

# A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo

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## SUMMARY

Cells of the cranial paraxial mesoderm give rise to parts of the skull and muscles of the head. Some mesoderm cells migrate from locations close to the hindbrain into the branchial arches where they undergo muscle differentiation. We have characterised these migratory pathways in chick embryos either by DiI-labelling cells before migration or by grafting quail cranial paraxial mesoderm orthotopically. These experiments demonstrate that depending on their initial rostrocaudal position, cranial paraxial mesoderm cells migrate to fill the core of specific branchial arches. A survey of the expression of myogenic genes showed that the myogenic markers *Myf5*, *MyoD* and *myogenin* were expressed in branchial arch muscle, but at comparatively late stages compared with their expression in the somites. *Pax3* was not expressed by myogenic cells that migrate into the branchial arches despite its expression in migrating precursors of limb

muscles. In order to test whether segmental plate or somitic mesoderm has the ability to migrate in a cranial location, we grafted quail trunk mesoderm into the cranial paraxial mesoderm region. While segmental plate mesoderm cells did not migrate into the branchial arches, somitic cells were capable of migrating and were incorporated into the branchial arch muscle mass. Grafted somitic cells in the vicinity of the neural tube maintained expression of the somitic markers *Pax3*, *MyoD* and *Pax1*. By contrast, ectopic somitic cells located distal to the neural tube and in the branchial arches did not express *Pax3*. These data imply that signals in the vicinity of the hindbrain and branchial arches act on migrating myogenic cells to influence their gene expression and developmental pathways.

Key words: Mesoderm, Branchial arch, Chick, Myogenesis, Muscle

## INTRODUCTION

During the development of the vertebrate embryo, populations of mesoderm cells assume distinct identities depending on their positions within the embryonic body plan. One important early difference concerns the organisation of the paraxial mesoderm of the head and trunk. Along the rostrocaudal axis, the trunk paraxial mesoderm is organised into metameric units – the somites, while the cranial paraxial mesoderm shows no obvious segmentation. Both cranial and trunk paraxial mesoderm eventually give rise to a range of tissue derivatives including muscle cell populations with common features, but with specific positional identities. The question therefore arises as to whether cranial and trunk paraxial mesoderm cells develop according to similar or different developmental principles. A related question concerns the degree to which the patterning of mesoderm cells at any axial level is intrinsic or is governed by interactions with other tissues.

While there have been few studies on the cranial paraxial mesoderm, somite development has been investigated in detail. Somites develop in a rostrocaudal sequence from the segmental plate mesoderm flanking the neural tube (reviewed in Keynes and Stern, 1988). As soon as the epithelial somite has formed,

a subdivision into rostral and caudal parts ensues. The migration of neural crest cells to form the dorsal root ganglia and outgrowth of motor axons is regulated by permissive and repellent cues originating in the rostral and caudal somite, respectively (Keynes and Stern, 1984; Rickmann et al., 1985; Aoyama and Asamoto, 1988; Kalchek and Teillet, 1989). It is therefore the metameric organisation of the somites that is critical in ensuring the periodic arrangement of the dorsal root ganglia and the segmentation of the spinal nerves. Initially the segmental plate and immature somites lack any dorsoventral specification, as 180° rotation (in a D/V direction) of either of these tissues has no effect on subsequent patterning (Aoyama and Asamoto, 1988). However, the somite matures and compartmentalises forming the dermomyotome dorsolaterally and the sclerotome ventromedially (giving rise to the axial skeleton). Further maturation of the dermomyotome leads to the formation of the dermatome, which gives rise to the dermis, and the myotome, which form the muscles of the back and striated muscle of the body wall. The regionalisation within the dorsoventral and mediolateral axes of the somite occurs as a result of interactions with neighbouring structures such as the notochord, lateral plate mesoderm, neural tube and ectoderm (e.g. Pourquié et al., 1996; Münsterberg et al., 1995; Spence et

al., 1996; Fan and Tessier-Lavigne, 1994; Pownall et al., 1996). At the level of the limbs and occipital somites, extensive migration of the myotome-derived cells gives rise to the limb and tongue musculature, respectively (Ordahl and Le Douarin, 1992; Bober et al., 1994; Couly et al., 1993; Noden, 1983a; Hazelton, 1970).

By contrast with the somites, the cranial paraxial mesoderm lying rostral to the first somite forms a continuous strip with no overt rostrocaudal segmentation. The subdivision of the cranial paraxial mesoderm into somite-like entities, or somitomeres, has been proposed (Meier, 1979; Meier and Tam, 1982). However, this 'segmentation' has been identified only using scanning electron microscopy (Meier and Tam, 1982) and there are no characterised molecular heterogeneities in this region. Moreover, the cranial paraxial mesoderm shows no obvious dorsoventral compartmentation into sclerotomal and dermomyotomal entities. In spite of this lack of overt subdivision, however, the cranial paraxial mesoderm gives rise to separate cell lineages, including craniofacial muscles and some bones of the chordal skull (e.g. supra occipital, sphenoid, pars canalicularis and cochlearis of the otic capsule; Couly et al., 1993; Noden, 1983a). By contrast with the cranial paraxial mesoderm, other cranial structures such as the neural tube and the branchial arches are arranged in a segmental manner (reviewed in Lumsden and Krumlauf, 1996). Neural crest cells migrate from specific axial levels of the dorsal neural tube in three separate streams to line the periphery of the first three branchial arches (Lumsden et al., 1991; Trainor and Tam, 1995) and later form much of the skull and connective tissue (Couly et al., 1993; Köntges and Lumsden, 1996). Cranial neural crest at premigratory stages is thought to possess some intrinsic patterning information that it conveys to the periphery. For example, heterotopic transplantation studies have shown that, when transplanted to an ectopic location, the crest forms branchial-arch-derived structures corresponding with its original axial position (Noden, 1983b, 1985, 1988). In addition to forming a segmental series of structures, the neural crest may thus be involved in patterning the muscles within the branchial arches. However, the possibility remains that the cranial paraxial mesoderm plays some role in setting up the segmental pattern of cranial structures or in conveying positional information to the periphery.

In the mouse, the cranial paraxial mesoderm migrates to fill the cores of the branchial arches. Injection of DiI (a lipophilic marker) into rostral cranial paraxial mesoderm labels cells that fill the first arch, whilst more caudal injections label cells that fill more caudal arches (Trainor and Tam, 1995). These data provide some evidence for spatially restricted migration patterns but, in this study, the migratory routes of the cranial paraxial mesoderm cells adjacent to the hindbrain were mapped at only three locations which lay at some distance from one another. It was therefore impossible to identify the extent of possible overlap between the migratory pathways of adjacent mesoderm populations. Experiments in zebrafish, mapping the migration at all levels of the cranial mesoderm, demonstrated that the mesodermal cells in the arches arise from overlapping populations within the cranial paraxial mesoderm (Schilling and Kimmel, 1994).

We have carried out a detailed investigation of the migratory behaviour of cranial paraxial mesoderm into the branchial arches using DiI injections in chick embryos and orthotopic

grafts of quail tissue into chick embryos. In order to identify molecular differences between mesoderm populations the expression patterns of the myogenic genes (*Myf5*, *MyoD* and *myogenin*) and of *Pax3* and *Pax7* has been investigated. We have investigated whether the cranial paraxial mesoderm contains any intrinsic patterning information, by grafting mesoderm populations heterotopically, and investigating whether both migration and molecular differences are maintained or altered in the ectopic location.

## MATERIALS AND METHODS

Rhode Island Red hens' eggs were incubated for 1-5 days at 38°C in a humidified incubator up to stages 7-25 (Hamburger and Hamilton, 1951). For all operations, host eggs were windowed and embryos made visible by sub-blastodermal injection of India ink (Pelikan, 1:20 dilution in Howard's Ringer). Microsurgery or DiI injection was performed through a small opening in the vitelline membrane made using flame-sharpened needles of 100 µm diameter tungsten wire.

