Development 129, 4855-4866 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2887

Axial progenitors with extensive potency are localised to the mouse chordoneural hinge

Noemí Cambray and Valerie Wilson*

Centre for Genome Research, Kings Buildings, West Mains Road, Edinburgh EH9 3JQ, UK
*Author for correspondence (e-mail: v.wilson@ed.ac.uk)

Accepted 11 July 2002

SUMMARY

Elongation of the mouse anteroposterior axis depends on a small population of progenitors initially located in the primitive streak and later in the tail bud. Gene expression and lineage tracing have shown that there are many features common to these progenitor tissues throughout axial elongation. However, the identity and location of the progenitors is unclear. We show by lineage tracing that the descendants of 8.5 d.p.c. node and anterior primitive streak which remain in the tail bud are located in distinct territories: (1) ventral node descendants are located in the widened posterior end of the notochord; and (2) descendants of anterior streak are located in both the tail bud mesoderm, and in the posterior end of the neurectoderm. We show that cells from the posterior neurectoderm are fated to give rise to mesoderm even after posterior neuropore closure. The posterior end of the notochord, together with the ventral neurectoderm above it, is thus topologically equivalent to the chordoneural hinge region defined in *Xenopus* and chick. A stem cell model has been proposed for progenitors of two of the axial tissues, the myotome and spinal cord. Because it was possible that labelled cells in the tail bud represented stem cells, tail bud mesoderm and chordoneural hinge were grafted to 8.5 d.p.c. primitive streak to compare their developmental potency. This revealed that cells from the bulk of the tail bud mesoderm are disadvantaged in such heterochronic grafts from incorporating into the axis and even when they do so, they tend to contribute to short stretches of somites suggesting that tail bud mesoderm is restricted in potency. By contrast, cells from the chordoneural hinge of up to 12.5 d.p.c. embryos contribute efficiently to regions of the axis formed after grafting to 8.5 d.p.c. embryos, and also repopulate the tail bud. These cells were additionally capable of serial passage through three successive generations of embryos in culture without apparent loss of potency. This potential for self-renewal in chordoneural hinge cells strongly suggests that stem cells are located in this region.

Key words: Mouse, Anteroposterior axis, Tail bud, Stem cell, Chordoneural hinge

INTRODUCTION

After the formation of the most rostral tissues, the extension of the mouse anteroposterior axis is undertaken by the primitive streak and subsequently by the tail bud (reviewed by Hogan et al., 1994). These two progenitor tissues share many common features. First, the morphology and topological relationship between the axial tissues that they produce is similar from the most rostral to the most caudal level. Some 60 somites flank two central tissues, the neural tube and notochord, and lie dorsal to the endoderm and lateral mesoderm. Secondly, the sites of mesoderm formation at gastrulation and in the tail bud later in axial elongation in vertebrates share expression of many genes (Chapman et al., 1996; Crossley and Martin, 1995; Dunwoodie et al., 1997; Gawantka et al., 1998; Ruiz and Robertson, 1994; Wilson et al., 1995). Third, several genes such as brachyury and *Wnt3a*, which have a crucial role in primitive streak morphogenesis revealed by null mutations, affect only tail development when function is partially lost (Chesley, 1935; Greco et al., 1996; Wilson et al., 1995). Thus, in these respects, the extension of the anteroposterior axis caudal to the head can be viewed, at least to some extent, as a continuum from its inception at 8.0 days postcoitum (d.p.c.) to its termination 5 days later.

In support of this, groups of 10-20 cells from the primitive streak and tail bud of 9.5 d.p.c.-13.5 d.p.c. embryos are able to incorporate in the streak of 8.5 d.p.c. embryos (Tam and Tan, 1992). However, there is evidence from the above study to suggest that cells in the primitive streak and tail bud are not completely interchangeable, as grafted cells from older tail buds contribute to more posterior somites than cells from the streak. In the past, it has been suggested that cells in the tail bud not only have different potency, but also proliferate according to different rules from those that pertain to the streak. Holmdahl (Holmdahl, 1925), from studies in chick, suggested that the vertebrate tail bud constitutes a blastema of undifferentiated cells with little or no regional specification of the progenitors. However, later fate mapping analysis in *Xenopus* showed regionalisation of distinct progenitors of neural tube, notochord and somites within a small area of the

blastopore (the *Xenopus* equivalent of the late primitive streak) and tail bud (Gont et al., 1993). In the tail bud, this region was termed the chordoneural hinge (CNH), and it is able to produce ectopic tails when grafted to host embryos. Gene expression within the tail bud is also strongly localised amongst the regionalised progenitors (Beck and Slack, 1998; Gawantka et al., 1998).

Intriguingly, however, it appears that there may be some cells in the tail bud whose fate is not specified, as marking very small groups of cells in the tail bud can result in descendants in more than one tissue type (Davis and Kirschner, 2000). Thus, while much of the tail bud in *Xenopus* is composed of regionalised progenitors, it is unclear whether these constitute all the axial progenitors. Alternatively, a second population of multi-fated progenitors may exist, which raises the possibility that these give rise to regionally specified progenitors.

In mouse, the descendants of single cells in the epiblast destined for the streak at early streak stage are not confined to any single tissue type (Lawson et al., 1991). Even later, mesoderm progenitors in the epiblast, although regionalised in fate, are not highly restricted in potency (Beddington, 1981). Whether the same multipotency is conserved in the later streak and tail bud in the mouse is unknown. The ontogeny of two of the axial tissues, the myotome, a paraxial mesoderm derivative, and spinal cord, derived from neural plate, has been studied using a retrospective single cell marking technique (Nicolas et al., 1996; Mathis and Nicolas, 2000). In these studies, descendants of single cells that have undergone a rare somatic recombination event and are located in either myotome or spinal cord are marked. In the myotome, those descendants of single cells that populate large anteroposterior axial distances are located bilaterally, showing that their progenitors originate in the primitive streak and tail bud (Nicolas et al., 1996). These studies indicate the existence of stem cell progenitors of both myotome and spinal cord. However, they do not give detailed information on their position and identity.

In lineage tracing experiments in cultured mouse embryos, most cells in the streak are destined for exit to differentiating axis tissues (Lawson et al., 1991; Tam and Beddington, 1987). However, a small proportion remain in the tail bud at the end of the culture period, in some cases after the formation of some 32 somites (Wilson and Beddington, 1996). Because we know that axial progenitors reside there, some or all of these may represent stem cells. Because stem cells are characterised by the ability to self-renew, they should be distinguishable from other cells in the axis by their capacity to contribute to both anterior and posterior differentiated tissues, and the ability to be serially passaged.

We have refined previous fate maps to show that the tail bud contains regionally separated descendants of cells in the streak using topically applied lipophilic dyes. We have exploited transgenic strains of mice that express green fluorescent protein (GFP) (Okabe et al., 1997) or *lacZ* (Munsie et al., 1998) ubiquitously to explore the potency of these cells. We show that cells in the vicinity of the node and their descendants are found in an equivalent structure to the *Xenopus* CNH. These cells fulfil the above criteria expected of stem cells. By contrast, cells in the more ventrally located tail bud mesoderm, which were found to be descended from the CNH, are more limited in their potency.

MATERIALS AND METHODS

Maintenance of mouse stocks and culture of embryos

MF1, *Zin40* (Munsie et al., 1998) and *TgN(beta-actEGFP)04Obs* (Okabe et al., 1997) (here termed 'GFP transgenic') mice were maintained on a 14 hour light, 10 hour dark cycle. Noon on the day of finding a vaginal plug was designated 0.5 days postcoitum (d.p.c.). Dissection and culture was performed as described (Cockroft, 1990).

Dissection of tissues for grafting

GFP transgenic×MF1 litters were dissected in M2 medium and those containing the transgene selected in a Nikon SMZ-U dissecting microscope with fluorescence attachment. The posterior half of the embryo containing the primitive streak was dissected using fine forceps. The primitive streak was dissected using an eyelash tool by making two longitudinal lateral cuts, isolating a thin strip of tissue containing the entire primitive streak and node and retaining both ectodermal and endodermal layers. Node and primitive streak fragments were further dissected by making transverse cuts with the eyelash tool. A schematic diagram of the sites dissected is shown in Fig. 1A.

Regions of the 10.5-12.5 d.p.c. tail bud were dissected by first isolating the whole tail bud using fine forceps. The CNH and tail bud mesoderm (TBM) were dissected using an eyelash tool or fine glass needles. First, the end of the tail was excised, and two dorsoventral longitudinal cuts made to remove the paraxial mesoderm. To dissect CNH, the hindgut and dorsal neurectoderm were then removed by similar longitudinal cuts in the mediolateral plane. The TBM was separated by a transverse cut posterior to the neural tube and hindgut. Dissected CNH was trimmed and divided into two to three pieces to graft to wild-type hosts. The surface ectoderm was removed from TBM and it was divided into two to three pieces for grafting. The position of these tissues in the intact and partially dissected tail is shown in Fig. 1A,B.

