesicle to the third branchial arch (Sham *et al.*, 1993). A similar pattern is also found in zebrafish, although the destination of the *Krox-20*-expressing neural crest cells was not apparent (Oxtoby and Jowett, 1993). In these detailed analyses, expression was not detected in neural crest precursors in r6 and it was suggested that *Krox-20*-expressing neural crest cells arise from r5 (Oxtoby and Jowett, 1993; Sham *et al.*, 1993), although this has not been examined rigorously by cell-marking studies. Studies of *Krox-20* expression in *Xenopus* present a less ambiguous picture of the relationship between segmentation and neural crest expression (Bradley *et al.*, 1992). In this system, the *Krox-20*-expressing neural crest cells are precisely aligned with r5;

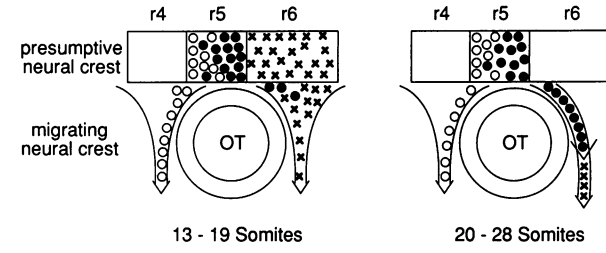

Fig. 9. Summary of the migration pattern of *Krox-20*-expressing neural crest. Pre-migratory neural crest in dorsal r5 and r6 is depicted in the upper part, and migrating neural crest shown below. r5 neural crest cells not expressing *Krox-20* are indicated with empty circles, r5 neural crest cells expressing *Krox-20* are indicated with filled circles and r6 neural crest cells are indicated with crosses. OT, otic vesicle.

this correspondence can be seen because, due to the location of the otic vesicle adjacent to r4 in *Xenopus*, these neural crest cells migrate in a direct ventral route to fill the third arch. It therefore seems highly likely that in *Xenopus* neural crest expression of *Krox-20* is specified coordinately with r5.

These data suggest that whereas the restriction of *Krox-20* expression to third arch neural crest is conserved, there is a diversity in the segmental origin of these cells. In *Xenopus*, all neural crest cells adjacent to r5 express *Krox-20*, but in the chick expressing and non-expressing precursors are found in r5. It is possible that this difference is associated with the location of the otic vesicle, which in the chick, but not *Xenopus*, may channel neural crest cells from rostral r5 towards the second branchial arch. The subsegmental specification of neural crest expression in r5 in the chick may therefore be required to establish the appropriate restriction to the third arch. In the mouse and zebrafish, the otic vesicle is located adjacent to r5, so it will be important to ascertain how the relationship between *Krox-20* expression in neural crest and the patterns of cell migration is established in these species.

A further evolutionary difference is the expression of *Krox-20* in neural crest precursors in r6 in the chick, but not in the other vertebrate species examined. The significance of this difference is obscure, but it is relevant to ask what mechanism might underlie this specification of *Krox-20* expression in r6 crest precursors. It is possible that *Krox-20* expression in r6 crest precursors has no mechanistic relationship with r5. However, another possibility is suggested by the physical continuity between *Krox-20* expression in prospective r5 and r6 from the onset of expression: all crest cells destined to express *Krox-20* may be specified in presumptive r5 and some move caudally within the neuroepithelium into presumptive r6. Similarly, it is possible that non-expressing cells in rostral r5 have been specified in presumptive r4. Indeed, preliminary results from cell-marking experiments indicate that neural crest precursors tend to shift caudally relative to more ventral neural epithelium (E.Birgbauer, J.Sechrist, M.Bronner-Fraser and S.Fraser, unpublished observations) and it will be interesting to analyse whether this contributes to the expression pattern of *Krox-20* in the hindbrain and its restriction to third arch crest.

Materials and methods

Preparation of embryos for microinjection with DiI

White Leghorn chick embryos were incubated at 38°C until they reached the desired stages of development [7–13 somites; stages 9–15 according to Hamburger and Hamilton (1951)]. A window was cut in the egg shell to gain access to the embryo, then India Ink (Pelikan Fount) was injected under the blastoderm to aid in visualization of the embryo. The vitelline membrane was deflected and the embryonic age was determined by counting the number of somites in the embryo.

Focal DiI labelling of rhombomeres 5 and 6

A solution of 0.5% w/v DiI (Molecular Probes) was made up in 100% ethanol. The injection micropipettes were back-filled with the DiI solution, mounted onto a Narashige micromanipulator and connected to a forced air pressure injection apparatus. The tips of the pipettes were broken to have an opening of ~20 μm.