### DiI injections

For DiI injections, stage 7<sup>+</sup> to 8 (2-4 somites) chick embryos were used. A solution of DiI C<sub>18</sub> (Molecular Probes, D-282) was made at 3 mg/ml in ethanol (Honig and Hume, 1986). Dye was injected using a pressure-injection unit as described in Lumsden et al. (1991). The rostrocaudal position of the injection site was recorded using an eyepiece graticule (one unit is equivalent to 25 µm) aligned to the rostral boundary of the first somite (position 0 in Fig. 1A), individual injections of DiI larger than 40 µm were discarded. The position of the injection spanned the extent of the cranial paraxial mesoderm adjacent to the hindbrain and midbrain (a region of 1000 µm, see results). The eggs were sealed with tape and incubated for a further 36-48 hours to stage 16-20, before being fixed in 3.5% paraformaldehyde for 2-12 hours at 4°C. After fixation, embryos were bisected sagittally and both halves mounted in a solution of 90% glycerol/2.5% DABCO anti-fade agent (BDH) in PBS beneath propped coverslips and viewed under epifluorescence. Fluorescent images were collected using confocal microscopy.

### Transplantation experiments

Using flame-sharpened needles, three tissues were dissected from quail embryos: cranial paraxial mesoderm, approximately one rhombomere in length was dissected at different hindbrain levels from 2-, 3- or 4-somite embryos (see Fig. 1B,C); somites and similarly sized pieces of segmental plate mesoderm at various axial levels from stage 8-12 embryos (see Fig. 1B). Care was taken to remove all surrounding tissues, i.e. endoderm, ectoderm and neuroepithelium. Dissections were aided by brief incubation (3-5 minutes) in Dispase (Boehringer; 1 mg/ml in 1× Hanks Balanced Salt Solution (HBSS; Gibco)). Tissue pieces were washed extensively and stored in 1× HBSS on ice.

A small incision in the surface ectoderm was made on the right-hand side of the host embryo and a region of the underlying cranial or somitic paraxial mesoderm corresponding in size to the donor tissue was removed. Quail tissue was transferred to the excision site and manoeuvred into position (Fig. 1B) in a random orientation. Operated eggs were sealed with tape and returned to the incubator for 36-60 hours to stage 16-22. Deformed or poorly developed embryos were excluded from further analysis. All remaining embryos were fixed in 3.5% paraformaldehyde for 2-24 hours at 4°C before being processed for immunohistochemistry or in situ hybridisation.

### In situ hybridisation

Whole-mount in situ hybridisation was performed as published (Henrique et al., 1995; detailed protocol available upon request); in

situ hybridisation on sections was performed essentially as described by Schaeren-Wiemers and Gerfin-Moser (1993). Probes used were *Myf5* (1108 bp *EcoRI* fragment; Saitoh et al., 1993), *MyoD* (900 bp *PstI-EcoRI* fragment; Dechesne et al., 1994), *myogenin* (1200 bp *EcoRI* fragment; Fujisawa-Sehara et al., 1990), *Pax3* (as in Goulding et al., 1993), *Pax7* (as in Goulding et al., 1994) and *Pax1* (as in Ebensperger et al., 1995).

After staining, embryos were imbedded in 20% gelatin and transverse sections cut at 75  $\mu\text{m}$  on a vibratome.

### Immunohistochemistry

Whole-mount immunostaining was carried out as described in Guthrie and Lumsden (1992) using monoclonal QCPN anti-quail antibody (developed by B. and J. Carlson, obtained from the Developmental Studies Hybridoma Bank of The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD).

For polyester wax sectioning, embryos were fixed in 3.5% paraformaldehyde for 2-4 hours before dehydration in ethanol. Embryos were then embedded in polyester wax according to Kent (1991). 7  $\mu\text{m}$  sections were mounted on 1% gelatin-covered slides and allowed to dry overnight. Sections were dewaxed by rehydration through an ethanol series before being washed extensively in PBS and blocked for 30 minutes with 10% sheep serum in PBS. Sections were incubated overnight at 4°C in primary antibody QCPN or anti-desmin polyclonal antibody (used at a dilution of 1:20; Sigma) in PBS containing 1% sheep serum and 0.1% Tween 20. Sections were further washed in PBS before application of secondary antibodies (anti-mouse Cy3-conjugated, 1:200; or anti-rabbit FITC-conjugated, 1:100; Jackson ImmunoResearch Laboratories) for 3 hours at room temperature. After washing in PBS, sections were mounted in 90% glycerol/2.5% DABCO and fluorescent images were collected by laser-scanning confocal microscopy (Biorad).

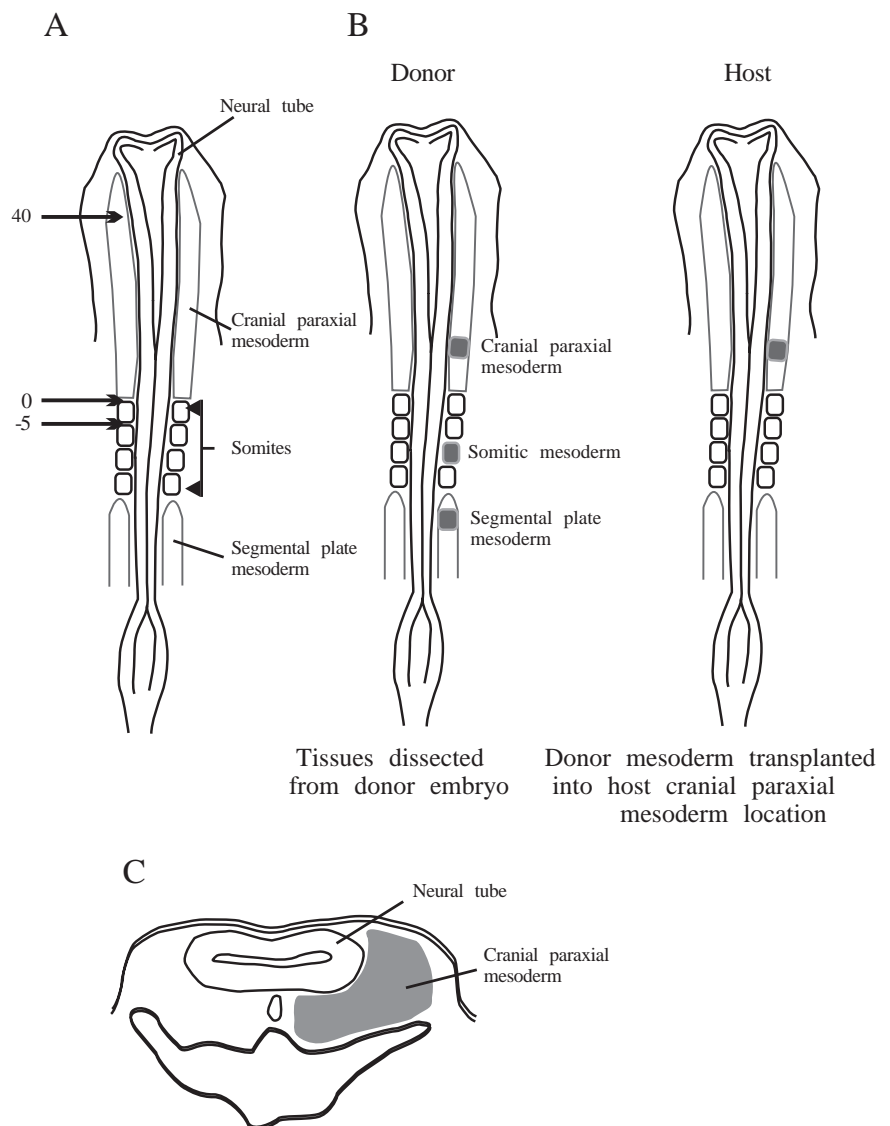
## RESULTS

### Mapping the migration of the cranial paraxial mesoderm

We have analysed the migration routes of the cranial paraxial mesoderm into the branchial arches using DiI, a lipophilic marker, which has been shown, in previous studies, not to transfer between cells (e.g. Trainor and Tam, 1995 and data not shown). Injections were made beneath the ectoderm into the paraxial mesoderm lateral to the hindbrain of chick embryos at between stage 7<sup>+</sup> and 8, just prior to the migration of the cranial neural crest (Lumsden et al., 1991). Thus these injections should label mesoderm cells exclusively and not neural crest. Injections were made extending from the rostral boundary of the first somite (position 0 in