DiI labelling

Embryos were labelled with CellTracker CM-DiI and CMFDA (Molecular Probes) as described previously (Wilson and Beddington, 1996). Dissected CNH and TBM were labelled by expelling DiI from a pipette held directly above the tissue to be labelled for a few seconds. The graft was then washed in fresh M2. Dissected 10.5 d.p.c. tail pieces were labelled in the neural tube by inserting a fine pipette into the lumen and expelling a small amount of dye, which covered most or all of the luminal surface. Labelling of the most posterior end was checked either by observing a faint pink colour under brightfield illumination, or by viewing in a dissecting microscope with fluorescence attachment. Label sites are shown in Fig. 1A.

Grafting labelled tissue

Grafts were performed using a hand-drawn micropipette. The embryo was held loosely in place with forceps while suction was gently applied with the micropipette to the anterior primitive streak immediately abutting the node to create an opening for the graft. The tissue to be grafted was then drawn into the pipette, and the pipette inserted in the opening. The graft was gently expelled as the pipette was drawn out of the embryo, leaving the tissue lodged in the opening (Fig. 1C). The embryos were then placed in a universal container in 50% rat serum, 50% GMEM or DMEM in an incubator gassed at 5% CO_2 in air for 30 minutes to allow the grafts to heal before sealing the Universals and placing them in a roller culture apparatus at 37°C overnight. Embryos which had developed normally were cultured for a further 24 hours in 75% rat serum in 40% oxygen, 5% CO_2, 55% nitrogen. At the end of the culture period fluorescence was assessed either in a Nikon SMZ-U dissecting microscope or, for more detailed analysis, in a Zeiss Axiovert inverted microscope. Images were captured using Improvision Openlab software and processed using Adobe Photoshop.

X-gal staining with in situ hybridisation

Embryos carrying the ubiquitously expressed *Zin40* gene trap integration were used as donors for experiments testing the gene expression of grafted cells. Grafted embryos were first stained with X-gal and then subjected to in situ hybridisation (Tajbakhsh and Houzelstein, 1995) with probes specific for *T* (Wilkinson et al., 1990), sonic hedgehog (Echelard et al., 1993), Delta like 1 (*Dll1*) (Dunwoodie et al., 1997) and *Pax3* (Goulding et al., 1991). Embryos were then dehydrated via a methanol series and processed for paraffin wax histology.

Histology

DiI-labelled embryos and embryos that received grafts of GFP transgenic cells were sectioned transversely in a Series 1000 Vibratome at 50 μm as and images obtained as described above. Embryos subjected to X-gal staining with in situ hybridisation were sectioned transversely at 7 μm and photographed in an Olympus Vanox compound microscope.

RESULTS

All host embryos used in this study were dissected for labelling or grafting at 8.5 d.p.c. (three to eight somites) and cultured for 48 hours, forming a total of 30-35 somites, as described previously (Wilson and Beddington, 1996).

Regionalisation of primitive streak descendants in the tail bud

Previous studies have shown that some descendants of cells in the node and primitive streak at 8.5 d.p.c. are present in the tail bud at 10.5 d.p.c. (Wilson and Beddington, 1996). To determine whether there is any relationship between origin of the cells in the streak and their subsequent location in the tail bud, two distinct sites were labelled: the ventral layer of the node and anterior primitive streak. In accordance with previous fate-mapping studies, the descendants of cells in the node were located in the notochord (Fig. 2A,E), whereas those of the anterior streak were predominantly somitic (Fig. 2B,G). Descendants of anterior streak were also located in the ventral neurectoderm, but not notochord (Fig. 2F). The anterior limit of labelling was around somite 12. In the tail bud after node labelling, the labelled notochord widened and ended abruptly beneath the neural tube, anterior to the end of the tail such that the mesoderm in the tail bud (Fig. 2A,K) was unlabelled. Descendants of the anterior streak were located in the tail bud in two domains: the posterior ectoderm continuous with the ventral posterior neural tube (termed posterior neural plate) and the TBM. (Fig. 2B,M).

We next compared this fate map information with the contribution of GFP transgenic node and primitive streak cells when grafted to stage-matched embryos. These were grafted to the anteriormost extreme of the primitive streak, touching the outer rim of the node (Fig. 1A,C), to allow incorporation of the grafted tissue in either the host node or streak. In general, these grafts mirrored the tissue contribution seen after DiI labelling, showing that, when grafted to this position, cells can incorporate efficiently in either tissue from this site, and that

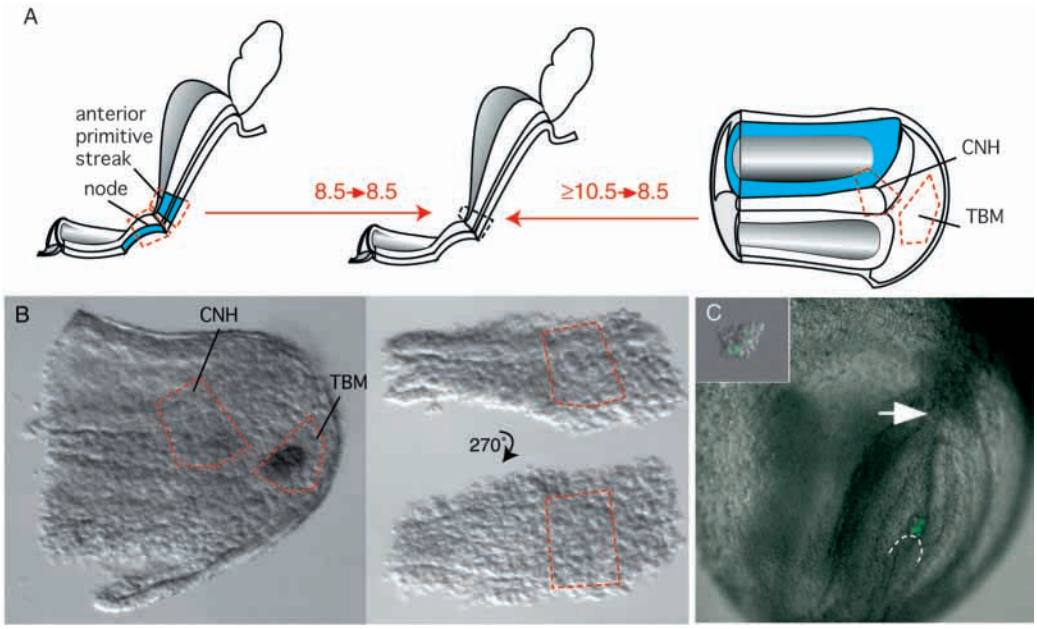

Fig. 1. Labelling sites, donor origin and graft sites. (A) Schematic showing labelling and grafting experiments. Blue fill denotes sites of DiI label. In 8.5 d.p.c. embryos, the ventral layer of node is exposed as a hiatus in the endoderm and can therefore be labelled separately from the ectoderm layer immediately above it, whereas anterior primitive streak is labelled by inserting a pipette through the endoderm and thus labels all layers. The entire neural ectoderm surface, including the posterior ventral neural plate that overlies the notochord is labelled in 10.5 d.p.c. cultured tail pieces. Broken red lines outline sites dissected for grafting. The broken black line outlines plug of tissue at the node/streak border replaced by graft in host embryo. (B) Dissection of 10.5 d.p.c. tail bud: (left) lateral view of tail bud after removal of paraxial mesoderm, overlaid with position of CNH and TBM (broken red lines); (right, top) the same embryo after removal of dorsal neural tube and hindgut; (right, bottom) the same piece rotated so that the widened end of the notochord is upwards. CNH is outlined in red. (C) Inset shows a dissected clump containing eight GFP-labelled cells amongst ~200 unlabelled cells from the CNH of the embryo in Fig. 2C,H,L. Main panel: posterior view of an embryo containing this clump grafted immediately posterior to the node (outlined by a broken white line) at the anterior of the primitive streak, the posterior limit of which is marked by a white arrow.

