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A Single Rostrocaudal Colonization of the Rodent Intestine by Enteric Neuron Precursors Is Revealed by the Expression of Phox2b, Ret, and p75 and by Explants Grown under the Kidney Capsule or in Organ Culture

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The colonization of the rodent gastrointestinal tract by enteric neuron precursors is controversial due to the lack of specific cellular markers at early stages. The transcription factor, Phox2b, is expressed by enteric neuron precursors (Pattyn *et al. Development* 124, 4065–4075, 1997). In this study, we have used an antiserum to Phox2b to characterize in detail the spatiotemporal expression of Phox2b in the gastrointestinal tract of adult mice and embryonic mice and rats. In adult mice, all enteric neurons (labeled with neuron-specific enolase antibodies), and a subpopulation of glial cells (labeled with GFAP antibodies), showed immunoreactivity to Phox2b. In embryonic mice, the appearance of Phox2b-immunoreactive cells was mapped during development of the gastrointestinal tract. At Embryonic Days 9.5–10 (E9.5–10), Phox2b-labeled cells were present only in the stomach, and during subsequent development, labeled cells appeared as a single rostrocaudal wave along the gastrointestinal tract; at E14 Phox2b-labeled cells were present along the entire length of the gastrointestinal tract. Ret and p75 have also been reported to label migratory-stage enteric neuron precursors. A unidirectional, rostral-to-caudal colonization of the gastrointestinal tract of embryonic mice by Ret- and p75-immunoreactive cells was also observed, and the locations of Ret- and p75-positive cells within the gut were very similar to that of Phox2b-positive cells. To verify the location of enteric neuron precursors within the gut, explants from spatiotemporally defined regions of embryonic intestine, 0.3–3 mm long, were grown in the kidney subcapsular space, or in catenary organ culture, and examined for the presence of neurons. The location and sequence of appearance of enteric neuron precursors deduced from the explants grown under the kidney capsule or in organ culture was very similar to that seen with the Phox2b, Ret, and p75 antisera. Previous studies have mapped the rostrocaudal colonization of the rat intestine by enteric neuron precursors using HNK-1 as a marker. In the current study, all HNK-1-labeled cells in the gastrointestinal tract of rat embryos showed immunoreactivity to Phox2b, but HNK-1 cells comprised only a small subpopulation of the Phox2b-labeled cells. In addition, in rats, Phox2b-labeled cells were present in advance of (more caudal to) the most caudal HNK-1-labeled cells by 600–700 μm in the hindgut at E15. We conclude that the neural crest cell population that arises from the vagal level of the neural axis and that populates the stomach, midgut, and hindgut expresses Phox2b, Ret, and p75. In contrast, the sacral-level neural crest cells that populate the hindgut either do not express, or show a delayed expression of, all of the known markers of vagal- and trunk-level neural crest cells.

Key Words: **neural crest; mouse; intestine; Phox2b; p75, Ret.**

INTRODUCTION

The neurons and glial cells of the enteric nervous system are derived from the neural crest. In both birds and mammals, the vast majority of enteric neurons in the stomach and intestine arise from vagal-level (somites 1–7) neural crest, particularly from the level of somites 2–6 (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Epstein *et al.*, 1994; Durbec *et al.*, 1996). Most of the enteric neurons in the esophagus do not arise from vagal-level neural crest (Epstein *et al.*, 1994), and recent evidence suggests that they arise from anterior trunk-level neural crest, which also gives rise to the

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superior cervical ganglion (Durbec *et al.,* 1996). Although most of the enteric neurons in the hindgut arise from vagal-level neural crest, studies using chick–quail chimeras and studies in which premigratory cells had been labeled with DiI or retroviruses have shown that some cells that populate the hindgut arise from sacral-level neural crest, which lies caudal to somite 28 in birds and somite 24 in mice (Le Douarin and Teillet, 1973; Pomeranz *et al.*, 1991; Serbedzija *et al.,* 1991). These cells colonize the hindgut well before the arrival of vagal-level-derived neural crest cells (Serbedzija *et al.,* 1991, Kapur *et al.*, 1992). In both mouse and chicken embryos, the vagal-level neural crest cells colonize the gastrointestinal tract rostrocaudally, and if segments of hindgut are removed prior to the arrival of the vagal neural crest cells but after the time the sacral neural crest cells are reported to have reached the hindgut, enteric neurons do not develop in the hindgut explants (Allan and Newgreen, 1980; Nishijima *et al.,* 1990; Kapur *et al.,* 1992). Moreover, following transection of the midgut prior to the arrival of the vagal neural crest cells, enteric neurons do not develop in the hindgut of chick embryos (Meijers *et al.,* 1989). Thus the contribution of the sacral neural crest to the formation of the enteric nervous system is controversial, and it appears that either the sacral neural crest cells do not give rise to enteric neurons or that they differentiate into enteric neurons only when vagal crest cells are present (see Allan and Newgreen, 1980; Gershon *et al.,* 1992).

While they are migrating, the precursor cells of the enteric nervous system do not express a neuronal or glial cell phenotype. In rodents, the expression of neurofilaments (Baetge *et al.*, 1990; Newgreen and Hartley, 1995), glial fibrillary acidic protein (GFAP, Rothman and Gershon, 1986), esterases (Webster, 1973), catecholaminergic markers (Cochard *et al.*, 1978; Teitelman *et al.*, 1978, 1981; Baetge and Gershon, 1989; Baetge *et al.*, 1990), and a variety of neurotransmitters (Rothman and Gershon, 1982; Rothman *et al.,* 1984; Branchek and Gershon, 1989) does not commence in a gut region until several hours to many days after the region has been first colonized. A major difficulty in determining in detail how the gastrointestinal tract is colonized by precursors of the enteric nervous system has been the deficiency of good markers of migratory-stage neural crest cells that recognize all enteric neuron precursors. In birds, the colonization of the gut by migrating neural crest cells has been studied using chick–quail chimeras (LeDouarin and Teillet, 1973) and the equivalent monoclonal antibodies, HNK-1 and NC-1 (Tucker *et al.*, 1986; Pomeranz and Gershon, 1990; Epstein *et al.,* 1991, Newgreen *et al.*, 1996). However, HNK-1 appears to recognize only a small subpopulation of migrating crest cells in the developing intestine of rats and has not been used successfully in mice. The colonization of the mouse gut by enteric neuron precursors has been studied using *DβH-nlacZ* mice (Kapur *et al.*, 1992) and by the expression of *c-ret* (Pachnis *et al.*, 1993). The *DβH-nlacZ* transgene is expressed by a subpopulation of enteric neurons (Kapur *et al.*, 1991). Using wholemount preparations of E9.5–E13 mouse gut, Kapur *et al.* (1992) demonstrated that there is a unidirectional, rostrocaudal wave of expression of the transgene during development, and this was inferred to represent the colonisation of the gut by vagal-level enteric neuron precursors. However, since DBH is only expressed by a subpopulation of enteric neurons, it is possible that the sacral-level enteric neuron precursors do not express this gene (Kapur *et al.*, 1992). The expression of c-*ret* also shows a rostrocaudal progression during mouse embryonic development (Pachnis *et al.*, 1993). c-*ret* encodes the receptor tyrosine kinase, Ret, which is a component of the receptor complex for glial-derived neurotrophic factor (GDNF; Jing *et al.*, 1996; Treanor *et al.*, 1996), and it is critical for the development of the enteric nervous system (Schuchardt *et al.*, 1994, Moore *et al.*, 1996, Pichel *et al.*, 1996, Sánchez *et al.,* 1996; see also Gershon, 1997, for review). Within the developing enteric nervous system, Ret is expressed by cells with neuronal and nonneuronal potential (Lo and Anderson, 1995), consistent with it being a neural stem cell marker, but it is unknown whether c-*ret* is expressed by all enteric neuron precursors. Since the expression of c-*ret* by cells in the embryonic mouse gut was only monitored in sections by Pachnis *et al.* (1993), expression of c-*ret* by a small number of cells in the caudal hindgut during early development could have been overlooked. p75 is the low-affinity nerve growth factor receptor, and like Ret, has been reported to be expressed by migratory-stage enteric neuron precursors (Lo and Anderson, 1995). However, the spatiotemporal pattern of expression of p75 has not been examined in the gastrointestinal tract of any species.