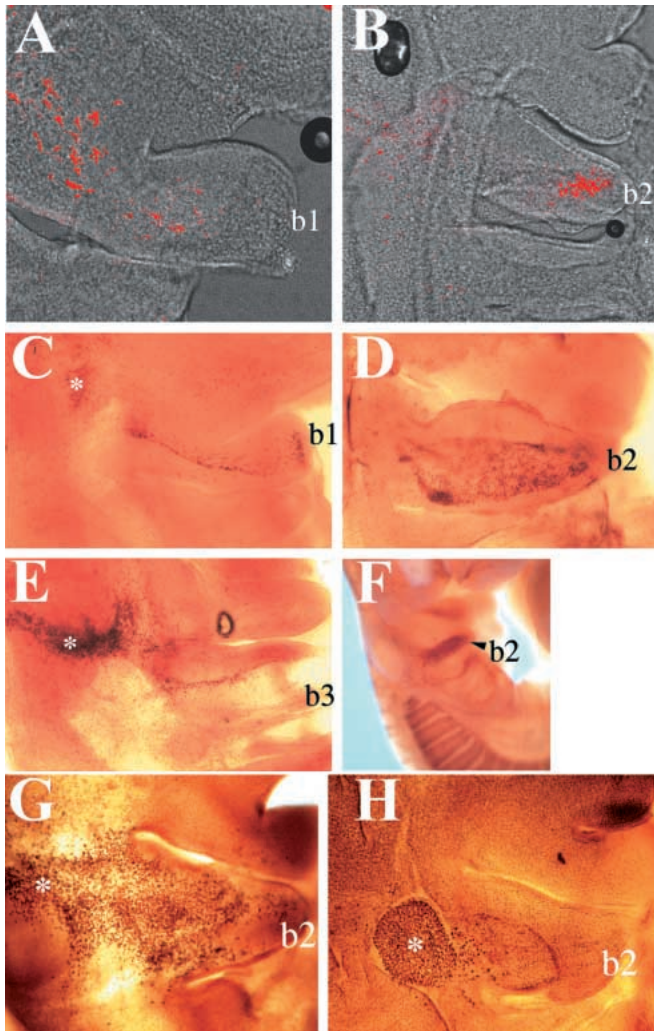
Fig. 1) into the mesoderm adjacent to the midbrain (position 40 in Fig. 1). We have therefore ignored other landmarks such as rhombomere boundaries, which do not appear until later, or somitomeres, which are not apparent using the light microscope (Meier and Tam, 1982).

We found that 36-48 hours after DiI labelling, cranial paraxial mesoderm cells were located in the cores of the branchial arches. In this analysis, it is unclear to what extent the cranial paraxial mesoderm actively migrates to fill the arches or whether mesoderm movements are more passive and perhaps dependent on the movements of neural crest cells. We

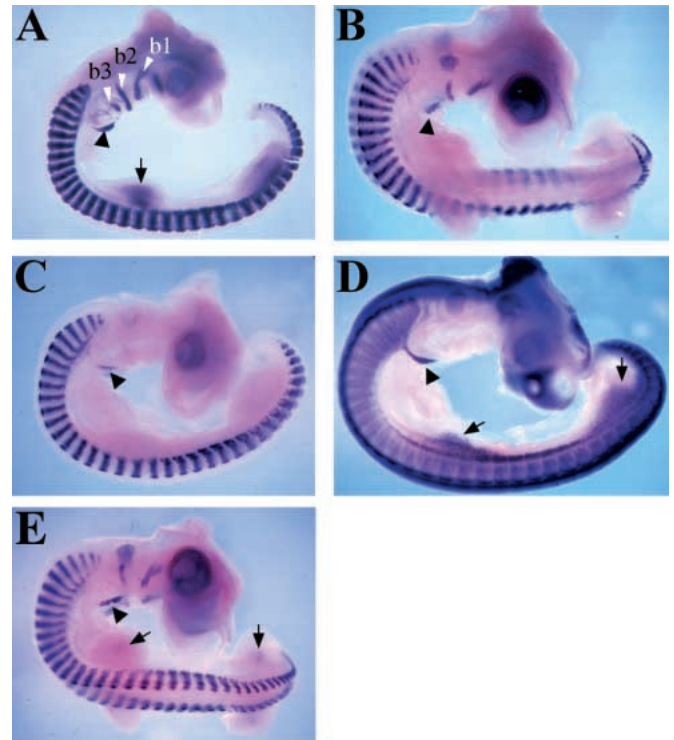


**Fig. 1.** (A) Diagram indicating the positions of DiI injections into the cranial paraxial mesoderm of 3- or 4-somite chick embryos. The rostrocaudal site of injection, between 0 and 40, was noted using an eyepiece graticule aligned to the rostral boundary of the first somite, position 0 (1 unit equals 25  $\mu\text{m}$ ). (B) Transplantation design: cranial paraxial mesoderm, somitic mesoderm or segmental plate mesoderm was dissected from donor 3- to 4-somite quail embryos and transplanted into host 3- to 4-somite chick embryos in the region of the cranial paraxial mesoderm. (C) Diagram showing a transverse section through the hindbrain of a 4-somite embryo; the shaded area represents the area of cranial paraxial mesoderm used for transplantations.





**Fig. 2.** Migration of paraxial mesoderm cells. (A) Region of the first branchial arch in a stage 20 embryo in which DiI was injected at position 25. Confocal image (false colour) of labelled cells shows that they have migrated into the central region of the first arch (b1). (B) Region of the second branchial arch in a stage 20 embryo in which DiI was injected at position 10. Confocal image (false colour) of labelled cells shows similar migration pattern as in A, but into the second branchial arch (b2), in addition some labelled paraxial mesoderm cells do not migrate and remain lying adjacent to the neural tube. (C-E) Orthotopic quail-to-chick grafts. Quail tissue dissected from a stage 8- to 8 embryo from between positions 20 and 25 (C), 7 and 12 (D), and 0 and 4 (E), transplanted to an orthotopic location in a chick embryo (the approximate location of the body of the graft is shown \* in C and E). Quail cells labelled with QCPN antibody are shown having migrated into the cores of the first (b1), second (b2) and third (b3) branchial arches, respectively. (F) The branchial arch region of a stage 20 chick embryo stained with an anti-desmin antibody. Strong immunoreactivity in second branchial arch (b2), to a lesser extent in the first and third branchial arch, also in the somites. (G, H) Heterotopic quail to chick grafts labelled with QCPN antibody. Region of second branchial arch in stage 20 embryos. (G) Somite and (H) segmental plate mesoderm transplanted into the cranial paraxial mesoderm at a presumptive second arch position. (G) Somitic graft (\*) disperses and quail cells migrate to fill the second branchial arch (b2). (H) The segmental plate graft (\*) fails to disperse and few quail cells migrate away or into the second arch (b2).