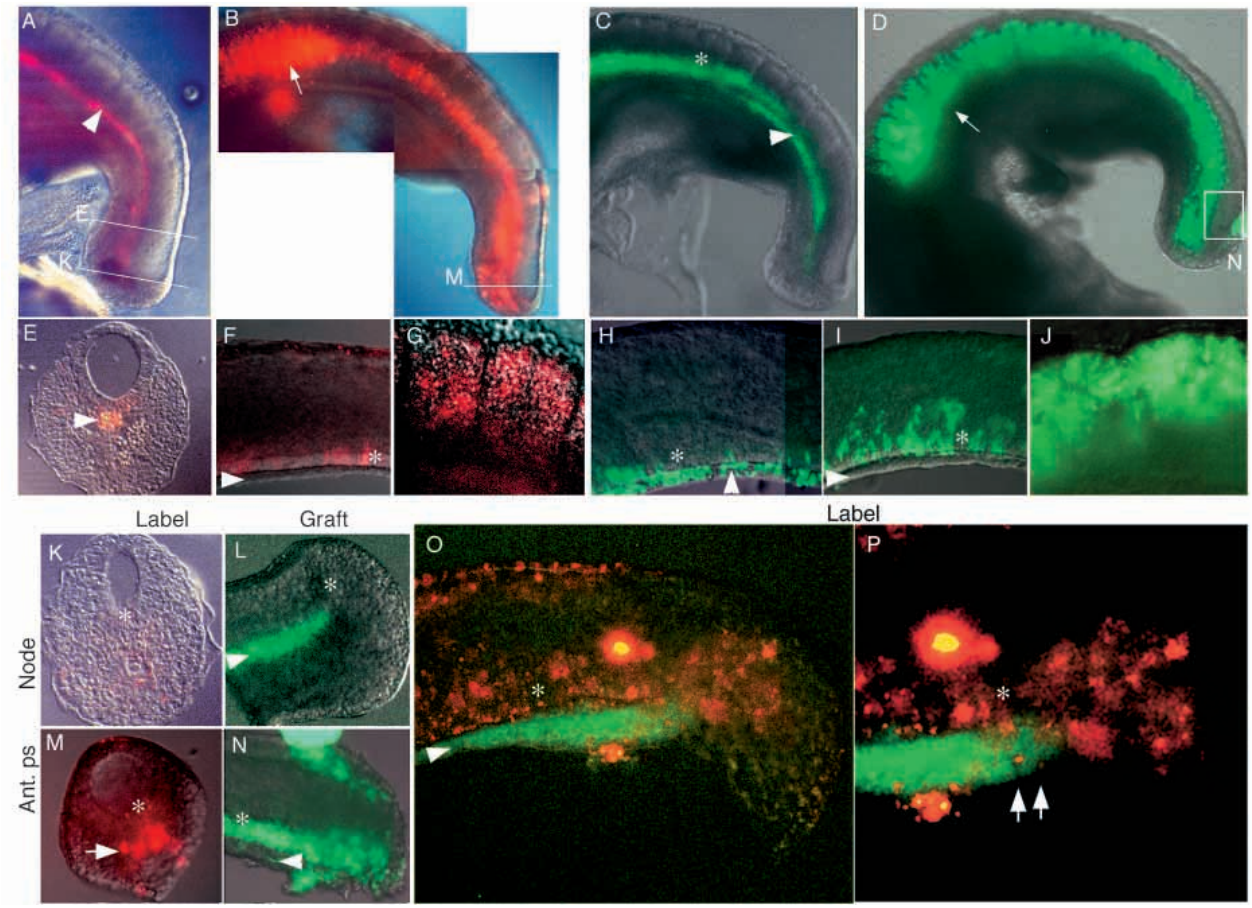

Fig. 2. Descendants of the anterior streak and node populate different regions of the tail bud. Embryos were DiI labelled or grafted with GFP-expressing cells at 8.5 d.p.c. and cultured for 48 hours. (A-D) Lateral views of the posterior ends of manipulated embryos. (A) Embryo labelled with DiI in the ventral node. Labelled cells populate the notochord (arrowhead) and end short of the tail tip, just anterior to the line showing the plane of section in K. (B) Embryo labelled with DiI in the anterior primitive streak. Labelled descendants colonise somites and are widespread in the tail bud. Arrow, position of somite 20. (C) Embryo grafted with 8.5 d.p.c. node. Label is similarly located to the label in A, but also includes the ventral neurectoderm (asterisk). (D) Embryo grafted with 8.5 d.p.c. anterior primitive streak. Label is similar to that in B. Arrow indicates position of somite 20. (E) Transverse section of the embryo shown in A, showing label in the notochord. (F) Neural tube and notochord, and (G) paraxial mesoderm of dissected embryo shown in B. Labelled cells are present in ventral neural tube, but not notochord (F), and in somites (G). (H) Dissected neural tube and notochord of embryo shown in C, where labelled cells populate notochord and ventral neural tube. (I) Dissected neural tube and notochord, and (J) paraxial mesoderm of the embryo shown in D. Labelling is similar to that in F,G. (K) Transverse section of distal tail bud of embryo in A. No labelling is seen. (L) Dissected tail tip of embryo shown in C. Labelling is confined to notochord and ends short of the tail tip. (M) Dissected neural tube and notochord (transverse view) of embryo in B. Labelled cells are present in posterior neurectoderm and mesoderm (arrow). (N) Dissected distal neural tube and underlying mesoderm of embryo in D. Labelled cells are present in ventral neurectoderm. (O,P) Embryo labelled with DiI in anterior primitive streak (red) and CMFDA in ventral node (green). (O) Fluorescent overlay on brightfield image, and (P) fluorescent image, of dissected neural tube and notochord. Node descendants end sharply under the neural tube. Anterior streak descendants populate the ventral neurectoderm and underlying mesoderm and, posteriorly, encroach on the notochord territory (arrows). Arrowheads indicate notochord; asterisks indicate ventral neural tube.

Table 1. Isochronic and heterochronic grafts

			Number (%) of incorporated embryos with label in				
			Axis			Tailbud	
	Total embryos	Total incorporated	NCH	NT	PXM	CNH	TBM
8.5 d.p.c. node	22	18 (82)	17 (94)	16 (89)	8 (44)	17 (94)	4 (22)
8.5 d.p.c. ant. streak	10	8 (80)	1 (12)	4 (50)	8 (100)	5 (62)	6 (75)
10.5 d.p.c. CNH	18	15 (83)	9 (60)	6 (40)	13 (87)	14 (93)	11 (73)
12.5 d.p.c. CNH	8	6 (75)	4 (66.7)	3 (50)	6 (100)	4 (67)	4 (67)
10.5-12.5 d.p.c. TBM	11	1 (9)	0	0	1 (100)	0	0

Contribution of DiI-labelled or GFP transgenic grafts to the axis of 8.5 d.p.c. (three to eight somite) embryos after grafting to the border of the node and anterior streak and culturing for 48 hours.

NCH, notochord; NT, neural tube; PXM, paraxial mesoderm; CNH, host chordoneural hinge; TBM, host tail bud mesoderm.

the pattern of incorporation reflects the site of origin of the cells. Grafts of node contributed predominantly to notochord (Fig. 2C,H; Table 1) and anterior streak to somites (Fig. 2D,J; Table 1). In eight embryos, contribution from grafted node to predominantly medial paraxial mesoderm was observed (Table 1). This is consistent with fatemaps of the chick node, where cells in lateral regions of the node contribute to somites (Psychoyos and Stern, 1996; Selleck and Stern, 1991). However, the majority of embryos receiving node grafts contained little or no contribution to somites, indicating that it is possible to physically separate somite from notochord progenitors. Both graft types contributed descendants to the ventral neural tube (Fig. 2H,I), although node grafts tended to contribute to more ventral descendants than anterior streak ones, consistent with existing fate maps. In the tail bud, the contribution from GFP transgenic cells (Fig. 2L,N) was essentially as seen with the fluorescent lineage tracers (Fig. 2K,M). In anterior streak grafts, GFP-labelled cells were absent from the notochord, except for a small number of cells at its posterior end in the CNH (Fig. 2N).

To confirm the distinct locations of streak and node descendants, anterior streak cells were labelled in situ with DiI (red) and ventral node with CMFDA (green). Here, the CMFDA-labelled notochord ends sharply beneath the neural plate, while anterior streak descendants are found in the posterior neural plate and mesoderm directly beneath it (Fig. 2O,P). Dorsal labelling in surface ectoderm is probably a result of DiI spreading in the amniotic cavity on initial labelling. Interestingly, although a sharp posterior border is seen in the notochord descended from ventral node, primitive streak descendants appear to encroach on this territory. This suggests that anterior streak descendants may contribute to posterior notochord.

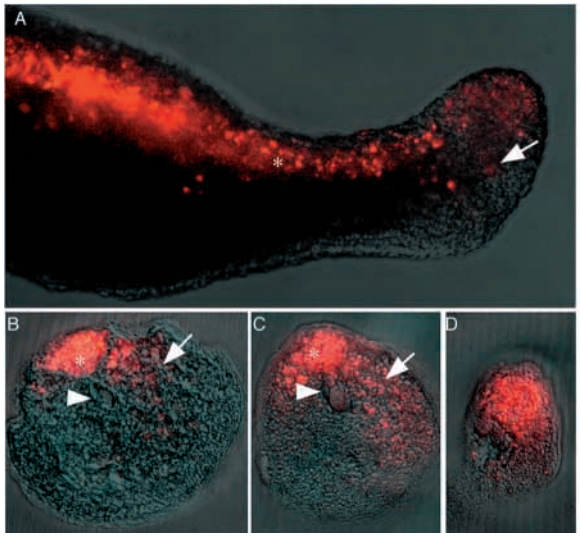

Fig. 3. The posterior neural plate generates mesoderm after posterior neuropore closure. (A) 10.5 d.p.c. tail piece labelled with DiI in neurectoderm and cultured for 48 hours. Labelled descendants are present in neurectoderm (asterisk) and posterior mesoderm (arrow). (B-D) successively more posterior transverse sections of a second embryo, showing label in neurectoderm (asterisks), mesoderm (arrow) and in the posterior, but not more anterior, notochord (arrowheads in B,C).