An ideal molecule to follow the migration and colonization of the gastrointestinal tract by enteric neuron precursors would be one that is expressed by migrating enteric neuron precursors and continues to be expressed by differentiated enteric neurons, so that it could be determined whether it is expressed by all, or a subpopulation, of enteric neurons. Transcription factors are often expressed before neuronal precursors begin to differentiate and can therefore be useful for monitoring the development of particular parts of the nervous system. For example, the transcription factor, Islet-1, is expressed by migrating, undifferentiated cholinergic amacrine cells in the retina; the development of the orderly spacing of cholinergic amacrine cells seen in mature animals has recently been examined by using an antibody to Islet-1 to map the behaviour of immature cholinergic amacrine cells (Galli-Resta *et al.*, 1997). The homeodomain transcription factors, Phox2a and Phox2b, are coexpressed in many parts of the peripheral autonomic nervous system, including the enteric nervous system, and in discrete parts of the central nervous system (Tiveron *et al.*, 1996; Morin *et al.*, 1997; Pattyn *et al.*, 1997). However, the two genes show some differences in onset and duration of expression, and while both *Phox2a* and *Phox2b* are expressed by developing enteric neurons, only *Phox2b* is expressed by migrating enteric neuron precursors (Pattyn *et al.*, 1997).

The aims of the current study were (i) to determine whether all, or only a subpopulation, of differentiated

TABLE 1
Primary Antisera Used

Antiserum	Concentration	Source of reference
Calretin (goat)	1:50	SWANT, Belinzona, Switzerland
Glial fibrillary acidic protein (GFAP) (rabbit)	1:1000	Dako, Botany, NSW, Australia; Code 20334
HNK-1 (mouse)	1:10	Hybridoma maintained at the Murdoch Institute, Royal Children's Hospital, Parkville, VIC, Australia
Neuron-specific enolase (NSE) (rabbit)	1:1000	Dako
Nitric oxide synthase (NOS) (sheep)	1:1000	Kind gift of Drs. P. Emson and I. Charles
p75 (rabbit)	1:250	Promega Corporation, Annandale, NSW, Australia
Phox2b (rabbit)	1:700 (or 1:7000 or 1:14,000 for tyramine signal amplification)	Pattyn *et al.*, 1997
Ret (rabbit)	1:50	Immuno-biological laboratories Co. Ltd., Tokyo, Japan

enteric neurons show immunoreactivity to Phox2b protein; (ii) to establish a detailed spatiotemporal timetable of the appearance of Phox2b-, Ret-, and p75-labeled cells within the gut of embryonic mice; and (iii) to examine the location of enteric neuron precursors by (a) explanting spatiotemporally defined regions of embryonic mouse intestine to the kidney subcapsular space of adult host mice for 2–3 weeks, and (b) growing spatiotemporally defined regions of embryonic mouse intestine in organ culture for 10 days. The explants grown under the kidney capsule and in organ culture were then processed, using immunohistochemical or histological techniques, to deduce if enteric neuron precursors were present at the time of explantation.

MATERIALS AND METHODS

Adult, neonatal, and embryonic BALB/c mice from an inbred colony and embryonic Sprague–Dawley rats were used. Embryos were obtained from timed, pregnant mice or rats; the date at which a copulatory plug was observed was designated E0, and the date of birth, P0. Embryonic mice were then staged precisely using the staging system of Theiler (1989; denoted by T) and rats with the staging system of Christie (1964; denoted by C). Segments of the gastrointestinal tract were fixed overnight in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, at 4°C. After washing, whole-mount preparations or frozen sections were processed for single or double-label fluorescence immunohistochemistry as described previously (Newgreen and Hartley, 1995; Young *et al.*, 1996). In some preparations, the primary antiserum was revealed using avidin–biotin–horseradish peroxidase and diaminobenzidine. The preparations were then embedded in resin and sectioned at 1 μm, and the sections were stained with a mixture of toluidine blue and methylene blue. The primary and secondary antisera used are shown in Tables 1 and 2. To perform double-label immunohistochemistry with two primary antisera raised in the same species [rabbit anti-Phox2b plus rabbit anti-neuron-specific enolase (NSE) or rabbit anti-Phox2b plus rabbit anti-glial fibrillary acidic protein (GFAP)], the tyramine signal amplification kit (NEN Life Science Products, Boston, MA) was used. Whole-mount preparations of fixed gut were preincubated in 0.5% blocking powder (from the kit) in 0.1 M Tris–HCl containing 0.15 M NaCl for 30 min at room temperature and then incubated in 1:7000 or 1:14,000 anti-Phox2b for 48 h. The tissue was then washed in 0.1 M Tris–HCl containing 0.15 M NaCl plus 0.05% Triton X-100 (TNT) and incubated in

TABLE 2
Secondary Antisera Used

Species in which primary antisera were raised	Seconary antisera	Concentration	Source
Rabbit	Donkey anti-rabbit Cy3	1:1000	Jackson Immunoresearch Inc., West Grove, Pa
Rabbit plus sheep (or goat)	Biotinylated donkey anti-rabbit followed	1:100	Jackson Immunoresearch Inc.
	by streptavidin–Texas red and donkey	1:100	Amersham
	anti-sheep FITC	1:50	Jackson Immunoresearch
Rabbit plus mouse	Biotinylated horse antimouse followed	1:200	Vector
	by streptavidin–Texas red and donkey	1:100	Amersham
	anti-rabbit FITC	1:25	Amersham

biotinylated donkey anti-rabbit (1:100; Jackson Immuno-research Inc., West Grove, PA) for 2 h. After washing in TNT, the tissue was incubated in streptavidin–HRP (from kit; 1:100 in phosphate-buffered saline, PBS) for 30 min in the dark, washed in TNT, incubated in biotinyl tyramine (1:100 in amplification diluent, both from kit), washed in TNT, and then incubated in streptavidin–Texas red (1:50, Amersham, Melbourne, VIC, Australia) for 1 h. After washing in PBS, the tissue was incubated in the second primary antiserum (rabbit anti-NSE or rabbit anti-GFAP, see Table 1) for 48 h and then in donkey anti-rabbit FITC (1:25; Amersham) for 90 min. Control pieces of tissue that had been incubated in anti-Phox2b (1:7000 or 1:14,000) for 48 h and then in donkey anti-rabbit FITC showed no staining. Control experiments in which whole-mount preparations of adult and embryonic myenteric plexus were exposed to secondary antisera only revealed no staining.

Explants to the kidney subcapsular space of mice. Pregnant mice with embryos at E9.5–E14 gestational age were killed by cervical dislocation, and the embryos were removed, placed in sterile tissue culture medium, and precisely staged (see above). Intestinal segments 0.3–3 mm long from carefully defined regions of the gastrointestinal tract were explanted to the subcapsular space of the kidney of adult host mice and allowed to grow for 2–3 weeks as described previously (Young *et al.,* 1996). The host animals were then killed and the explants removed. Some explants were fixed overnight in 4% formaldehyde in 0.1 M phosphate buffer, and then frozen sections were cut on a cryostat and processed for Phox2b, neuron-specific enolase (NSE), or nitric oxide synthase (NOS) immunohistochemistry. Other explants were fixed in 2.5% formaldehyde plus 3.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 1 h prior to dehydration through alcohol and embedding in glycol methacrylate. Sections, 4 μm thick, were cut and stained with Lee's methylene blue–basic fuchsin (Bennett *et al.,* 1976).