**Fig. 3.** Comparison of expression patterns of myogenic genes in various regions of the developing chick embryo. Whole-mount in situ hybridisations; (A-D) Stage 20 chick embryo stained for *Myf5* (A), *MyoD* (B), *myogenin* (C) and *Pax3* (D); (E) Stage 22 chick embryo stained for *MyoD*. Diffuse staining in head and eye is indicative of background. White arrowheads in A indicate the location of the first (b1), second (b2) and third (b3) branchial arches for reference. Black arrows indicate expression in the limb buds. Black arrowheads indicate the migrating tongue muscle precursor cells.

here define the movement of cranial paraxial mesoderm cells as a 'migration', without attributing this process to any specific mechanism.

DiI injections made between positions 13 and 40 led to labelled cells filling the first arch (Fig. 2A shows an injection at position 25). In addition, in the case of the injections between positions 28 and 40, mesoderm cells also migrated anteriorly into the head and around the optic vesicle. Injections made between positions 6 and 16 yielded fewer cells in a rostral location, with labelled cells located in the core of the second arch (Fig. 2B shows an injection at position 10). Injections made even further caudally (position 0 to 5) led to cells filling the core of the third branchial arch. Thus mesoderm cells from successively more caudal locations migrated into successively more caudal arches. There are clearly regions along the rostrocaudal axis where cells have the ability to contribute to either the first and second or the second and third branchial arches. Very few DiI injections led to cells filling exclusively the third arch. These data are summarised in Table 1.

To investigate further the migration patterns of the cranial paraxial mesoderm, orthotopic grafts were carried out. Using the results obtained from the DiI injections, regions of cranial paraxial mesoderm were dissected from donor quail embryos (stage 8- to 8). Paraxial mesoderm pieces of approximately a

rhombomere in length (100  $\mu\text{m}$ ), which were known to contribute cells to the first, second and third arches, were transplanted into chick hosts of a similar age in an orthotopic location. Care was taken to remove as much of the mesoderm from the host embryo at the location of the graft as possible. Quail cells filled the positionally appropriate arch as determined by DiI injections (Table 1). Transplantation of a piece of cranial paraxial mesoderm located between positions 20 and 25 led to quail cells populating the first arch (Fig. 2C). Transplantation of mesoderm from positions 7-12 yielded cells in the second arch (Fig. 2D) and from positions 0-4 yielded cells in the third arch (Fig. 2E). In the latter case, at least some quail cells were expected to fill the second arch in accordance with the data obtained from the DiI injections (see Table 1). However, in only one case were quail cells found in the second arch. These results may reflect differences between the two labelling techniques and a potential limitation of grafting. For example, although transplantations were carried out at stage 8<sup>-</sup> to 8, the time taken for the graft to integrate into the surrounding tissue may result in less cell mixing and a more focused migration into a single branchial arch.

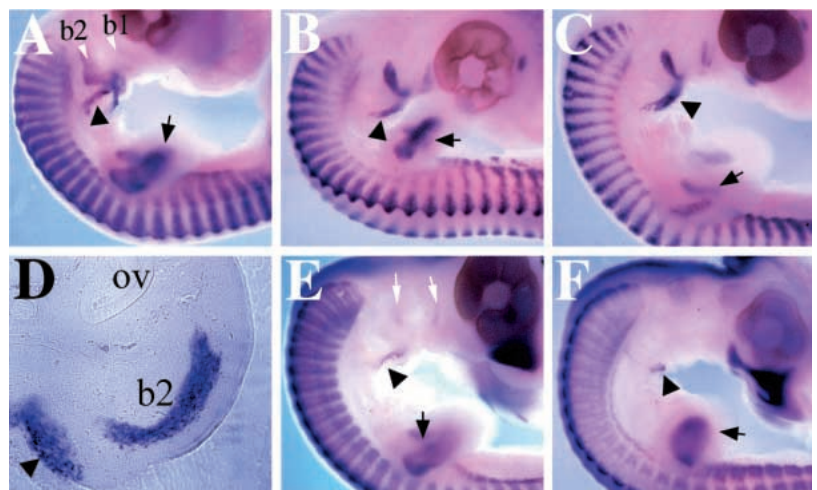
The expression pattern of the intermediate filament protein desmin is known to be associated with early myogenic differentiation (Schaart et al., 1989). Immunostaining using an anti-desmin antibody demonstrated that this protein is expressed in the core of the branchial arches (Fig. 2F), a region corresponding to the muscle mass. A direct comparison of the desmin expression pattern (Fig. 2F) with the location of the quail cells in the orthotopic grafts (Fig. 2C-E) reveals that the patterns are very similar. This alignment of the quail cells to the desmin-positive muscle mass was shown by double-labelling transplanted embryos with the QCPN anti-quail antibody and an anti-desmin antibody (see Fig. 5D). Thus, mesoderm cells that migrate into the branchial arches contribute to the muscle plate.

### Patterns of myogenic gene expression in the branchial arches

A detailed analysis of the expression patterns of the myogenic regulatory genes *Myf5*, *MyoD* and *myogenin* was carried out by in situ hybridisation to determine the timing of expression in different mesoderm populations, including the somites, branchial arches and the limbs. We found that in all these tissues *Myf5* was expressed first, followed by *MyoD* and then *myogenin*. In the somites; *Myf5* is expressed first at stage 8, then *MyoD* at stage 9 followed by *myogenin* at stage 12 (Table 2). This cascade is therefore triggered over a period of 3/4 of a day. The initial expression of each gene appears in a number of somites simultaneously and so is not dependent on the maturity of the somite but on the age of the embryo. Subsequent expression, as more somites develop, is dependent on their maturity. Expression is maintained in the somites at least up to stage 25 (see Fig. 4A-C). In the limbs, *Myf5* turns on at stage 20 (arrow Fig. 3A), followed by *MyoD* at stage 22 (Fig. 3E) and *myogenin* at stage 25 (Fig. 4C), a time period of about 1 day. As with the somites, expression of these three

genes is maintained in the limb buds up to stage 25, by which time expression is visible in two distinct parallel domains, corresponding to the differentiating muscle masses (black arrows Fig. 4A-C). The time taken to turn on these three genes in the branchial arches is more protracted, approximately 2.25 days. Expression of *Myf5* is first seen in the arches at stage 14<sup>+</sup>, *MyoD* at stage 19 and *myogenin* at stage 25 (Table 2). A comparison of the expression of *Myf5* in the branchial arches at stage 20 (Fig. 3A) with expression of Desmin (Fig. 2F) and the migration of transplanted quail cells into the branchial arches (Fig. 2C-E) shows that all three markers are expressed in the same cells, the muscle masses of the arches. This is exemplified in a vibratome section through the muscle mass of the second branchial arch of a stage 25 embryo in situ hybridised for *myogenin* (Fig. 4D).

We have also examined the expression of the paired box-containing genes *Pax3*, which has been shown to be important in mesoderm development (Williams and Ordahl, 1994; Maroto et al., 1997; Tajbakhsh et al., 1997) and *Pax7*, which has not been shown to be directly involved in muscle differentiation but shares an overlapping expression domain with *Pax3* (Jostes et al., 1991). These genes are very early markers of the somitic dermomyotome. By stage 17, *Pax3* is expressed in the myogenic cells that migrate from the ventrolateral edge of the dermomyotome into the developing limb buds and contribute to the limb musculature (arrows Fig. 3D). *Pax7* is expressed later than *Pax3*, at stage 21, in the developing muscle masses of the limbs (data not shown). The expression of *Pax3* and *Pax7* in the limb buds is maintained until at least stage 25 (arrows Fig. 4E,F). Neither *Pax3* nor *Pax7* are early markers for the developing muscle masses of the branchial arches. *Pax3* is not expressed at all in the cranial mesoderm over the stages analysed (Table 3, Fig. 4F) and *Pax7* is only beginning to be expressed by stage 25 (Fig. 4E white arrows). Expression in the hindbrain region is restricted to the



**Fig. 4.** Expression patterns of myogenic genes at stage 25. (A-C,E,F) Whole-mount in situ hybridisations, close-up of limb and branchial arch regions, *Myf5* (A), *MyoD* (B), *myogenin* (C), *Pax3* (E) and *Pax7* (F). (D) A transverse vibratome section through the second branchial arch (b2) of embryo in C showing that expression is confined to the core of the branchial arch, a region corresponding to the muscle mass. Annotations as in Fig. 3 but also white arrows in E indicate low levels of *Pax7* expression in the branchial arches; ov, otic vesicle.