The neural crest at the level of r5 or r6 was labelled by inserting a micropipette into the dorsal neural tube and expelling a very small amount of the DiI solution, as described in Sechrist *et al.* (1993). At the time of the injection, the injection site was visible as a small red spot of dye in the tissue through the dissecting microscope. In some embryos, the location of the dye label was verified under the epifluorescence microscope immediately after injection and recorded photographically. After injection, the eggs were sealed with cellophane tape and returned to the incubator until the indicated times of fixation.

Rhombomere rotations

Using fine glass needles, a lateral cut was made between the neural tube and adjacent mesenchyme as well as under the notochord. After separating the neural tube/notochord from adjacent tissue, they were cut transversely at the borders between selected rhombomeres. The neural tube plus the notochord was freed from adjacent tissue and rotated 180° rostrocaudally, while maintaining dorsoventral polarity. This resulted in transposition of the positions of rhombomeres.

Cryostat sections

After fixation and photography, embryos were washed in phosphate-buffered saline (PBS), and then placed sequentially in 5 and 15% sucrose in PBS for 4–24 h at 4°C. Embryos were embedded in 15% sucrose and 7.5% gelatin (Sigma, 300 Bloom) for 4 h at 37°C, followed by embedding in fresh gelatin, which was allowed to solidify. Immediately before sectioning, embryos were quick frozen in liquid nitrogen and allowed to equilibrate to −25°C prior to cutting transverse 10 μm sections on a Zeiss Microm cryostat. Sections of DiI-labelled embryos were viewed without a coverslip shortly after sectioning. In some cases, they were stained with antibodies against neurofilament proteins using a monoclonal antibody against the non-phosphorylated form of the intermediate molecular weight neurofilament protein (kindly provided by Dr Virginia Lee; Lee *et al.*, 1987). Cryostat sections were warmed to 25°C prior to antibody staining. Approximately 20 μl of antibody solution, diluted 1:300 in PBS containing 0.1% bovine serum albumin (BSA), were applied to each section and incubated overnight in a humidified chamber either at 4 or 25°C. After incubation with primary antibodies, sections were washed in PBS for 5 min and incubated for 1–1.5 h with fluorescein isothiocyanate (FITC)-conjugated antibodies against mouse IgGs. Sections were washed in PBS and coverslipped with gelmount (Biomeda). Both whole mounts and sections were imaged processed using the Vidim software system (S.Fraser, J.Stolberg and B.Belford, unpublished).

Photoconversion of DiI

Embryos were fixed in 4% paraformaldehyde at 4°C overnight. They were subsequently rinsed with PBS (pH 7.4), followed by 0.1 M Tris−HCl (pH 7.4) for 30 min at 4°C. After removing the Tris buffer, embryos were placed in a solution of 5 mg/10 ml diaminobenzidine in 0.1 M Tris−HCl (pH 7.4) at 4°C for 30 min. They were then placed in well slides, coverslipped and illuminated for 10–20 min under the epifluorescence microscope (rhodamine filter set), refocusing every 5 min. Care was taken to stop illumination when a brown precipitate was first observed, so as to not obscure the signal for *in situ* hybridization; thus, the photoconverted signal provides an underestimate of the number and intensity of DiI-labelled cells. After photoconversion, the embryos were rinsed in 0.1 M Tris−HCl (pH 7.4) for 30 min and finally placed in methanol, in which they are stable for up to a week.

In situ hybridization

Chick *Krox-20* sequences (Nieto *et al.*, 1991) were used for whole-mount *in situ* hybridization, essentially as described by Nieto *et al.* (1995).

Flat mounting and sectioning of whole-mount hybridized embryos

Embryos were equilibrated in 3:1 glycerol/PBT (PBS, 0.1% Triton X-100), and flat mounts of the neural epithelium prepared by cutting through the dorsal and ventral midline of the hindbrain and mounting under a coverslip. Flat mounts to view the neural crest were prepared by dissecting out the branchial region and slitting through the ventral part of the neural tube and underlying tissue with a tungsten needle. This was then mounted under a coverslip with the ventral tissue splayed out and the dorsal neural tube at the midline. To prepare sections, embryos were incubated overnight at 4°C in 4% formaldehyde in PBT in order to fix the *in situ* hybridization stain. The embryos were then washed with PBT twice for 10 min, with methanol for 5 min, with isopropanol for 10 min, with tetrahydronaphthalene for 15 min, with tetrahydronaphthalene plus an equal volume of paraffin wax at 60°C for 10 min, and finally with three changes of paraffin wax at 60°C for 15 min each. The embedded tissue was orientated in an embryological dish, and 10 μm sections cut in the transverse plane, placed on TESPA-

subbed slides and dried overnight at 37°C. The wax was removed by two 10 min incubations in Histoclear and the sections mounted under a coverslip with DPX mounting agent (BDH).

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