Organ culture. All of the organ culture experiments were performed using two regions of the gastrointestinal tract of E10.5–late E11.5 (T17–T19+) embryos: (a) midgut, which was defined as the region of gut rostral to the caecal swelling; and (b) hindgut, which included the entire gut caudal to the cecal swelling. The gut segments were 0.3–0.7 mm long and were dissected out in Dulbecco's modified eagle medium (DME). The ends of each segment were attached to the two sides of a V-shape notch which had been cut into small squares (about 3 mm^2) of black Millipore paper (type HA; Millipore, North Ryde, NSW, Australia). The filter paper had been sterilized using ethanol. The pieces of filter paper, with the gut segments suspended catenary-fashion from the sides of the V-shaped notch, were then laid across the top of Terasaki wells (60-well plate; Disposable Products, Technology Park, SA, Australia), containing 14 μl of culture medium. The culture medium consisted of DME containing 20 mM glutamine, 6 mg/ml penicillin, 10 mg/ml streptomycin, and 10% heat-inactivated fetal calf serum. The cultures were maintained in a 5% CO_2 environment at 37°C and cultured for 10 days before being fixed in 4% formaldehyde and processed as whole-mounts for immunohistochemistry as described above.

Microscopy. Preparations were viewed on a Zeiss Axioskop or Axioplan microscope, and video images were recorded using an ImagePoint cooled CCD camera (Photometrics Ltd., Tucson, AZ) and V for Windows imaging software (Digital Optics Ltd., Auckland, New Zealand). Each image was processed using a sharpen filter and contrast adjustment. Plates were made using Corel PhotoPaint and Corel Draw (Corel Corp., Dublin, Ireland). Some preparations were scanned on a Bio-Rad MRC 1024 confocal laser scanning microscope installed on an Axioplan fluorescence microscope with a krypton/argon laser. The images were processed using Confocal Assistant and Corel PhotoPaint and Corel Draw software.

RESULTS

Identification of Phox2b-Stained Cells in the Gastrointestinal Tract of the Late Embryonic, Neonatal, and Adult Mice

In the small and large intestine of adult mice, strong Phox2b immunoreactivity was shown by the nuclei of cells within the myenteric (Fig. 1A) and submucosal plexuses (Fig. 1B). Stained nuclei were found only within the ganglia and along the internodal strands. The interstitial cells of Cajal, which form a plexus between the myenteric ganglia (Torihashi *et al.,* 1995), and muscle cells were not stained. Many of the Phox2b-immunoreactive nuclei were round in shape with prominent, unstained nucleoli; other stained nuclei were oval in shape, without prominent nucleoli and were less strongly stained than the round nuclei (Fig. 1B). In preparations that had been processed for double-label immunohistochemistry using antisera to NSE and Phox2b, almost all of the NSE-stained cells showed Phox2b immunoreactivity (Figs. 1C and 1C′); of 1000 NSE-positive cells from two preparations of myenteric plexus from the large intestine, 999 were Phox2b positive. Some of the Phox2b-stained cells did not show immunoreactivity to NSE. These cells appeared to have only a small amount of cytoplasm and hence may have been glial cells (Figs. 1C and 1C′). All of the NOS- (Fig. 1D) and calretinin-immunoreactive neurons, which together constitute over 70% of all myenteric neurons in the mouse small intestine (Sang and Young, 1996), also showed Phox2b staining. In preparations that had been double-stained to reveal GFAP and Phox2b immunoreactivities, some, but not all, of the GFAP-stained cells showed Phox2b immunostaining (Figs. 1E and 1E′). The intensity of the Phox2b immunostaining exhibited by the GFAP-positive cells was usually lower than that of neuronal nuclei. Whole-mount preparations of the external muscle from the esophagus (Fig. 1F) and intestine from late embryonic (E16, T24) and neonatal mice were also processed to reveal Phox2b immunoreactivity, and the pattern of staining observed was identical to that seen in the intestine of adult mice.

Identification of Ret- and p75-Stained Cells in the Gastrointestinal Tract of Adult Mice

In the enteric nervous system of adult mice, no specific Ret immunostaining was observed. A reticular pattern of p75 immunostaining was observed, but it was not possible, even using a confocal microscope, to determine if the stained structures were the cell membranes of nerve cell bodies or of neuronal and glial cell processes that were passing around the nerve cell bodies (Fig. 1G). We were therefore unable to determine if individual enteric neurons were p75 immunoreactive.