**Table 1. Summary of DiI injection and transplantation data**

DiI injection site rostral of first somite	Location of DiI Labelled Cells					
	Head & B1	B1	B1 & B2	B2	B2 & B3	B3
1 unit = 25microns						
40-28	7	5				
27						
26						
25		3				
24						
23						
22						
21		1				
20		2				
19						
18						
17		3				
16		4	2			
15		4	4	6		
14				3		
13			4	1		
12				2		
11				2		
10				5		
9				3		
8				4		
7				3		
6						
5				2	2	
4					4	
3					3	
2						
1					5	1
0					1	1

	Position of transplant	Migration into arch by wholemound QCPN	Expression of marker	
			In arch	In body of graft
Orthotopic grafts	Presumptive first arch level	6/7 <sup>(1)</sup>		
	Presumptive second arch level	6/7 <sup>(2)</sup>	Desmin 3/3	Desmin 3/3
	Presumptive third arch level	6/6 <sup>(3)</sup>		
Heterotopic grafts	Somite to presumptive second arch level	19/19	Desmin 3/3 <i>Pax3</i> 0/8 <i>MyoD</i> 12/12 <i>Pax1</i> 0/6	Desmin 3/3 <i>Pax3</i> 7/8 <i>MyoD</i> 5/12 <i>Pax1</i> 5/6
	Segmental plate mesoderm to presumptive second arch level	1/16	N/A	<i>MyoD</i> 0/10 <i>Pax1</i> 3/19
	Cranial paraxial mesoderm to somite	N/A	N/A	<i>Pax3</i> 12/12 Desmin 5/5

(1) The remaining graft had moved rostrally and showed migration into the head; in addition one of the grafts that did migrate led to cells filling the first and second arch.

(2) The graft that failed to migrate was small (part had prolapsed out of the embryo).

(3) One of the grafts led to cells filling the second and third arches.

dorsal neural tube. These data again imply that the programme of gene expression in the muscle masses of the branchial arches may be different from the pattern in other myogenic populations.

Further differences are revealed in expression of these genes within populations of myogenic cells, in the process of migration. Cells migrating from the cranial paraxial mesoderm into the branchial arches express none of the markers examined. Paraxial mesoderm cells that migrate from the lateral part of the somite into the limbs express none of the myogenic markers but do express *Pax3* (Fig. 3D arrow). We have also observed a population of apparently migrating cells that is initially associated with somites 2 to 6. These cells express all the markers examined starting from stage 17 to 19, and form a stream from the occipital somites extending both ventrally and rostrally. These cells are likely to be myogenic

precursors that migrate to form the tongue musculature (Couly et al., 1993; Noden, 1983a; Hazelton, 1970). During their migration, these cells express all markers examined, i.e. *Myf5*, *MyoD*, *myogenin*, *Pax3* and *Pax7* (highlighted in Figs 3 and 4 by black arrowheads). These data are summarised in Table 3.

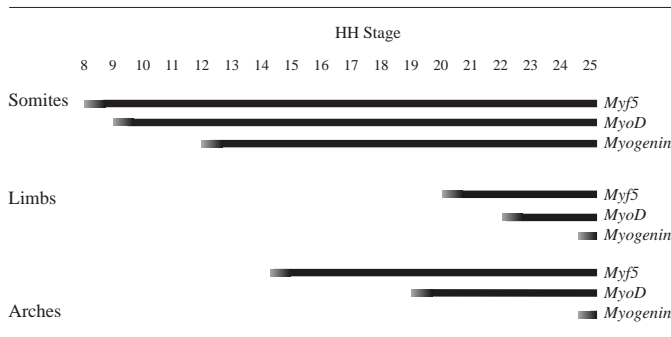
### Heterotopic grafting of mesoderm from different axial levels

#### (a) Migration

Having established the migratory pathways of the cranial paraxial mesoderm and the profile of gene expression, we have investigated the ability of trunk mesoderm, which expresses a different subset of genes, to substitute for the cranial paraxial mesoderm. We have dissected a range of individual somites I to X, using the numbering system of Ordahl (1993) where Roman numeral I denotes the most recently segmented and



**Table 2. Timecourse of expression of myogenic genes in the somites, limbs and branchial arches of the developing chick embryo**



least mature somite, from quail embryos of between stage 8 and stage 12. Somites dissected from quail embryos were transplanted into the cranial paraxial mesoderm of stage 8- or stage 8 host-chick embryos at the presumptive second arch level. After re-incubation for a further 36 to 48 hours, the distribution of quail cells was examined by whole-mount immunostaining using the QCPN antibody. In all cases ( $n=19$ ), at least some of the grafted cells were located within the second branchial arch (Fig. 2G). When segmental plate mesoderm was transplanted into a chick host, very little or no migration was observed into the second branchial arch ( $n=15$  out of 16) (Fig. 2H). In four cases we have eliminated, as far as possible, the differences in maturity of the somite and segmental plate by transplanting the youngest somite (somite I) into a cranial paraxial mesoderm position. In all four cases, grafted somitic cells migrated into the second arch. We therefore conclude that cells are capable of migrating into the branchial arches from a somite graft but not from a graft of segmental plate.

We have also examined these segmental plate mesoderm grafted embryos by in situ hybridisation for *MyoD* and *Pax1*, both markers of somite maturation. The grafted quail cells failed to express either of these markers ( $n=0$  out of 10 and  $n=3$  out of 19 respectively, data not shown). These data imply that the segmental plate mesoderm fails to mature normally in the cranial environment.

### (b) Gene expression of heterotopically grafted cells

Given that somitic mesoderm can migrate into the branchial arches, how do these cells behave? Do they become incorporated into the muscle mass region of the arch and maintain their previous characteristics, e.g. the expression of *Pax3*? Both orthotopic presumptive second arch cranial paraxial mesoderm and heterotopic somite transplantations were carried out into a presumptive second arch mesoderm position, followed by incubation for 48 to 60 hours. Operated embryos were embedded in polyester wax and sectioned transversely before analysis by immunofluorescence with both QCPN and anti-desmin antibodies. Sections were taken at the level of the body of the graft and at the level of the muscle mass of the arch (Fig. 5A). In the orthotopic grafts, quail cells (in red) are distributed and have integrated well into the surrounding mesenchyme (Fig. 5B). In the arch, quail cells are present in the desmin-positive muscle mass (in green) and are restricted to that area (Fig. 5D). By contrast, when somites

were present in a cranial paraxial position, the graft often did not disperse and integrate into the surrounding tissue and ectopic muscle masses were apparent in the form of desmin-positive areas (Fig. 5C). The somitic cells, as has been seen in previous experiments, migrated into the arch and integrated into the desmin-positive muscle mass, although cells were not completely confined to this area (Fig. 5E). Cells that lay outside the desmin-positive muscle mass could be trunk neural crest or rogue mesoderm cells that failed to show the strict localisation to the muscle mass exhibited by the cranial paraxial mesoderm.