Before posterior neuropore closure, the posterior neural plate is a source of mesoderm for somites (Wilson and Beddington, 1996). As labelled anterior streak contributed descendants to both posterior neurectoderm and mesoderm, it was of interest to determine whether the posterior neural plate continues to produce mesoderm after posterior neuropore closure. To test this, the entire neurectoderm of dissected 10.5 d.p.c. tail pieces that had undergone posterior neuropore closure was labelled using DiI. After 48 hours, labelled mesoderm was detected in the posterior region of six out of six cultured tail pieces (Fig. 3). Here, too, there is some evidence that the most posterior notochord is populated by ectoderm descendants (compare notochord in Fig. 3B with that in 3C) Therefore, a region continuous with the neurectoderm – most probably the posterior ventral neurectoderm, which is descended from the streak (Fig. 2M) – contributes to the mesoderm of the tail. The region composed of the posterior neural plate and the posterior end of the notochord is thus topologically equivalent to the CNH defined in *Xenopus*.

In the CNH, the ventral node descendants identified by lineage labelling are morphologically indistinguishable from more posterior axial mesoderm beneath the neurectoderm in the tail bud. By contrast, mesoderm located in more posterior, ventral and paraxial regions in the tail bud is composed of loose mesenchyme. It was therefore possible to dissect apart the loose tail bud mesoderm (TBM) from the CNH. As shown above, the CNH contains descendants of ventral node and anterior streak, while the TBM contains only anterior streak descendants. The location of these cells in the tail bud suggests that they may constitute a self-renewing subset of the labelled or grafted tissue. This has been tested in two ways: (1) we have grafted CNH and TBM from tail buds up to 12.5 d.p.c. into 8.5 d.p.c. embryos, and (2) labelled 10.5 d.p.c. CNH or TBM have been serially passaged into successive 8.5 d.p.c. embryos. In each case, a self-renewing population would be expected to contribute descendants both to the differentiated axial tissues formed by the host and the tail bud itself.

Potency depends on location in the tail bud

We compared the capacity of dissected 10.5-12.5 d.p.c. CNH or TBM to differentiate relative to control isochronic grafts, when grafted to the 8.5 d.p.c. primitive streak/node border (Table 1). The donor tissues were derived either from GFP transgenic embryos, *Zin40* embryos or wild-type tissue labelled with DiI. Although a high proportion of control isochronic grafts had incorporated well in the axis (Table 1), we observed a reduction in the proportion of grafts from the TBM that incorporated correctly, with cells remaining predominantly as morphologically undifferentiated clumps (Fig. 4A,B). Differentiation of only one out of 11 heterochronic grafts of TBM, derived from a 10.5 d.p.c. embryo, was observed. In this embryo, labelled cells were restricted to a short unilateral stretch of somitic mesoderm and did not populate the tail bud (data not shown).

By contrast, a high proportion of embryos receiving up to 12.5 d.p.c. CNH cells showed extensive contribution to the axis (Fig. 4C-G; Table 1). Most successfully grafted embryos contained label in somites (Fig. 4C,D,G), with a lower proportion showing label in notochord and/or neural tube (Fig. 4E,F). All embryos showed bilateral contribution from the labelled cells. In general, the anterior limit of contribution

tended to be more posterior than in isochronic grafts (approx. somite 17 onwards; Fig. 4C). Unlike TBM, they also populated the tail bud with high frequency (Fig. 4C,E; Table 1), and were located in both the CNH and in TBM.

To verify that these apparently well-integrated donor tissues indeed differentiated appropriately, a subset of the embryos were grafted with cells expressing the transgenic marker *Zin40*, a developmentally neutral gene trap integration containing a ubiquitously expressed *lacZ* gene (Munsie et al., 1998). This histologically stable marker allowed processing of grafted embryos for in situ hybridisation to markers of differentiation in axial tissues (Tajbakhsh and Houzelstein, 1995). Co-expression of *lacZ* and the differentiation marker was scored in serial transverse sections.

Within the axis, brachyury (*T*) and sonic hedgehog (*Shh*) are expressed in the notochord, and Shh is additionally expressed in the floorplate (Echelard et al., 1993; Wilkinson et al., 1990). In embryos that received an 8.5 d.p.c. node graft, both *T* and *Shh* were expressed appropriately in donor cells in the notochord, and graft-derived cells in the floorplate expressed *Shh* but not *T* (Fig. 5A-D; Table 2). Cells

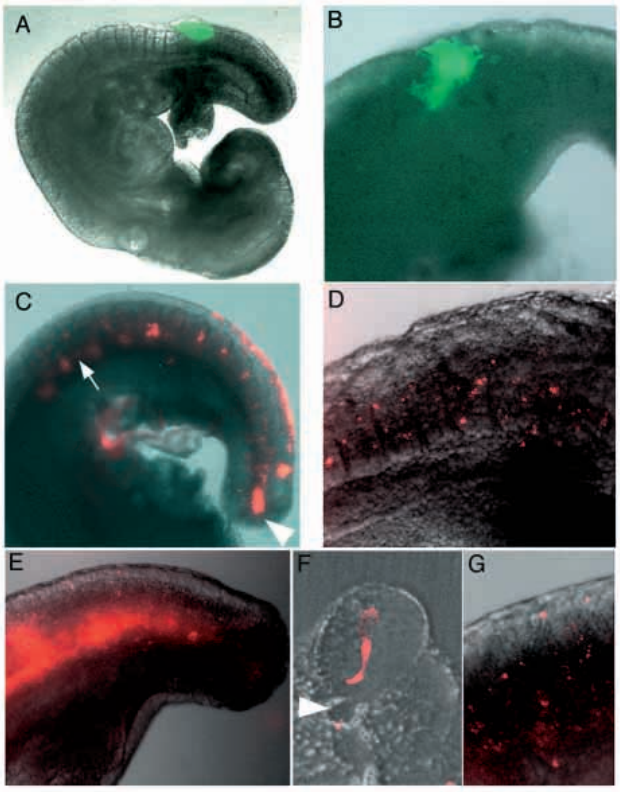

Fig. 4. The CNH, but not the TBM, generate labelled descendants in axis, CNH and tail bud. (A,B) Cultured embryos that received a graft of 10.5 d.p.c. (A) and 12.5 d.p.c. (B) TBM. Grafts remain as distinct clumps and do not incorporate in the host. (C,D) Whole embryo (C) and dissected paraxial mesoderm from a second embryo (D) that received a graft of DiI-labelled 10.5 d.p.c. CNH. Labelled cells are present in CNH (arrowhead) and paraxial mesoderm. Arrow indicates position of somite 20. Labelled cells are also present along the dorsal neural tube, possibly because of incomplete incorporation of the graft during posterior neuropore closure. (E-G) Embryos receiving a graft of DiI-labelled 12.5 d.p.c. CNH. Tail (E) and transverse section (F) of a second embryo showing incorporation in notochord (arrowhead). (G) Dissected paraxial mesoderm of a third embryo showing label in somites.