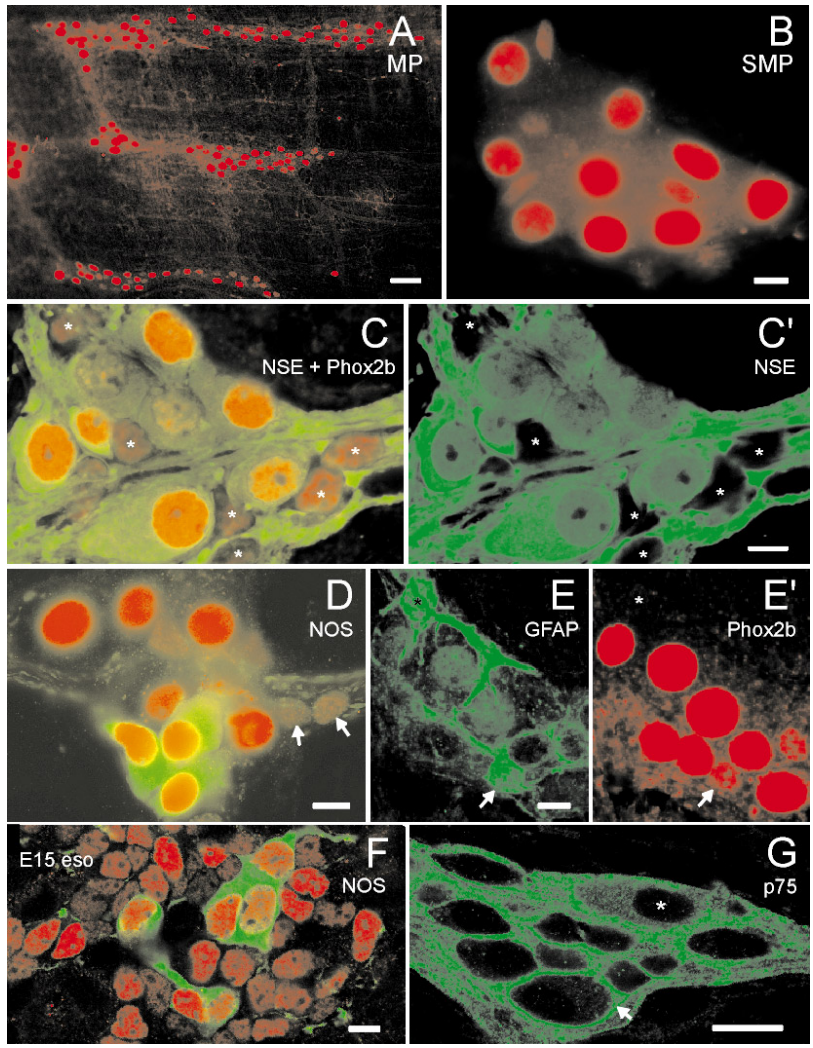

FIG. 1. Low-power micrograph of a whole-mount preparation of longitudinal muscle/myenteric plexus (*MP*) from the small intestine of an adult mouse showing the pattern of Phox2b immunoreactivity. Stained nuclei are present only in the ganglia and internodal strands. Scale bar, 50 μm. (B) Whole-mount preparation of a submucosal ganglion (*SMP*) from the small intestine of an adult mouse showing Phox2b-stained nuclei. Many of the strongly stained nuclei are round with prominent nucleoli. There are also oval nuclei present that are less intensely stained; these may be the nuclei of glial cells. Scale bar, 10 μm. (C and C′) Confocal microscope images (single optical sections) of a whole-mount preparation of the myenteric plexus of the large intestine of an adult mouse following processing for Phox2b-specific (red) and neuron-specific enolase immunoreactivity (green). C is an overlay of the NSE and Phox2b immunostaining, and C′ shows the pattern of NSE staining only. All of the NSE-immunostained cells have Phox2b-stained nuclei, but some Phox2b-stained cells (*asterisks*) do not show NSE immunostaining; most of the Phox2b-stained cells that are NSE-negative (*asterisks*) have very little cytoplasm and are likely to be glial cells. Scale bar, 10 μm. (D) Whole-mount preparation of the myenteric plexus of the small intestine of an adult mouse following processing for Phox2b (red) and nitric oxide synthase immunoreactivity (*NOS*, green). All of the NOS-immunostained cells show Phox2b staining, but some Phox2b-stained cells do not show NOS immunostaining. Some of the Phox2b-stained cells that are NOS-negative (*arrows*) have very little cytoplasm and are located along the internodal strands. Scale bar, 10 μm. (E and E′) Confocal microscope images (projections of 15 optical sections, 0.5 μm *z* steps) of a whole-mount preparation of myenteric plexus from the small intestine of an adult mouse showing GFAP (E) and Phox2b (E′) immunostaining. There are two GFAP-immunostained cells in the field of view, one of which shows Phox2b immunostaining (*arrow*), and the other (*asterisk*) is Phox2b-negative. Scale bar, 10 μm. (F) Confocal microscope image (single optical section) of a whole-mount preparation of esophagus from an E15 mouse showing Phox2b (red) and NOS (green) immunoreactivities. All of the NOS cells have Phox2b-stained nuclei, but NOS cells comprise only a small proportion of all of the Phox2b-positive cells. Scale bar, 10 μm. (G) Confocal micrograph of a myenteric ganglion from an adult mouse showing the reticular pattern of p75 immunoreactivity. There are rims of staining outlining the cells (*arrow*). There is also weak cytoplasmic staining in one of the neurons (*asterisk*). Scale bar, 20 μm.

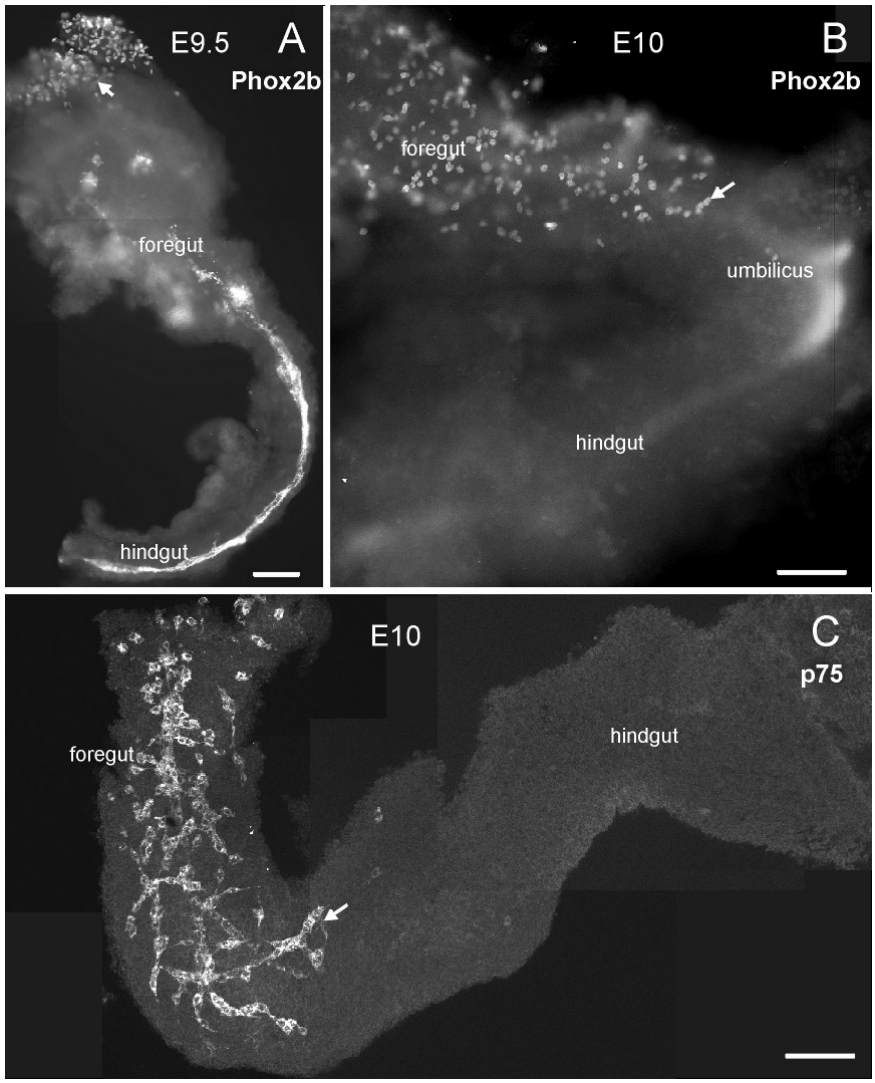

FIG. 2. Whole-mount preparations of gastrointestinal tract from E9.5 and E10 mice showing the pattern of Phox2b or p75 immunoreactivity. (A) Mid- and hindgut plus tissue attached to the foregut from an E9.5 (T15, 27 somite) mouse. Phox2b-stained cells (*arrow*) are present adjacent to the foregut. Scale bar, 100 μm. The line of fluorescence down the middle of the preparation is nonspecific staining in the gut lumen. (B) At E10 (T16), Phox2b-stained cells extend throughout the foregut. The most caudal Phox2b-stained cell (*arrow*) is rostral to the umbilicus. There are no stained cells present in the hindgut. Scale bar, 100 μm. (C) At E10 (T16), p75-stained cells also extend throughout the foregut, but there are no stained cells present in the hindgut. The most caudal p75-stained cell (*arrow*) is in the midgut. Scale bar, 100 μm.

Spatiotemporal Pattern of Appearance of Phox2b-Immunoreactive Cells in the Early Developing Gastrointestinal Tract

In E9.5 mice [Thieler stage 15 (T15), 25–27 somites] Phox2b-stained cells were present in the mesenchyme adjacent to the foregut (Fig. 2A). By E10 (T16), Phox2b-stained cells were present in the mesenchyme of the foregut and rostral midgut and extended to within 100–200 μm of the umbilicus (Fig. 2B), but stained cells were not observed within the mesenchyme of the hindgut. At E11 (T18), the most caudal Phox2b-stained cells were at the level of the umbilicus (Fig. 9A). By E11.5 (T19), the most caudal stained cells were at the level of, and sometimes just caudal to, the caecal swelling. By E12 (T20) the cecum had been colonized and stained cells were also present in the rostral one-third of the hindgut (Fig. 3A). By E13 (T21) the wavefront of stained cells was in the caudal half of the hindgut. By E14 (T22) mice Phox2b-stained cells were present along the entire gut. In E9.5–E13 (T15–21) mice, we did not observe any Phox2b-stained cells within the mesenchyme of the hindgut prior to (i.e., caudal to) the wave of labeled cells spreading rostrocaudally from the foregut. However, in