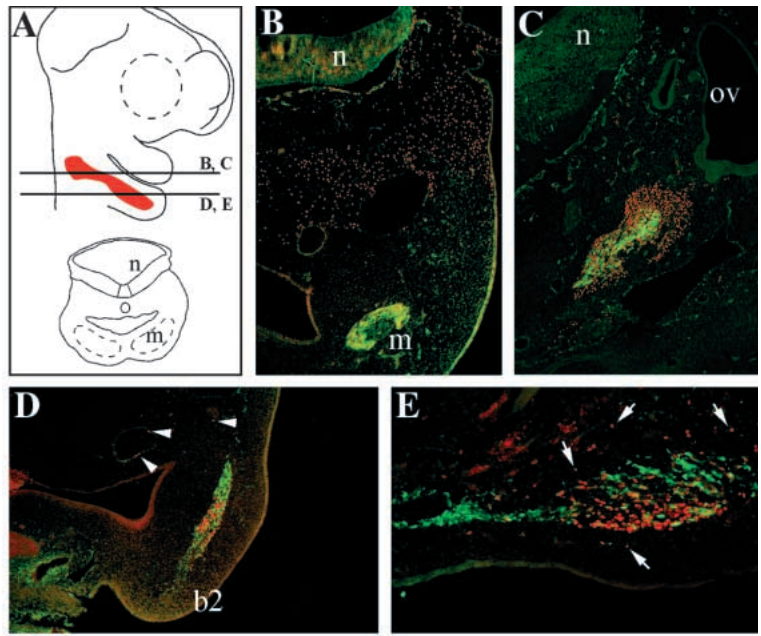
A more detailed analysis of the somite grafts has demonstrated that certain molecular characteristics are maintained in the ectopic location. Whole-mount in situ hybridisation for *Pax3* (Fig. 6A,B), *MyoD* (Fig. 7A,B) and *Pax1* (Fig. 7C,D), followed by QCPN immunohistochemistry to identify the transplanted quail cells, demonstrated that, in sections through the body of grafts, all these markers were expressed but at different levels. *Pax1* was expressed at very high levels, *Pax3* at lower levels (although higher in grafts lying adjacent to the neural tube and surface ectoderm (Fig. 6E-G)) and *MyoD* at lower levels or not at all (see Table 1 for summary). There was no correlation between the maturity of the somite and expression of any of the markers. Cells located more distally and in the branchial arches did not express *Pax3* (Fig. 6A-G) or *Pax1* (data not shown). We have also examined operated embryos incubated for 18 hours, a time at which migration of mesoderm cells into the branchial arches is occurring. In these embryos, cells located away from the body of the graft, and in the process of contributing to the formation of the second branchial arch, did not express *Pax3* ( $n=6$  out of 6).

In the converse experiment, cranial paraxial mesoderm from a 4-somite embryo was transplanted into an immature somitic location of a 10-somite embryo. In frozen sections, quail cells lying in a dermomyotomal location expressed *Pax3* (Fig. 6H,I), a marker that these cells would not normally express, and also desmin (data not shown). In addition, some cranial paraxial mesoderm cells were found in more medial/sclerotomal locations that did not express *Pax3* (Fig. 6H, I arrows). These data imply that external signals are required to maintain *Pax3* expression in migrating mesoderm cells. In the head, these signals are absent, leading to downregulation of *Pax3*. The cranial paraxial mesoderm is similarly labile, since the trunk environment is capable of activating the *Pax3* program in ectopic cranial paraxial mesoderm.

**Table 3. Expression of genes in different mesoderm populations**

	<i>Myf5</i>	<i>MyoD</i>	<i>Myogenin</i>	<i>Pax3</i>	<i>Pax7</i>
Migrating mesoderm populations (st. 15-20)					
to the branchial arches	-	-	-	-	-
to the limbs	-	-	-	+	-
to the tongue	+	+	+	+	+
Mesoderm populations at st. 25					
Branchial arch muscle	+	+	+	-	-/+
Limb mesoderm muscle	+	+	+	+	+

**Fig. 5.** Orthotopic and heterotopic quail grafts labelled with QCPN (red) and anti-desmin (green) antibodies. (A) Schematic diagram of the head and branchial arch region of a stage 22 chick embryo, showing the location of the transverse sections B-E. The location of the graft and the approximate route taken by migrating quail cells is shown in red. Lower panel shows a transverse section through the second branchial arch (similar to section in D or E), showing the location of the muscle mass (m) and neural tube (n). (B-E) Transverse sections of stage 22 embryos. (B) Orthotopic graft of cranial paraxial mesoderm, section taken at the level of the body of the graft. Quail cells have integrated into the surrounding tissue. The proximal edge of the second arch muscle mass is visible (m). (C) Heterotopic graft, quail somite transplanted into a cranial paraxial mesoderm location, in this section at the level of the graft quail cells did not disperse and an ectopic muscle mass developed; ov, otic vesicle. (D) Transverse section at the level of the second branchial arch (b2) of the embryo shown in B in which quail cells have migrated into the branchial arch and are almost completely confined to the desmin-positive muscle mass (green), a few quail cells also contribute to blood vessels (white arrowheads). (E) Same embryo as shown in C but section was taken at the level of the second branchial arch. Quail cells fill the core of the arch but are not confined exclusively to the desmin-positive muscle mass (white arrows).



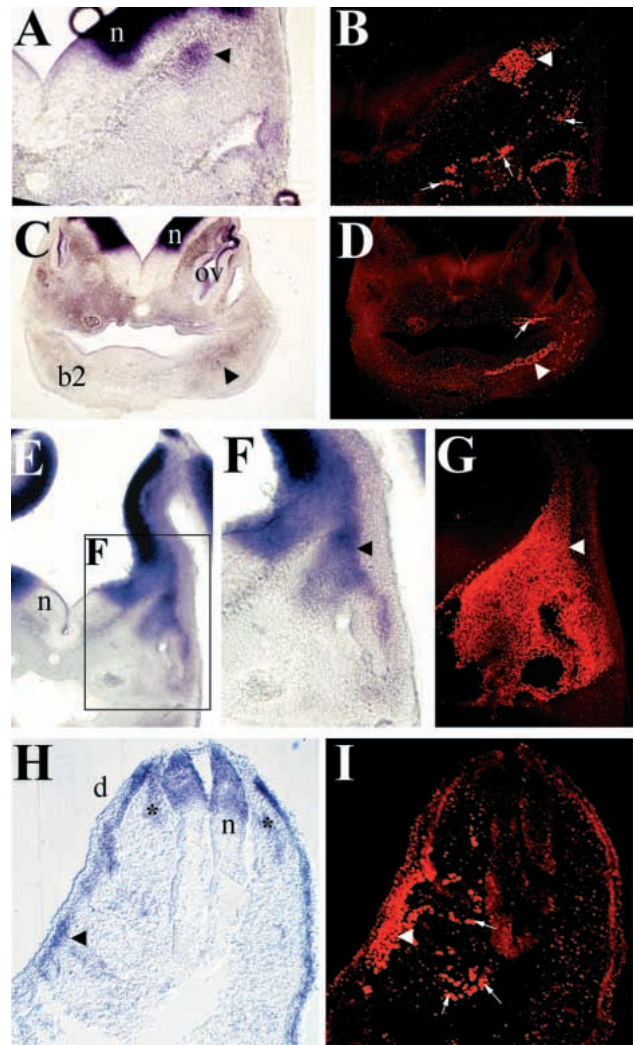
## DISCUSSION

In this study, we have mapped the migration of the cranial paraxial mesoderm into the branchial arches using both Dil labelling and the quail-chick chimaera system. Transplantation of somitic mesoderm into a cranial paraxial mesoderm location led to migration of cells into the core of the arches and downregulation of *Pax3*. Our data imply that cranial and somitic mesoderm pursue distinct developmental pathways that depend on patterning signals from the surrounding environment.