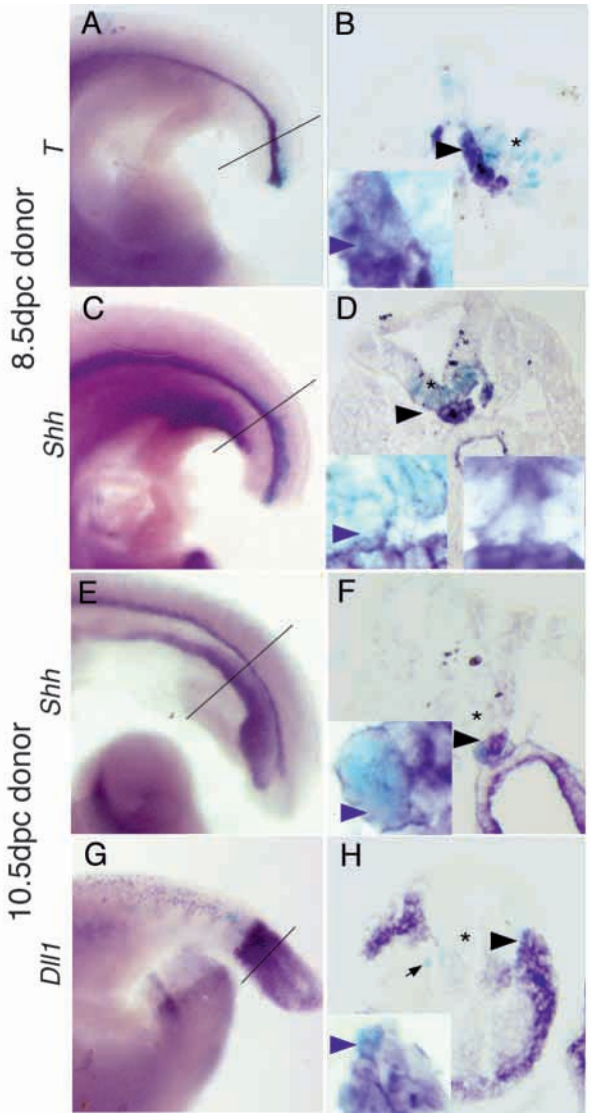

Fig. 5. Grafted cells express markers of differentiation correctly. Embryos are doubly stained with X-gal (light blue; donor cells), and antisense riboprobes as indicated (purple). (A,C,E,G) Whole embryo; (B,D,F,H) transverse section of the adjoining embryo at the level shown by the lines in A,C,E,G. Left hand insets in B,D,F,H show high power images of the regions indicated by black arrowheads. Co-expressing cells are indicated by blue arrowheads. (A-D) Embryos resulting from graft of 8.5 d.p.c. node. (A,B) Embryo hybridised with *T* riboprobe. Like unlabelled host cells, donor cells in the notochord express *T*, while those in the ventral neural tube (asterisk and upper right-hand corner of inset) do not. (C,D) Embryo hybridised with *Shh* riboprobe. Donor cells correctly express *Shh* in the floorplate (compare floorplate in B with that in D). A comparable high magnification image in a control unlabelled embryo is shown (right-hand inset in D). (E-H) Embryos receiving grafts of 10.5 d.p.c. CNH. (E,F) Embryo hybridised with a *Shh* riboprobe. Donor cells in the notochord express *Shh*. (G,H) Embryo hybridised with *Dll1* riboprobe. Donor cells in dorsal paraxial mesoderm express *Dll1*, while those outside the region of host cells expressing *Dll1* do not (arrow). Asterisks indicate ventral neural tube.

Table 2. Gene expression in grafted cells anterior to the tail bud

Graft type	Embryo	Probe	Expression of marker by donor cells in NCH	NT	PXM
8.5 d.p.c. node	1	*T*	Yes	No	No
	2	*Shh*	Yes	Yes	No
	3	*Pax6*	No	Yes	No
10.5 d.p.c. CNH	4	*T*	Yes	No	No
	5	*Shh*	Yes	No	No
	6	*Shh*	Yes*	Yes*	No
	7	*Dll1*	No	No	Yes
	8	*Pax3*	No	Yes	Yes

*Rodlike groups of donor cells near the notochord could be followed through serial sections in this embryo. When lying beside the notochord, they did not express *Shh*, but became physically incorporated over several sections in notochord and ventral neural tube, and concomitantly expressed *Shh*.

NCH, notochord; NT, neural tube; PXM, paraxial mesoderm; CNH, host chordoneural hinge.

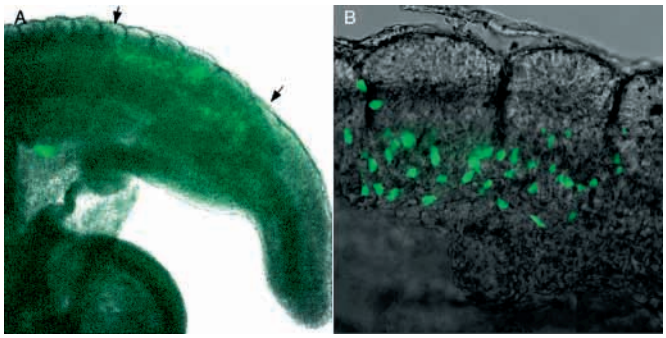

Fig. 6. Regrafting of GFP-labelled TBM (derived from an anterior streak graft) results in contribution to short stretches of somites, not the tail bud. (A) Whole embryo and (B) dissected paraxial mesoderm (enlarged) of embryo shown in A. Cells have incorporated over a distance of six somites unilaterally. Arrows indicate anterior and posterior borders of labelled cell incorporation.

immediately dorsal to the floorplate express the neural marker *Pax6*, and graft-derived cells populating this region also appropriately expressed *Pax6* (Table 2). Medially located donor cells in the paraxial mesoderm showed no ectopic *T* expression (data not shown). The tail buds of the embryos shown in Fig. 5A-D had been removed prior to processing and were not assayed for marker gene expression. This apparently normal differentiation therefore correlates well with the morphological assessment of incorporation in tissue derived from isochronic grafts. To determine whether this was true of grafted 10.5 d.p.c. tissue, the expression of *T*, *Shh* and two additional markers of paraxial mesoderm differentiation, *Dll1* (Dunwoodie et al., 1997) and *Pax3* were assayed. Within the axis, where donor cells appeared morphologically incorporated in a tissue, they correctly co-expressed all differentiation markers assayed (Fig. 5E-H; Table 2). Furthermore, the incorporated cells did not ectopically express differentiation markers (Fig. 5H, arrow). In the tail bud mesoderm and CNH, many donor cells also expressed *T*, showing that these cells also express markers appropriate for tail bud (data not shown).

Although differentiation towards somites was apparent in many of the embryos, the grafted tissue did not always intersperse well with host tissue. Typically, some regions of the grafted embryos contained small groups of medially located somitic tissue, sometimes out of register with those of the host (Fig. 4C). However, it is clear that some regions – even in embryos where this abnormal differentiation was apparent – do mix well with host tissue (Fig. 4D,G). Taken together, these results suggest that the grafted CNH is at least partially equivalent to its earlier counterpart in the node and anterior streak. These results show that the CNH has the potential both to contribute widely to the axis, and to repopulate the CNH itself and the TBM.

The CNH, but not TBM, is serially transplantable

The population of host CNH by grafted CNH cells separated by up to 4 days in developmental stage from the host (Table 1; Fig. 4), together with their ability to participate in differentiated axial tissue formation, suggested that axial stem cells reside there, and not in the TBM. Such stem cells should also contribute cells to the axis and repopulate CNH on multiple passages through host embryos. We therefore tested this by regrafting GFP-labelled CNH and TBM to 8.5 d.p.c. embryos.

In second generation grafts, groups of cells containing labelled TBM derived from initial anterior streak grafts were also disadvantaged relative to CNH from incorporating in the axis (Table 3). Similar to the results above, when they did incorporate in the axis, they did so only over short axial

Table 3. Serial grafting of CNH and TBM

	Total embryos	Total incorporated	No. (%) of incorporated embryos with label in Axis NCH	NT	PXM	Tailbud CNH	TBM
2nd generation							
CNH	10	8 (80)	2 (25)	1 (12)	6 (75)	6 (75)	4 (50)
TBM	11	6 (55)	0	0	5 (83)	0	1 (17)
3rd generation							
CNH	10	4 (40)	3 (75)	2 (50)	3 (75)	3 (75)	2 (50)
TBM	4	0					

Contribution of CNH or TBM derived from original GFP transgenic node and anterior streak grafts to the axis of 8.5 d.p.c. (three to eight somite) embryos after grafting to the border of the node and anterior streak and culturing for 48 hours..

NCH, notochord; NT, neural tube; PXM, paraxial mesoderm; CNH, host chordoneural hinge; TBM, host tail bud mesoderm.