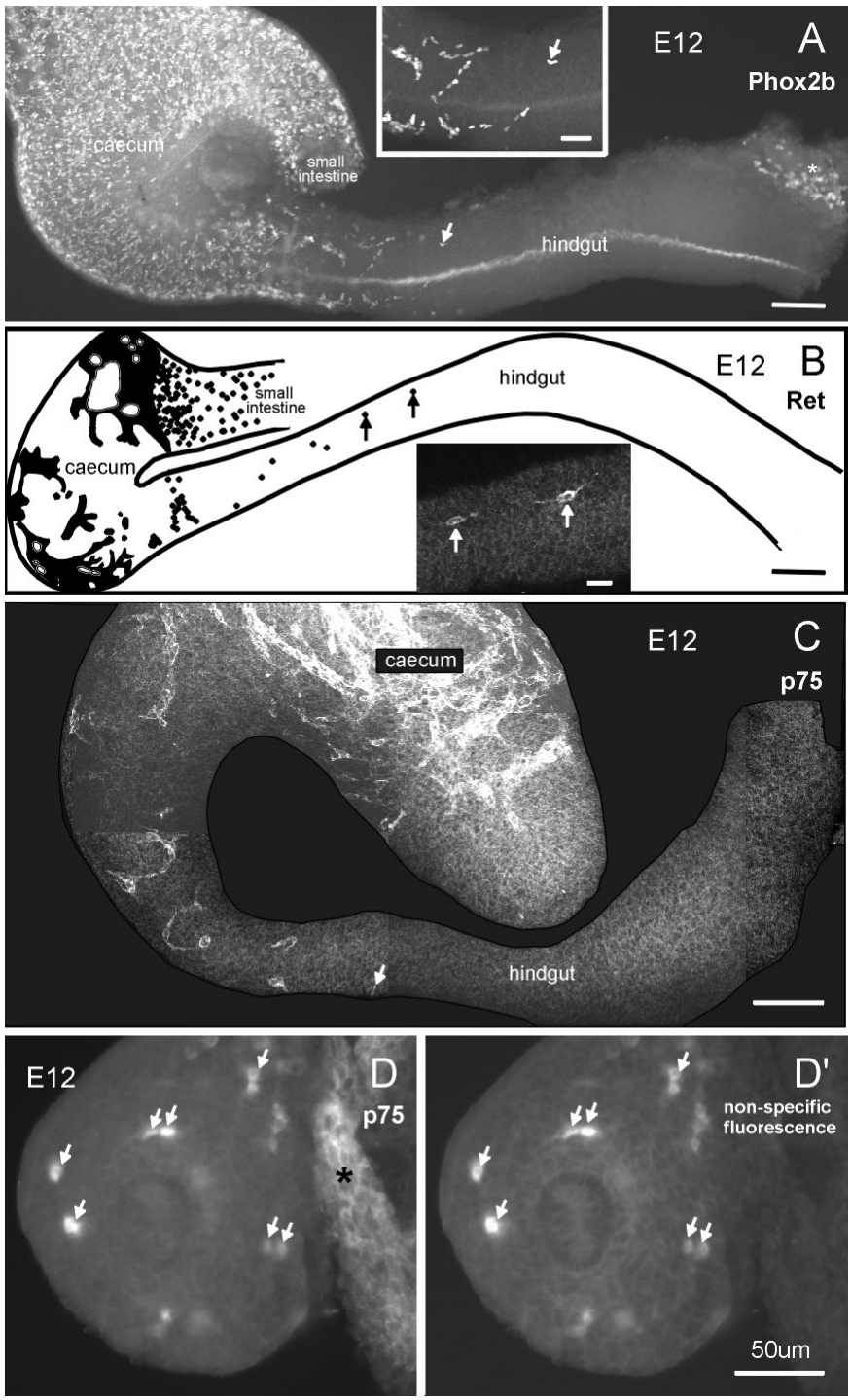

FIG. 3. (A–C) Whole-mount preparations of cecum and hindgut from E12 (T20) mice stained for Phox2b (A), Ret (B), and p75 (C). (A) Phox2b cells are present in the caecum, and extend into the hindgut. The most caudal Phox2b-stained cell within the gut mesenchyme (*arrow*) is in the rostral half of the hindgut. A group of stained cells (*asterisk*) is present external to the mesenchyme of the caudal hindgut. (Inset) Higher magnification of the most caudal Phox2b-stained cells. Note that Phox2b-stained cells near the wavefront are often aligned in rows that spiral, or run longitudinally, along the gut. Scale bars, 100 and 50 μm (inset). (B) Tracing of the most caudal part of the hindgut of an E12 (T20) mouse that had been processed for Ret immunohistochemistry. The Ret immunostaining was too weak to be illustrated in a low-power fluorescence micrograph, and thus this drawing was obtained by photographing the entire hindgut using a $\times$40 objective, making a montage, and then plotting the location of the Ret-positive cells on an overlay. The locations of Ret-positive cells are indicated

some preparations from E10–12 (T16–20) mice, groups of Phox2b-stained cells were present adjacent to, but clearly outside of, the mesenchyme of the caudal hindgut (Fig. 3A). These groups of cells were probably the primordia of the pelvic plexus. Within the mesenchyme of the gut wall, some fluorescent cells were present. However, these cells were identified as nucleated embryonic blood cells because they showed nonspecific fluorescence (the fluorescence was visible through the FITC, Texas red, and UV filters, while the fluorescence of the Phox2b-stained cells was only visible through a single filter).

Spatiotemporal Pattern of Appearance of Ret- and p75-Immunoreactive Cells in the Early Developing Gastrointestinal Tract

Since at least some enteric neuron precursors express c-*ret* (Pachnis *et al.*, 1993) and p75 (Lo and Anderson, 1995; Lo *et al.*, 1997), the spatiotemporal appearance of the Phox2b-positive cells was compared to that of Ret- and p75-immunoreactive cells. At E10 (T16), E10.5 (T17), and E11 (T18), the location of p75-positive cells was very similar to that of Phox2b-positive cells in that p75-stained cells were present in the gut rostral to the umbilicus, but not in the hindgut (Figs. 2C and 9B); no immunoreactivity to Ret could be detected in the gut at these stages. At E12 (T20), Ret- and p75-immunostained cells were present throughout the foregut and cecum; the most caudal Ret- and p75-positive cells were in the rostral half of the hindgut (Figs. 3B and 3C), which was very similar to the location of the most caudal Phox2b-stained cells at this stage (Fig. 3A). No Ret- or p75-positive cells were observed within the mesenchyme of the caudal end of the hindgut at E12, although groups of p75-stained cells were observed adjacent to, and clearly outside of, the caudal hindgut in some specimens (Figs. 3D and 3D′). At E13 (T21), Ret- and p75-stained cells extended down to the caudal third of the hindgut, and at E14 (T22), Ret- and p75-stained cells were present throughout the entire gastrointestinal tract. Thus, Ret- and p75-positive cells were found at the same rostrocaudal locations as the Phox 2b-positive cells.

Arrangement of Cells at or near the Wavefront

The arrangement of Phox2b-, Ret-, and p75-stained cells at the wavefront was similar. In E10 (T16) preparations, Phox2b- and p75-stained cells appeared randomly and densely distributed with no obvious differences between the density and arrangement of cells at the "front" and those behind (i.e., rostral to) them (Figs. 2B and 2C); this gave a sharp cutoff of the labeling. In E10.5 (T17) and older embryos, the density of stained cells at the wavefront was always considerably lower than that 500 μm or more rostral, giving a graded decline of density of labeled cells. At these stages, many of the most caudal Phox2b-, Ret-, and p75-stained cells were arranged in chains that ran longitudinally down the gut, or that spiralled around the gut [Figs. 3A (inset), 4A, and 4B). The Phox2b-positive nuclei forming these chains were separated by less than 10 μm from each other (Fig. 4A). Ret and p75 immunostaining revealed that the processes of neighboring cells forming the chains were in contact with each other (Fig. 4B). Rostral to the wavefront, Phox2b-, Ret-, and p75-positive cells all appeared to be evenly distributed (Figs. 4C–4E), and there appeared to be less Ret-stained cells than Phox2b- or p75-stained cells.