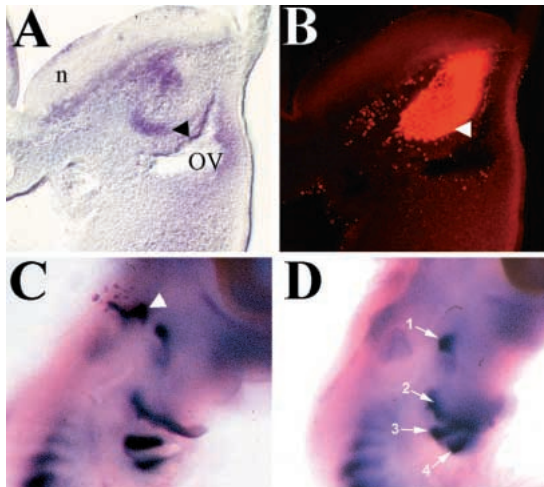
### Cranial paraxial mesoderm migrates to core of the nearest branchial arch

The results of the Dil labelling and transplantation experiments showed that cells of the cranial paraxial mesoderm migrate into the nearest branchial arch in streams. Thus cells at the level of r1 migrate to the first branchial arch, and cells at the level of r6 migrate to the third arch (summarised in Fig. 8). There are

**Fig. 6.** Expression of *Pax3* in heterotopic grafts. (A-G) Somite transplanted to a cranial paraxial mesoderm location. Vibratome sections at the level of the otic vesicle, sectioned after whole-mount in situ hybridisation for *Pax3* (A,C,E,F) and QCPN immunohistochemistry (B,D,G, red). The body of the graft (arrowhead) adjacent to the neural tube (n) expresses *Pax3* (A,B,F,G). (C,D) At the level of the second branchial arch (b2), same embryo as A,B, quail cells (red) are located in the core of the arch (arrowhead in D) but do not express *Pax3* (arrowhead in C). Some cells can also be seen contributing to blood vessels (white arrow in D). (H,I) Cranial paraxial mesoderm from a 4-somite quail embryo transplanted into an immature somitic location of a stage 10 embryo. Frozen sections after whole-mount in situ hybridisation for *Pax3* (H) and QCPN immunohistochemistry (I, red). Quail cell located in a dermomyotomal (d) location express *Pax3*, arrowhead provides reference. In addition some cells are located more medially that do not express *Pax3* (arrows in B and I). \*, spinal ganglia.







**Fig. 7.** Expression of *MyoD* (A) and *Pax1* (C,D) in heterotopic somite to cranial paraxial mesoderm grafts. (A,B) Vibratome section at the level of the otic vesicle (ov) shows low level of *MyoD* expression (A, arrowhead) in the grafted quail cells (B, QCPN staining, arrowhead). Whole-mount in situ hybridisation demonstrates that *Pax1* is ectopically expressed at a high level in the grafted side (C, arrowhead), but not in the unoperated side of the same embryo (D). For reference, white arrows show the location of the branchial clefts that express *Pax1*.

differences between the migration patterns of neural crest and mesoderm cells. The crest populations in r3 and r5 are depleted due to extensive cell death (Lumsden et al., 1991), which has the effect of creating separated streams of migrating cells. Each stream colonises a separate branchial arch (see Fig. 8). By contrast, within the paraxial mesoderm there appear to be no depleted regions and cells do not migrate in separated streams. The result is that there are extensive regions of overlap, where cells can migrate to either the first and second or the second and third branchial arches. There is little evidence for migration that is directed to a particular axial level, as transplantation of presumptive first arch mesoderm into a

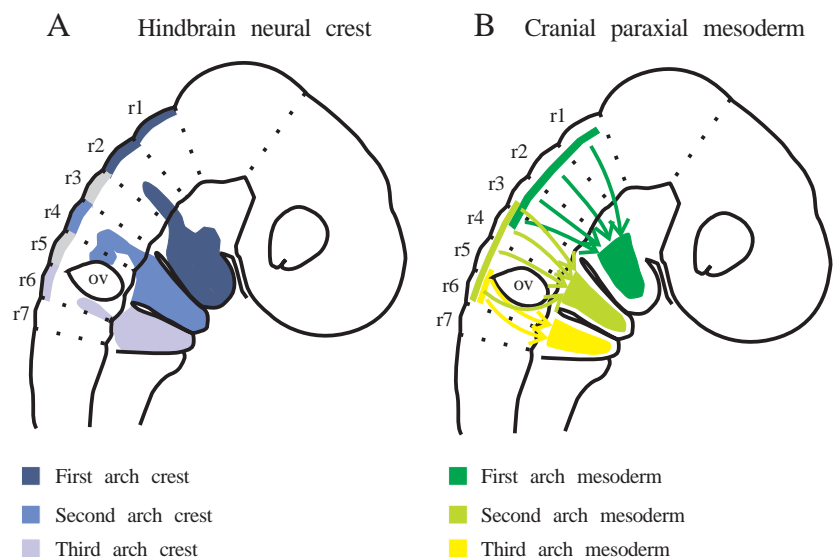
presumptive second or third arch position leads to migration away from the neural tube to the closest arch (data not shown).

The cranial paraxial mesoderm enters the developing branchial arch and colonises its core, a region known to correspond to the muscle mass (our data and Trainor and Tam, 1995). This is in contrast to the regions occupied by the migrating neural crest in the periphery of each arch (Fig. 8, Lumsden et al., 1991; Trainor and Tam, 1995).

### Transplantations of mesoderm into an ectopic cranial or trunk location lead to the adoption of a new fate

We have demonstrated that grafting early mesoderm tissue from quail to chick can be used to assess the migration pattern of these cells. Orthotopic transplantation of cranial paraxial mesoderm resulted in migration into the branchial arches in a similar pattern to that of resident mesoderm in unoperated embryos. The transplantation of an epithelial somite adjacent to the hindbrain also led to cells migrating into the arches. Segmental plate mesoderm in such an heterotopic location failed to migrate.

Previous studies have investigated the potential of mesoderm populations to substitute for each other (e.g. Noden, 1986; Chevallier et al., 1977). Noden (1986), for example, grafted somites or segmental plate adjacent to the mesencephalon. These grafts contributed to normal extra-ocular and jaw muscles as well as ectopic structures (skeletal, connective and muscle tissue). These experiments were limited for a number of reasons. The grafts contained the overlying ectoderm, which could have interfered with the experimental results, giving rise to ectopic feathers for example. In addition, grafts were carried out into host embryos at stage 9<sup>+</sup>, at a time when neural crest cells have begun their migration (Lumsden et al., 1991). In this case, the presence of the ectopic somite or segmental plate could act as a physical barrier preventing and interfering with the correct migration of neural crest. In our experiments care was taken to remove non-mesoderm tissue, grafts were undertaken earlier before neural crest migration, between stage 7<sup>+</sup> and 8, and tissue was transplanted into a different location, adjacent to the hindbrain.



**Fig. 8.** Comparison of the migration of the cranial neural crest (A, adapted from Lumsden et al., 1991) and cranial paraxial mesoderm (B, our data). The original locations of the labelled cells prior to migration are shown along the neural tube in A and adjacent to the neural tube in B. (A) cranial neural crest migrate in three separate streams from rhombomeres r1-r2, from r4 and from r6 to line the periphery of the first, second and third branchial arches, respectively. (B) The cranial paraxial mesoderm migrate in three overlapping streams to fill the cores of the branchial arches. Cells adjacent to r1-r4 migrate to fill the first arch, those adjacent to r3-r6 fill the second arch and those adjacent to r5-r6 migrate to the third arch. The original injection sites in relation to the position of rhombomeres were determined retrospectively, by recording the location of labelled mesoderm that failed to migrate into the branchial arches.