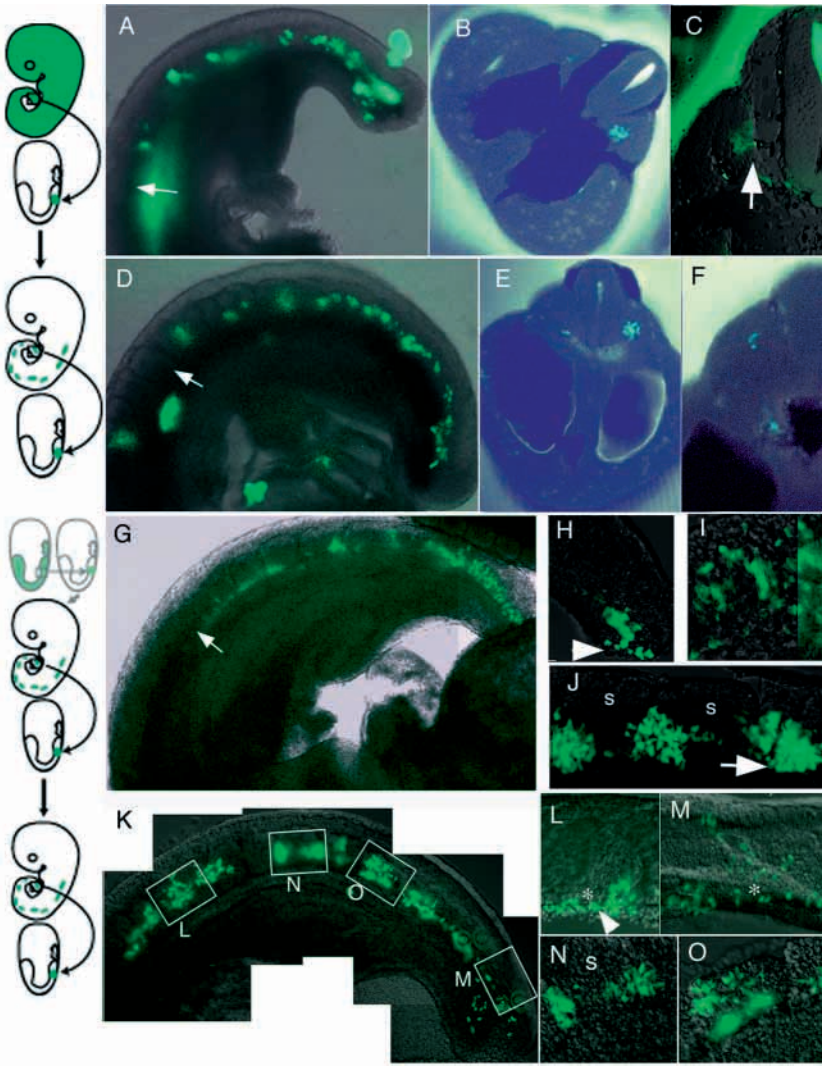

Fig. 7. Regrafting of GFP-labelled CNH results in contribution to both the axis and tail bud in up to three generations. Diagrams illustrate the history of grafted cells in the cultured embryo shown immediately to the right. (A-C) Whole mount (A) and transverse sections (B,C) of an embryo that received a graft of 10.5 d.p.c. GFP-labelled CNH. In the axis, cells populated the paraxial mesoderm exclusively and either formed small medial graft-derived somites (B), or incorporated into wild-type tissue (C, arrow). (D-F) Whole mount (D) and sections (E,F) of an embryo grafted with CNH cells from the embryo in A-C. Grafted cells populate axial derivatives that are identical to the parent graft. (G-J) Whole mount (G), dissected neural tube/notochord (H) and paraxial mesoderm (I,J) from embryos that had received a graft of 10.5 d.p.c. CNH, derived from an initial 8.5 d.p.c. node graft. Labelled cells populate the posterior end of the notochord and CNH (arrowhead, H), incorporate in paraxial mesoderm (I), but also form small medially located somites that are epithelial posteriorly (arrow in J) and disperse anteriorly, and are located out of register with the endogenous somites (s). (K-O) A third generation graft. Whole mount (K), dissected neural tube and notochord (L,M), and paraxial mesoderm (N,O), showing contribution to notochord (arrowhead in L), posterior neurectoderm (asterisks in L and M), and both ectopic somites located between host somites (s) (N) and interspersed GFP-labelled cells (O) in host somites. Arrows in A,D,G indicate the position of somite 20.

distances (Fig. 6), and only one embryo showed label in the tail bud. In this embryo, no contribution to more anterior positions in the axis were observed, and it is therefore impossible to determine whether these grafted cells truly retained potential to contribute to the axis. Thus, even though these cells were now retained in the tail bud 48 hours after transplant to the anterior primitive streak, this did not select for greater ability to generate descendants both in axis and tail bud.

As shown above, labelled CNH from 10.5 d.p.c. embryos grafted to 8.5 d.p.c. primitive streak resulted in contribution throughout the axis and in the tail bud. Labelled cells from the CNH of the embryo shown in Fig. 7A were regrafted to an 8.5 d.p.c. host, which contributed to the same axial tissues and the CNH, although there may be some depletion of cells from the CNH itself (Fig. 7D). Sections revealed contribution to somites in both generations (Fig. 7B,C,E,F). Similar results were obtained when the grafted 10.5 d.p.c. CNH was derived from an initial 8.5 d.p.c. node graft. These second generation embryos predominantly showed contribution to somites, but also to notochord and ventral neural tube (Fig. 7G-J; Table 3). Although intermingling of host and wild-type cells could be observed in paraxial mesoderm (Fig. 7H), formation of small, medial, graft-derived somites within the somite territory was also apparent (Fig. 7J). The majority of grafted embryos showed repopulation of both CNH and TBM, supporting the hypothesis that TBM is derived from CNH. These second generation CNH were grafted a third time, and incorporation was observed both in axial tissues and the tail bud, in CNH and TBM (Fig. 7K-O). The grafted tissue shows a somewhat reduced rate of incorporation (Table 3). However, the pattern of incorporation in notochord, somites and neural tube was similar in these 3rd generation grafts to that observed in the second generation and in the grafts of 10.5-12.5 d.p.c. CNH described above. TBM derived from second generation CNH grafts showed similar properties to other 10.5 d.p.c. TBM grafts (Table 3).

In general, the anterior limit of contribution (approx. somite 17 onwards; Fig. 7A,D,G) was similar for the 1st, 2nd and 3rd generations of CNH grafts, showing that the stage of the donor tail bud (not the absolute age of the cells) determined this anterior border. No difference in contribution was obvious between CNH derived from anterior streak versus that from node. However, it was striking that contribution to 3 generations was seen only where the first generation grafts were from nodes that contributed not only to notochord, but also to paraxial mesoderm. This suggested that a population of axial progenitors with capacity for self-renewal and extensive contribution to somites, notochord and neurectoderm were located close to the node at 8.5 d.p.c., and that these continued to be associated with the CNH in successive generations.

DISCUSSION

Regionalisation in the tail bud

We have shown that the descendants of primitive streak and node populate different regions in the tail bud. The descendant of the mouse anterior primitive streak in the posterior neural plate is composed of both neural and somitic progenitors that overlie the posterior end of the notochord. This layout is similar to that in the *Xenopus* tail bud (Tucker and Slack, 1995), where neural (N) progenitors abut precursors of mesoderm within the posterior neural plate (M) and notochord (C) progenitors underlie both. Thus, it is valid to term the mouse posterior neural plate and notochord region 'CNH'. In *Xenopus*, cells from the M region in the CNH pass posteriorly in the tail bud before exiting laterally in the paraxial mesoderm. Similar posterior and lateral movement from the equivalent chick CNH have been observed (Catala et al., 1996; Catala et al., 1995). Thus, the passage of cells from the ectoderm of the CNH towards the more posterior TBM is conserved among vertebrates.

The dramatic involution movements during *Xenopus* gastrulation cease by the neural plate stage (Gont et al., 1993), as does the transit of a large part of the epiblast through the streak and node/organiser to generate mesoderm in mouse by the equivalent headfold stage (Kinder et al., 2001; Snow, 1981). In chick, passage of lateral epiblast cells early during gastrulation through Hensen's node ceases prior to node regression (Joubin and Stern, 1999). Thus, at the start of somitogenesis in vertebrates, the neural, mesodermal and notochordal precursors are no longer in mass transit from the ectoderm but are contained in the region of ingression. We have extended previous studies in the mouse to show that this ingression of cells to the mesoderm layer continues even after posterior neuropore closure around the 35-somite stage. In chick, ingression movements after posterior neuropore closure have also been observed (Knezevic et al., 1998). These can apparently occur from the dorsal surface, perhaps indicating subtle differences in the organisation and/or movements of vertebrate tail bud tissues. In *Xenopus* and chick therefore, as in mouse, the posterior neural plate may merely represent a localised remnant of the outer layer of the marginal zone/primitive streak, which continues a form of ingression after gastrulation.

The mouse ventral node has been identified previously as a putative self-renewing progenitor region for the notochord (Beddington, 1994; Wilson and Beddington, 1996), as its posterior extremity contains labelled cells after culture. In the present study, the apparent population of the posterior end of the notochord by anterior streak derivatives and the posterior neural plate (Fig. 2N,P; Fig. 3C) suggests that the ventral node itself may not contain all notochord progenitors. Instead, the notochord may be supplied from cells in the ectoderm layer that represent more primitive notochord precursors. In chick, too, there is evidence that some notochord progenitors reside in the ectodermal layer, rather than in the ventral node region (Catala et al., 1996; Catala et al., 1995; Psychoyos and Stern, 1996). Furthermore, while passage through Hensen's node is a prerequisite for incorporation in the notochord, some notochord progenitors originate outside Hensen's node in the anterior primitive streak and are only incorporated there later, presumably during node regression (Psychoyos and Stern, 1996). The hypothesis that the mouse ventral node does not contain all notochord progenitors is supported by the relative quiescence of cells in the node and notochord (as few as 10% appear to cycle), while cells around the node are dividing rapidly (Bellomo et al., 1996). Furthermore, the ability of grafted 8.5 d.p.c. node to give rise to notochord in the present study, even when grafted to the anterior streak, suggests that the ventral node contains committed notochord precursors. Therefore the ventral node may contain cells destined only for exit to the notochord, and although dramatic elongation of this structure occurs, mediolateral intercalation (Wilson and Keller, 1991) may account for much of this elongation. This therefore suggests that notochord, somite and neural progenitors are located close together in the ectoderm layer near the node at 8.5 d.p.c., and we show that these progenitors remain closely apposed in the later CNH.