Formation of Enteric Ganglia and the Location of Enteric Neuron Precursors within the Mesenchyme of the Gut

The first indication of the formation of groups of cells that form ganglia was observed in the small intestine of E13 (T21) mice, where groups of Phox2b-labeled cells and areas lacking such cells could be identified (Figs. 4F and 4G). Subsequently, the proportion of Phox2b-stained cells that were present in groups increased, and by birth the vast majority of stained cells were present in groups aligned circumferentially around the gut that were similar in shape and arrangement to myenteric ganglia present in adult mice.

In sections through the embryonic gut, Phox2b- and p75-stained cells formed a layer, one to two cells thick in the outer mesenchyme, with usually only a single layer of unstained cells present external to the stained cells (Figs. 4H, 4I, and 5A). Since the longitudinal muscle layer does not form until E18 in mice (Torihashi *et al.*, 1997), the cells external to the Phox2b-stained cells are likely to be serosal cells.

Presence of Neurons in Explants of Embryonic Mouse Intestine Grafted to the Kidney Subcapsular Space and in Explants of E11 Mid- and Hindgut Grown in Organ Culture

Segments of embryonic intestine explanted to the kidney subcapsular space grew and differentiated (Fig. 6)

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with black dots or black regions. The most caudal Ret-stained cells (*arrows*) are in the rostral half of the hindgut. (Inset) Micrograph of the two most caudal Ret-stained cells indicated in the drawing. Scale bars, 100 μm and 20 μm (inset). (C) p75-stained cells are present in the cecum, and the most caudal stained cell (*arrow*) is in the rostral hindgut. There are no stained cells in the caudal hindgut. Scale bar, 100 μm. (D and D′) Frozen section through the hindgut of an E12 mouse after processing for p75 immunohistochemistry using a secondary antibody conjugated to Cy-3 and viewed through a rhodamine/Texas red/Cy-3 filter (D) and a FITC filter (D′). Cells that are p75-positive are present outside of the mesenchyme of the hindgut (*asterisk* in D), but the only fluorescent cells within the mesenchyme of the hindgut are blood cells (*arrows*) that can be seen through both filters. Scale bar, 50 μm.

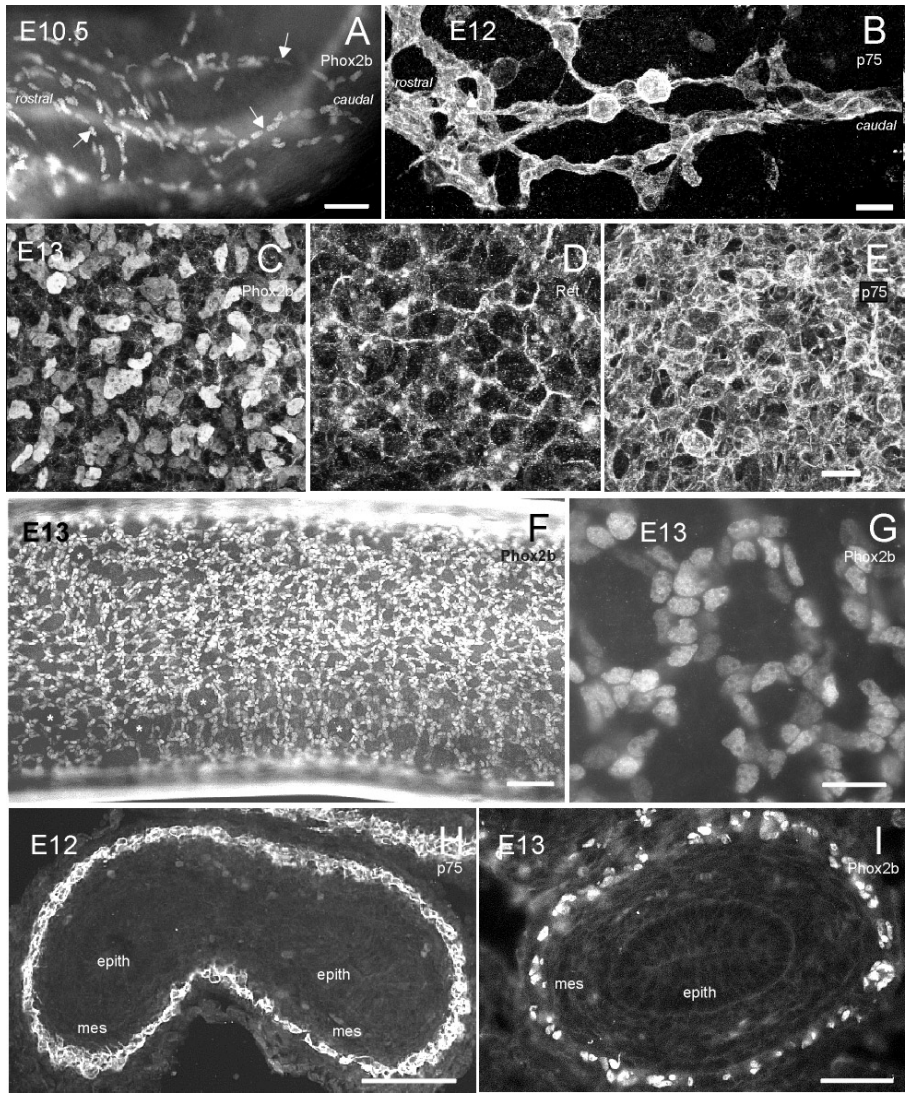

FIG. 4. Phox2b-stained cells close to the wavefront (which is close to the umbilicus) in a whole-mount preparation of E10.5 (T17) mouse gut. The Phox 2b-stained cells often occur in chains (*arrows*) that run longitudinally down, or spiral around, the gut. Scale bar, 50 μm. (B) Chain of p75-stained cells near the wavefront in the rostral hindgut of an E12 (T20) mouse. Scale bar, 20 μm. (C–E) Fluorescence micrographs of whole-mount preparations of gut from E13 (T 21) embryos to show the distribution of Phox2b-, and Ret-, and p75-immunoreactive cells just behind the wavefront, in the rostral hindgut (just caudal to the cecum). At this stage, the most caudal Phox2b-, and Ret-, and p75-stained cells are in the caudal half of the hindgut (see Fig. 8). Scale bar, 20 μm. (F and G) Low-power (F) and high-power (G) micrographs of Phox2b-stained cells in whole-mount preparations of the small intestine from an E13 mouse (T21). Some of the cells are clustered into groups and cell-free areas can be identified (*asterisks*). Scale bars: 100 μm (F) and 20 μm (G). (H) Frozen section of an E12 mouse embryo showing the distribution of p75-stained cells, which are present in the outer mesenchyme (*mes*). *epith*, epithelium lining the lumen. Scale bar, 10 μm. (I) Phox2b-stained cells in a frozen section of an E13 mouse. Stained cells are present in the outer part of the mesenchyme (*mes*). *epith*, epithelium lining the lumen. Scale bar, 50 μm.

and were very similar histologically to tissue from mice at an equivalent developmental age. Explants from spatiotemporally defined regions of intestine from E9.5–E14 mice (Fig. 7) were grown in the subcapsular space for 2–3 weeks. To determine if the explants contained neurons, antisera to NSE (Figs. 6A and 6B), Phox2b (Figs. 6D and 6E), or NOS (Fig. 6C) or conventional histology (Fig. 6F) was used. The regions from where the explants were taken and the presence or absence of neurons in the explants are shown in Fig. 7. Nine of the explants of foregut or midgut from E10–E12 mice, and six of the explants of hindgut from E10–E12 mice were processed