Migrating cells filled the core of the arch and expressed desmin. In addition the somitic mesoderm in an ectopic arch location downregulated *Pax3*, a dermomyotome marker which is not usually expressed by the muscle mass of the branchial arch. The body of the somite graft appeared to maintain *Pax3* and *Pax1* expression, whilst among cells that had migrated away both genes were downregulated. This may be due either to the presence in the arch of signals that directly downregulate *Pax3* expression, or the absence of maintenance signals normally present in the somite, body wall or limb tissues. The ability to migrate into the arches is clearly not a property of paraxial mesoderm cells, per se, as the segmental plate mesoderm placed in a similar location failed to migrate. Nor is it dependent on *Pax3*, which is not expressed by the cranial paraxial mesoderm but is required, in the trunk, for cell migration from the somites into the developing limbs (Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996).

Following transplantation cranially, some of the somitic cells did not migrate but remained in a cluster. These ectopic cells may represent the non-migrating epaxial or sclerotomal components of the somite that maintain some of their characteristics and express *Pax3*, *Pax1* and *MyoD*. Differentiation into ectopic muscle masses, at least as judged by desmin expression, has been observed (our data and see also Noden (1986)). Based on morphology, these cells may have also formed cartilage (data not shown). The cells that did migrate from the somitic graft must express the appropriate molecules that allow their aggregation with chick myogenic cells within the muscle mass. Some are not confined to this region and have been observed in more peripheral regions of the arch. These may be trunk neural crest cells present in the more mature somites prior to grafting (trunk neural crest begins emigrating from the dorsal neural tube approximately three somites rostral to the most recently formed somite (Bronner-Fraser, 1986)).

When a piece of segmental plate mesoderm was transplanted to the cranial mesoderm position, very little or no cell migration occurred. This is in striking contrast to similar somite grafts. In at least some of our experiments, the transplanted somite was immature, only about 2 hours older than the transplanted segmental plate, yet this striking difference in cell behaviour was maintained. At this stage, in isolated culture experiments of immature somites or segmental plate mesoderm, no myogenic programme has been shown to be triggered (Buffinger and Stockdale, 1994; Gamel et al., 1995; Stern and Hauschka, 1995). The somite requires influences originating from the neural tube, floorplate and notochord to trigger myogenesis (e.g. Gamel et al., 1995; Münsterberg and Lassar, 1995). One key difference between the somite and the segmental plate mesoderm may be the epithelial nature of the somite. It seems that, in order to be capable of migrating, the morphological changes associated with the mesenchymal-to-epithelial transition of somitic cells and the accompanying molecular changes are required. Culture of segmental plate mesoderm with adjacent ectoderm and endoderm and lateral plate mesoderm has been shown to result in the formation of epithelial somites (Buffinger and Stockdale, 1994). The signals necessary for this transition may be absent in the vicinity of the hindbrain. This is supported by the fact that, in our segmental plate mesoderm grafts into the head, early markers of somite differentiation, *Pax1* and *MyoD*, fail

to be expressed. The cranial paraxial mesoderm may be fundamentally different from the trunk mesoderm, with no requirement for a mesenchymal-to-epithelial transition in order for cell migration to be initiated.

### The program of gene expression is different in different mesoderm populations

The activation of the myogenic genes has been investigated in both mammalian and avian embryos. In the mouse, the first myogenic factor to be detected is *Myf5* in the dermomyotome of the developing somite (Ott et al., 1991). Both *myogenin* and *Mrf4* follow within 6 to 12 hours (Hinterberger et al., 1991; Bober et al., 1991). *MyoD* is the last myogenic factor to be expressed, approximately 24 hours later. *Myf5* gene expression is initiated first in the developing muscle masses of the limbs and branchial arches, with the expression of *myogenin* and *MyoD* following together (Bober et al., 1991; Sassoon et al., 1989). The timing of onset of expression in each tissue is different, with *Myf5* turning on in the arches before the limbs (Ott et al., 1991). *Mrf4* is not expressed in the branchial arches and is only expressed late and transiently in the limbs (Bober et al., 1991; Hinterberger et al., 1991).

In the quail, examination of the maturing somite has revealed a different sequence of events: *qmf1* (homolog of *MyoD*) is expressed first, followed by *qmf3* (*Myf5*) and then followed later by *qmf2* (*myogenin*) (Pownall and Emerson, 1992). RT-PCR analysis of expression in the developing chick limb gives a sequential order of *Myf5* followed by *MyoD* and *myogenin* (Lin-Jones and Hauschka, 1996).

Our study does not agree with the published order of gene expression in either mice or quail. The potential controversy regarding the sequence of *Myf5* and *MyoD* expression in the somites may derive from the assay of different axial levels. We have concentrated on the earliest time of expression in the embryo, rather than the onset of expression in the maturing somite as does the study of Pownall and Emerson (1992). We see that *Myf5* is the first myogenic factor to be expressed in the somites of the developing chick embryo (stage 8) while, by stage 9, *MyoD* begins to be expressed and *myogenin* is expressed even later. This order is recapitulated in the limb buds (in agreement with the study of Lin-Jones and Hauschka, 1996) and the branchial arches. The timing of the cascade of gene expression in each of these tissues is different. Expression is activated rapidly in the somites and is more protracted in the branchial arches, taking 2.25 days for *myogenin* to turn on after *Myf5*. A similar delay has also been observed in the mouse (Ott et al., 1991). This may imply that different regulatory pathways are responsible for triggering gene expression in the arches, limb buds and somites. Indeed, this is in agreement with an experiment carried out in the mouse, where the use of a *Myf5-lacZ* construct gave good expression in the branchial arches but initially no expression in the somites (Patapoutian et al., 1993). In a similar experiment examining the expression and regulation of *myogenin*, the regulatory elements required for the expression in the somites were separated from those responsible for expression in the limb buds and branchial arches (Cheng et al., 1993). These data are consistent with the myogenic determination in head mesoderm being under separate control from that of the trunk mesoderm.

The three migrating mesoderm populations that we have examined are different with respect to the genes that they

express. The tongue muscle precursors express all markers examined, while limb muscle precursors express only *Pax3* and the cells migrating to the branchial arches express none of these markers. These data demonstrate that different myogenic pathways have been activated in each of these three mesoderm populations. The mesoderm may be naive, receiving signals from the surrounding tissues, which activate expression. The cells may then become responsive to signals that permit or trigger migration. *Pax3*, for example, is a prerequisite for migration of dermomyotome into the limb and migration of the hypoglossal myogenic precursors (Hogan et al., 1992; Tajbakhsh et al., 1997). Migration could be an active process, with mesoderm cells responding to a diffusible factor. HGF/SF, for example, is responsible for the migration of cells from the lateral dermomyotome into the limb buds (Théry et al., 1995; Brand-Saberi et al., 1996). Alternatively, migration could occur along the routes taken by other cell types (perhaps neural crest cells in the head and circumpharyngeal crest in the occipital somites). Paraxial mesoderm cells may be incapable of responding to differentiation cues until they have migrated into their target locations. Once the mesoderm cells have reached their target external signals trigger the myogenic program and *Myf5*, *MyoD* and *myogenin* are expressed. In the limbs, *Pax3* and *Pax7* expression is maintained. In the branchial arches, at least by stage 25, *Pax7* is only just beginning to be expressed. These data again imply the existence of different myogenic programmes. Consistent with these findings in the chick, a recent study by Tajbakhsh et al. (1997) has demonstrated that in *Myf5/Pax3* double mutants, the muscles of the head, except for the hypoglossal musculature, were unaffected whereas the body muscles, including those of the limb, were absent. This implies that there may be a gene that substitutes for *Pax3* in the head that has a role in triggering the migration of the cranial paraxial mesoderm into the developing branchial arches.

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