In the *Xenopus* tail bud, there is also evidence from labelling very small groups of around one to three cells in the CNH itself during tail elongation that the progenitors of notochord, muscle and neural tube are located close together, or represent single multilineage cells (Davis and Kirschner, 2000). Earlier, during blastopore closure at the neural plate stage, there appear to be more regionally separated cells (Gont et al., 1993). However, if multilineage cells were located in only a small proportion of the blastopore, lineage labelling larger groups of cells using DiI may not have highlighted such a population.

A stem cell population in the CNH ectoderm?

It has been hypothesised in chick and mouse that cells remaining in the streak or tail bud at the termination of prospective lineage labelling studies represent a minority population composed of self-renewing stem cells in the streak (Beddington, 1994; Psychoyos and Stern, 1996; Tam and Beddington, 1987; Wilson and Beddington, 1996). Evidence for stem cell precursors of myotome (Nicolas et al., 1996) and spinal cord (Mathis and Nicolas, 2000) located in or near the posterior midline of the embryo strengthens this hypothesis. However, so far, evidence showing that the prospective lineage labelled cells in the streak are indeed stem cells, and not dead or quiescent cells, has been lacking. We tested whether the two major areas colonised by primitive streak descendants in the tail bud, the TBM and CNH, fulfilled criteria expected of axial stem cells by grafting GFP transgenic cells to 8.5 d.p.c. embryos.

Potency of tail bud in contributing to anterior axial positions

In this study we have shown that the tissues in the tail bud are not developmentally equipotent. Unlike TBM, CNH cells can efficiently incorporate in the axis, differentiating into somites, ventral neural tube and notochord, and giving rise to descendants in the tail bud itself. Within the tail bud, CNH descendants are found in both CNH and TBM, consistent with the observation that the posterior neural plate continues to generate mesoderm long into axial elongation. There was no apparent difference between 10.5 and 12.5 d.p.c. CNH in the anterior extent of labelling or the tissue types colonised. It is therefore likely that this region contains self-renewing progenitors.

The strong bias towards contribution to somites by CNH cells compared with their node-derived antecedents is

intriguing. This may result from the composition of the graft. Somites form most of the bulk of the tail, while the neural tube and notochord are a relatively minor population. Alternatively, cells or growth factors at the graft site, at the anterior of the primitive streak, may influence the CNH population to differentiate towards somites. A further characteristic of the graft-derived somites was the occurrence of medially located ectopic somites, sometimes in embryos that also had bona fide intermingling of grafted cells with wild type cells in somites. It is possible that the cluster of grafted cells in the streak may retain information on the periodicity of somites to be formed. An alternative possibility is that as the CNH ectoderm is much smaller than the primitive streak, the grafted cells may include the progenitors of entire somites, effectively creating a heterotopic graft of lateral somite precursors to a location where cells normally exit to medial somites.

In contrast to CNH, TBM is only capable of populating short axial stretches that corresponds to a distance of a few somites. It shows a low frequency of incorporation in axial tissue, and fails to contribute to tail bud. The capacity of TBM cells to contribute to anterior axial positions has also been studied by Tam and Tan (Tam and Tan, 1992), who grafted of small numbers of cells from the tail bud of embryos up to 13.5 d.p.c. These grafts are capable of contributing to much more anterior positions than they would have done in situ. As these authors do not distinguish CNH from TBM in the grafts, it may be that it is a small population of CNH cells included in their grafted population that retain potency, especially to contribute to the tail bud. The relatively low frequency observed by these authors of grafted cell retention in the tail bud (around 20% of embryos) supports this idea. Alternatively, the smaller number of grafted cells used by Tam and Tan (Tam and Tan, 1992) may intermingle more extensively with the host cells than the TBM grafts in the present study. Larger TBM grafts may therefore be subject to greater community effects that preserve either specification as mesoderm or anteroposterior information. As recently ingressed mesoderm earlier in gastrulation is more restricted in potency than the cells from the ectoderm that produced it (Tam et al., 1997), it is likely that TBM cells that have undergone ingression from the posterior neural plate, are also restricted in potency.

Serial passage of axial progenitors

When CNH cells are passaged through a second and third generation of embryos in culture, at least some cells retain the capacity to colonise the axis in an identical manner to first generation CNH. These results show that the primitive streak/tail bud can retain descendants of cells initially located near the node over a total of around 90 somites made by the hosts, and strongly suggests that a stem cell population resides in the CNH. When we tested TBM in the same way, this showed the same pattern of incorporation as grafts from fresh 10.5 d.p.c. TBM, albeit a slight increase in the frequency of grafts that incorporate in the axis (compare TBM grafts in Tables 1 and 3). However, this subpopulation of TBM cells that originated from anterior streak does not show any greater tendency for retention in the tail bud than the bulk population of TBM, and argues against stem cells residing in any part of the TBM.

It is interesting to note that the grafts that gave rise to three generations of incorporation in the axis and CNH were descended from initial node grafts that showed contribution to both notochord and paraxial mesoderm. This, together with the observation that many node grafts did not show such contribution, suggests that it is not the node itself, but cells immediately abutting it that were included with the grafted tissue, that demonstrate stem cell-like properties. This would correspond well with the hypothesis, discussed earlier, that the ventral layer of the node contains committed notochord progenitors, while cells in its vicinity in the ectoderm layer constitute less committed (stem cell) progenitors. An interesting parallel to these experiments is seen in studies of the chick node and anterior streak (Charrier et al., 1999). The junction of Hensen's node and the anterior primitive streak (the axial-paraxial hinge) shows overlapping expression of genes characteristic of the node (*Hnf3b* and chordin) and those characteristic of the streak (chicken *Tbx6l*). Cells from this region are capable of generating notochord, neural tube or somites. Normally this contribution is limited to small regions of the axis, and cells are retained in the tail bud. Deletion of the bulk of Hensen's node (excluding the axial-paraxial hinge) results in the interruption of notochord formation, but this resumes further posteriorly. However, deletion of the axial-paraxial hinge results in embryos in which notochord formation continues for a short distance, but is followed by axial truncation. These results imply that this region is important as a signalling centre allowing maintenance of axial elongation, and/or that it contains stem cells for the axis. This is consistent with lineage data in the chick that places progenitors contributing to the entire mediolateral extent of all somites at the anterior end of the streak, in a region overlapping with notochord precursors (Psychoyos and Stern, 1996).

The hypothesis that stem cells are highly localised at the anterior streak, however, presents a paradox. If stem cells are so highly localised, why do fate maps show that most cells in

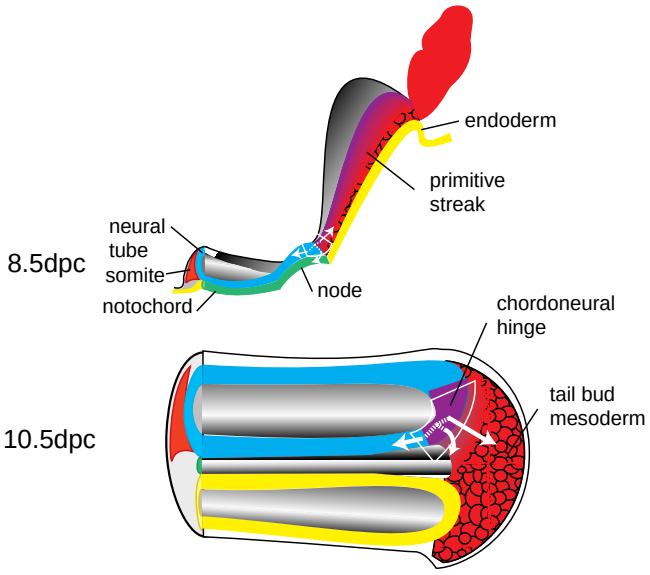

Fig. 8. Location of the progenitor cells during axis elongation. White boxes represent the regions in 8.5 d.p.c. and 10.5 d.p.c. embryos where the stem cell-like population resides. Descendants populate notochord, neural tube and somites (white arrows), and may originate from a common stem cell axial progenitor (broken lines).

the anterior streak close to the node give rise only to the medial portion of somites? The precursors of lateral somites lie more posteriorly in the streak at 8.5 d.p.c. (Wilson and Beddington, 1996). Descendants of these also contribute to the tail bud after 24-48 hours culture (Wilson and Beddington, 1996) (results not shown). However, they do not contribute to the CNH, but instead lie ventrally in the TBM. In contrast, the CNH can generate ventral tail bud mesoderm (Fig. 3A,D; Fig. 7A,K). Therefore, the cells in more posterior regions of the 8.5 d.p.c. streak may represent progenitors already committed to a mesoderm fate, which have themselves arisen earlier from a stem cell population near the node. Nonetheless, the contribution of cells in posterior regions of the 8.5 d.p.c. streak to relatively long axial distances without generating a population in which we can demonstrate extensive potency leaves open the possibility that not all of the postcranial axis is generated by node/streak border and CNH derived-stem cells.