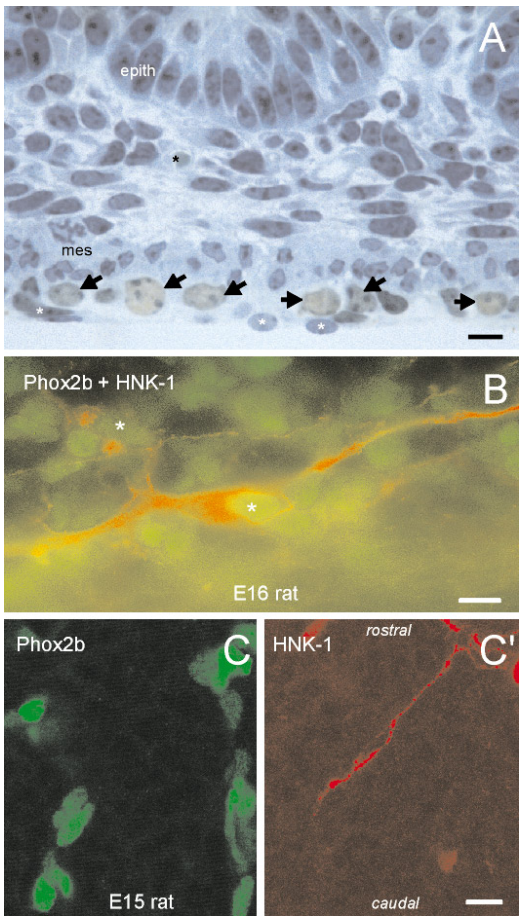

FIG. 5. Transverse, 1-μm section of resin-embedded small intestine from an E15 mouse in which the Phox2b antiserum had been revealed using avidin–biotin–horseradish peroxidase and DAB. The stained cells (*arrows*) are in the outer mesenchyme (*mes*), with only a single layer of cells (*white asterisks*) external to the stained cells. The brown cell (*black asterisk*) in the mucosa is a blood cell that shows endogenous peroxidase activity. *epith*, epithelium. Scale bar, 10 μm. (B, C and C′) Whole-mount preparations of intestine from embryonic rats following processing for Phox2b (green) and HNK-1 (red) immunoreactivities. (B) Hindgut from an E16 rat. Both of the HNK-1-stained cells (*asterisks*) in this field of view show Phox2b staining, but HNK-1-stained cells compose only a small proportion of the Phox2b-stained cells. Scale bar, 10 μm. (C and C′) Hindgut from an E15 rat. Paired confocal micrograph images of the region of hindgut caudal to the cecum where the most caudal HNK-1-positive cell was observed (C′). Phox2b-stained cells were found caudal to this point (C). Scale bar, 10 μm.

for both Phox2b and NSE or NOS and NSE immunohistochemistry. In every case, the results obtained with the different neuronal markers were identical; that is, if neurons could be detected with one marker, then they were also detected with the other and vice versa. The presence of neurons in the explants suggested a single rostrocaudal wave of colonization of the gastrointestinal tract by enteric neuron precursors. As shown in Fig. 8, the locations of enteric neuron precursors at different embryonic stages revealed by Phox2b immunostaining and by explants grafted to the subcapsular space were similar. Both techniques suggested a single rostrocaudal wave of colonization of the gastrointestinal tract by enteric neuron precursors, and neither technique revealed a caudal–rostral wave of colonisation of the gastrointestinal tract.

Segments from two different regions of gut from E10.5–late E11.5 (T17-T19+) mice were grown in organ culture for 10 days. The two regions were (i) midgut, which was defined as the region of gut rostral to the caecal swelling; and (ii) the hindgut, caudal to the cecal swelling (Figs. 9A and 9B). Experiments with *DBH-LacZ* transgenic mice (Kapur *et al.*, 1992) and Phox2b (Fig. 9A) and p75 (Fig. 9B) antisera have shown that enteric neuron precursors derived from the vagal-level neural crest would be present in segments of E11 midgut, but not in the hindgut, and cells derived from the sacral-level neural crest would be present in E11 hindgut (Serbedzija *et al.*, 1991). The catenary organ culture system developed for these experiments preserves the linear tubular gut morphology while allowing for growth and elongation. After 10 days in culture, the explants had grown in diameter and length (Figs. 9C, 9E, 9G, and 9H) and were up to 3 mm long. To determine if the organ-cultured explants contained neurons, antisera to NSE (Figs. 9C, 9D, and 9G) and NOS (Figs. 9E, 9F, and 9H) were used. Most of the explants were processed for double-label immunohistochemistry, but some were processed for NOS alone. The results obtained using the two antisera were identical, that is, if NOS-immunoreactive neurons were present, NSE-immunoreactive neurons were also present and vice versa. The results are shown in Table 3. After 10 days in organ culture, NSE- and NOS-immunoreactive nerve cell bodies and nerve fibers were present in all (n = 46) of the explants of midgut (Figs. 9C–9F; Table 3). In addition, all of the midgut explants had NSE- and NOS-immunoreactive nerve fibers extending from the ends of the explants onto the filter paper (Figs. 9D and 9E). Groups of immunostained neurons, which had migrated out of the explant, were also present on the filter paper (Fig. 9D). None of the 15 hindgut explants from E10.5 (T17) or E11 (T18) mice had any NSE- or NOS-stained cells within the explant or nerve fibers extending from the explant onto the filter paper (Table 3). The gut of E11.5 (T19) and late E11.5 (T19+) mice could be differentiated by changes in the shape of the caecum. Of 23 hindgut explants from E11.5 (T19) mice processed for NOS/NSE double staining or NOS alone, 22 did not possess any stained neurons or exhibit nerve fiber outgrowth (Figs. 9E and 9G). However, in one of the E11.5 hindgut explants, neurons were present along only about 25% of the length of the explant. Of the 10 explants of hindgut from late E11.5 (T19+) mice, 3 also had NSE- and NOS-stained cells within the explants and fibers growing from the explants (Table 3). For comparison, when the same regions of E11.5 mid- or hindgut that were used for the organ culture experiments were grown under the kidney