The stem cell progenitors suggested by Nicolas and colleagues for the myotome and spinal cord may therefore reside close together in the regions we have identified: the junction of the node and streak at 8.5 d.p.c., and the CNH at 10.5-12.5 d.p.c. (Fig. 8). Their close physical proximity raises the possibility that a single multipotent axial stem cell type may exist, consistent with the observation that some cells in the *Xenopus* CNH appear to contribute to neurectoderm, somites and notochord (Davis and Kirschner, 2000). In the myotome, stem cell-derived clones are almost exclusively bilateral (Nicolas et al., 1996), consistent with our observation that CNH cells contribute bilaterally to host embryos. Our analysis also suggests a location for progenitor cells that contribute to up to six consecutive somites (either unilaterally or bilaterally) in the myotome observed by Nicolas et al. (Nicolas et al., 1996). The present study would locate such clones posterior to the axial-paraxial hinge at 8.5 d.p.c., and in the TBM from 10.5 d.p.c. Like these progenitors identified by Nicolas et al. (Nicolas et al., 1996) TBM derived cells contributed to one or both sides of the midline (results not shown). Our identification of the position of putative stem cells in the node/streak junction and the CNH will make it possible to characterise their potency in more detail via single cell transplantation or other forms of clonal analysis.

The authors thank Ronald Wilkie for technical assistance, and Elena Tzouanacou, Austin Smith, Ruth Arkell and Josh Brickman for helpful comments on the manuscript. V. W. gratefully acknowledges the mentoring of the late Rosa Beddington, who provided early inspiration for this work. This work was funded in part by an MRC Career Development Award (G120/215).

REFERENCES

Beck, C. W. and Slack, J. M. (1998). Analysis of the developing Xenopus tail bud reveals separate phases of gene expression during determination and outgrowth. *Mech. Dev.* **72**, 41-52.

Beddington, R. S. (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-620.

Beddington, S. P. (1981). An autoradiographic analysis of the potency of embryonic ectoderm in the 8th day postimplantation mouse embryo. *J. Embryol. Exp. Morphol.* **64**, 87-104.

Bellomo, D., Lander, A., Harragan, I. and Brown, N. A. (1996). Cell proliferation in mammalian gastrulation: the ventral node and notochord are relatively quiescent. *Dev. Dyn.* **205**, 471-485.

Catala, M., Teillet, M. A. and le Douarin, N. M. (1995). Organization and development of the tail bud analyzed with the quail-chick chimaera system. *Mech. Dev.* **51**, 51-65.

Catala, M., Teillet, M. A., de Robertis, E. M. and le Douarin, M. L. (1996). A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls. *Development* **122**, 2599-2610.

Chapman, D. L., Agulnik, I., Hancock, S., Silver, L. M. and Papaioannou, V. E. (1996). Tbx6, a mouse T-Box gene implicated in paraxial mesoderm formation at gastrulation. *Dev. Biol.* **180**, 534-542.

Charrier, J. B., Teillet, M. A., Lapointe, F. and le Douarin, N. M. (1999). Defining subregions of Hensen's node essential for caudalward movement, midline development and cell survival. *Development* **126**, 4771-4783.

Chesley, P. (1935). Development of the short-tailed mutant in the house mouse. *J. Exp. Zool.* **70**, 429-459.

Cockroft, D. L. (1990). Dissection and culture of postimplantation embryos. In *Postimplantation Mammalian Embryos: A Practical Approach* (ed. A. J. Copp and D. L. Cockroft), pp. 15-40. Oxford: Oxford University Press.

Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Davis, R. L. and Kirschner, M. W. (2000). The fate of cells in the tailbud of Xenopus laevis. *Development* **127**, 255-267.

Dunwoodie, S. L., Henrique, D., Harrison, S. M. and Beddington, R. S. (1997). Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **124**, 3065-3076.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Gawantka, V., Pollet, N., Delius, H., Vingron, M., Pfister, R., Nitsch, R., Blumenstock, C. and Niehrs, C. (1998). Gene expression screening in Xenopus identifies molecular pathways, predicts gene function and provides a global view of embryonic patterning. *Mech. Dev.* **77**, 95-141.

Gont, L. K., Steinbeisser, H., Blumberg, B. and de Robertis, E. M. (1993). Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip. *Development* **119**, 991-1004.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10**, 1135-1147.

Greco, T. L., Takada, S., Newhouse, M. M., McMahon, J. A., McMahon, A. P. and Camper, S. A. (1996). Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. *Genes Dev.* **10**, 313-324.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Holmdahl, D. E. (1925). Experimentelle Untersuchungen über die Lage der Grenze zwischen primarer und sekundarer Körperentwicklung beim Hühn. *Anat. Anz.* **59**, 393-396.

Joubin, K. and Stern, C. D. (1999). Molecular interactions continuously define the organizer during the cell movements of gastrulation. *Cell* **98**, 559-571.

Kinder, S. J., Tsang, T. E., Wakamiya, M., Sasaki, H., Behringer, R. R., Nagy, A. and Tam, P. P. (2001). The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development* **128**, 3623-3634.

Knezevic, V., de Santo, R. and Mackem, S. (1998). Continuing organizer function during chick tail development. *Development* **125**, 1791-1801.

Lawson, K. A., Meneses, J. J. and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113**, 891-911.

Mathis, L. and Nicolas, J. F. (2000). Different clonal dispersion in the rostral and caudal mouse central nervous system. *Development* **127**, 1277-1290.

Munsie, M., Peura, T., Michalska, A., Trounson, A. and Mountford, P. (1998). Novel method for demonstrating nuclear contribution in mouse nuclear transfer. *Reprod. Fertil. Dev.* **10**, 633-637.

Nicolas, J. F., Mathis, L., Bonnerot, C. and Saurin, W. (1996). Evidence in the mouse for self-renewing stem cells in the formation of a segmented longitudinal structure, the myotome. *Development* **122**, 2933-2946.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313-319.

Psychoyos, D. and Stern, C. D. (1996). Fates and migratory routes of primitive streak cells in the chick embryo. *Development* **122**, 1523-1534.

Ruiz, J. C. and Robertson, E. J. (1994). The expression of the receptor-protein tyrosine kinase gene, eck, is highly restricted during early mouse development. *Mech. Dev.* **46**, 87-100.

Selleck, M. A. and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112**, 615-626.

Snow, M. H. (1981). Autonomous development of parts isolated from primitive-streak-stage mouse embryos. Is development clonal? *J. Embryol. Exp. Morphol.* **65 Suppl**, 269-287.

Tajbakhsh, S. and Houzelstein, D. (1995). In situ hybridization and beta-galactosidase: a powerful combination for analysing transgenic mice. *Trends Genet.* **11**, 42.

Tam, P. P. and Tan, S. S. (1992). The somitogenetic potential of cells in the primitive streak and the tail bud of the organogenesis-stage mouse embryo. *Development* **115**, 703-715.

Tam, P. P. and Beddington, R. S. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99**, 109-126.

Tam, P. P., Parameswaran, M., Kinder, S. J. and Weinberger, R. P. (1997). The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation. *Development* **124**, 1631-1642.

Tucker, A. S. and Slack, J. M. (1995). Tail bud determination in the vertebrate embryo. *Curr. Biol.* **5**, 807-813.

Wilkinson, D. G., Bhatt, S. and Herrmann, B. G. (1990). Expression pattern of the mouse T gene and its role in mesoderm formation. *Nature* **343**, 657-659.

Wilson, P. and Keller, R. (1991). Cell rearrangement during gastrulation of Xenopus: direct observation of cultured explants. *Development* **112**, 289-300.

Wilson, V. and Beddington, R. S. (1996). Cell fate and morphogenetic movement in the late mouse primitive streak. *Mech. Dev.* **55**, 79-89.

Wilson, V., Manson, L., Skarnes, W. C. and Beddington, R. S. (1995). The T gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. *Development* **121**, 877-886.