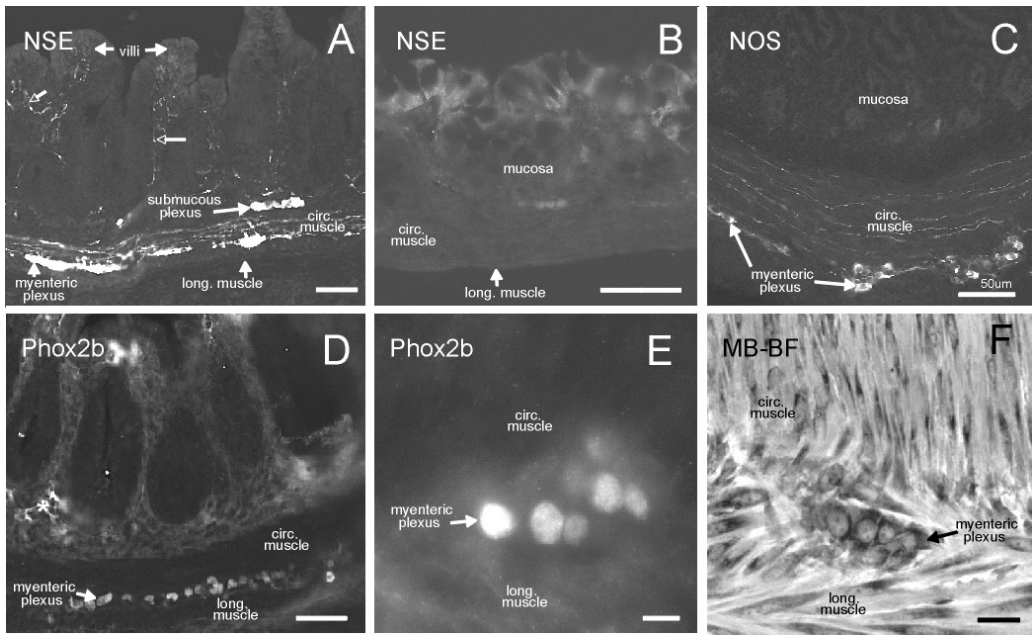

FIG. 6. Explants of embryonic mouse intestine following growth in the renal subcapsular space of host adult mice for 2–3 weeks. The explants illustrated in A, and C–F possessed neurons, whereas the explant illustrated in B did not possess neurons. (A) NSE immunostaining in an explant of E10 mouse foregut after growing in the subcapsular space for 2.5 weeks. NSE-stained cell bodies are present in the myenteric and submucosal plexuses and stained fibers (*open arrows*) are present in the circular muscle layer (*circ. muscle*) and mucosa. Scale bar, 50 μm. (B) NSE-immunostaining in an explant of E10 mouse hindgut after growing in the subcapsular space for two weeks. No NSE-immunoreactive structures are present. Scale bar, 50 μm. (C) NOS immunostaining in an explant of E10 mouse foregut after growing in the subcapsular space for three weeks. NOS-stained cell bodies are present in the myenteric plexus and stained fibers are present in the circular muscle layer (*circ. muscle*), but not the mucosa. Scale bar, 50 μm. (D and E) Low-power (D) and high-power (E) micrographs of an explant of E10 mouse foregut after growing in the renal subcapsular space for 2 weeks. Phox2b-stained cells are present in the myenteric plexus. There is some staining of the lamina propria (*asterisk* in D) which is possibly cross-reactivity with a component of the extracellular matrix. *circ. muscle*, circular muscle layer; *long. muscle*, longitudinal muscle layer. Scale bars, 50 μm (D) and 10 μm (E). (F) Explant of a segment of hindgut taken from just caudal to the caecum of an E13 mouse, cultured in the renal subcapsular space for 2 weeks, and then stained for methylene blue/basic fuchsin (*MB-BF*). Stained neurons are present in the myenteric plexus. Scale bar, 20 μm.

capsule, neurons were present in the explants of midgut (n = 2), but not in the explants of hindgut (n = 4; Fig. 7).

Comparison Between Phox2b- and HNK-1 Immunostaining in the Embryonic Rat Gut

The pattern of Phox2b immunoreactivity observed in the gut of embryonic rats was very similar to that observed in embryonic mice. In preparations of embryonic rat gut that had been stained to reveal both Phox2b and HNK-1 immunolabeling, all of the HNK-1-stained cells possessed Phox2b-positive nuclei, but HNK-1-positive cells comprised only a small proportion of all Phox2b-stained cells (Fig. 5B). In E14.75 (C26) rats, Phox2b-stained cells were present in the rostral but not caudal hindgut. HNK-1-stained cells were also present in the rostral but not caudal hindgut. However, the most caudal Phox2b-stained cells were 600–700 μm in advance of (i.e., caudal to) the most caudal HNK-1-stained cell processes (Figs. 5C and 5C′).

DISCUSSION

This study used a variety of methods to examine the colonization of the mouse gastrointestinal tract by enteric neuron precursors: (i) Antisera to the homeodomain transcription factor, Phox2b, the receptor tyrosine kinase, Ret, and the low-affinity nerve growth factor receptor, p75, were used on whole-mount preparations of embryonic mouse gut to map in detail the spatiotemporal appearance of Phox2b-, Ret-, and p75-positive cells. (ii) Explants from spatiotemporally defined regions of embryonic intestine were grown in the kidney subcapsular space for 2–3 weeks and then examined for the presence of differentiated neurons. (iii) Segments of E10.5–E11 midgut (which previous studies had shown contain vagal-level neural crest cells) and E10.5–E11 hindgut (which previous studies had shown would contain sacral-level, but not vagal-level, neural crest cells) were grown in organ culture for 10 days, before they were examined for the presence of neurons.

All of the methods revealed a progressive, unidirectional,

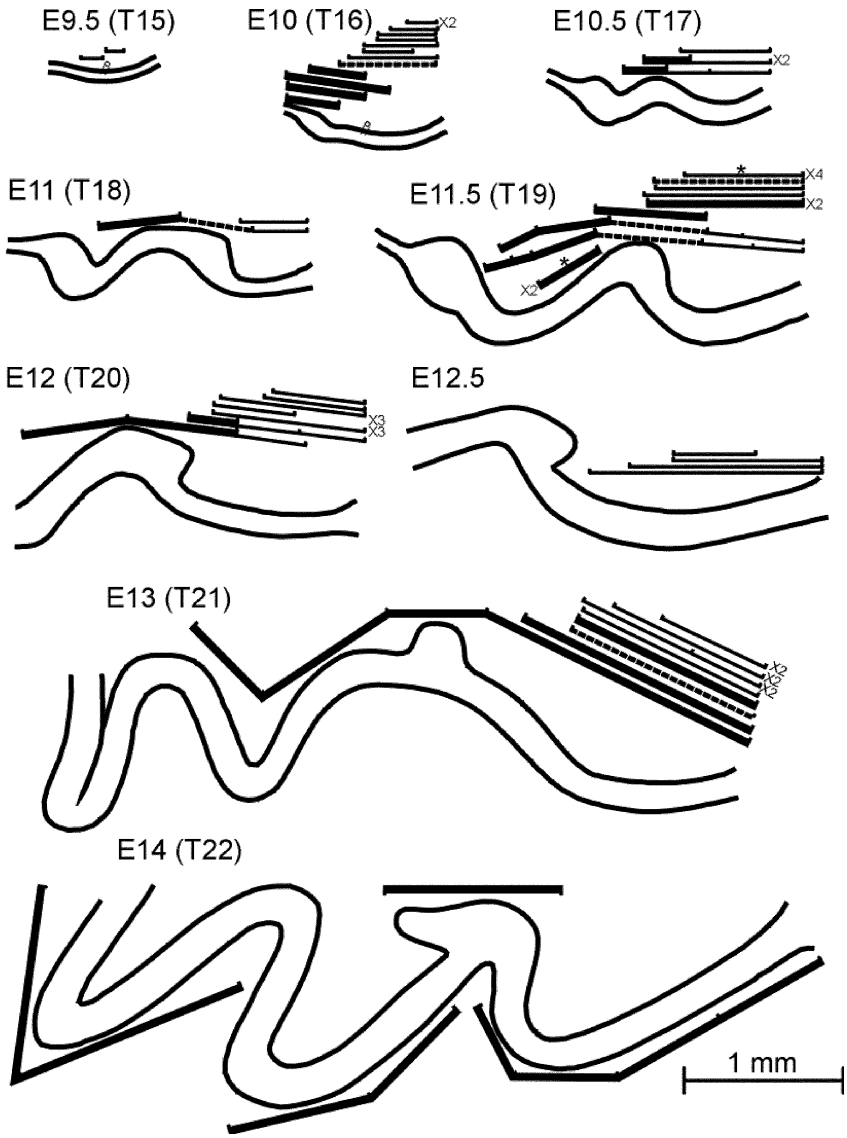

FIG. 7. Locations from where segments of embryonic mouse intestine were taken and then explanted to the kidney subcapsular space. The presence of neurons forming well-developed ganglia in the explants is indicated with the thick black line, and an absence of neurons is indicated with a thin line. Some explants, indicated with a dotted line, possessed only sparse neurons. The E11.5 explants indicated with asterisks correspond to the regions of mid- and hindgut used in the organ culture experiments.

rostrocaudal colonization of the gastrointestinal tract by enteric neuron precursors which was very similar to that described using the transgene, *DβH-nlacZ*, as a marker of vagal-level enteric neuron precursors (Kapur *et al.*, 1992). Phox2b-, Ret-, and p75 staining and the explants grown under the kidney capsule or in organ culture also showed similar locations for the most caudal enteric neuron precursors at each embryonic stage in the mouse. The colonization of the embryonic rat gastrointestinal tract by Phox2b-stained cells also occurred rostrocaudally. Since Phox2b, Ret, and p75 are expressed by migratory-stage or immediate post-migratory-stage enteric neuron precursor cells, the rostrocaudal appearance of Phox2b-, Ret-, and p75-stained cells in the embryonic gut of rodents represents the colonization of the gut by vagal neural crest cells. No Phox2b, Ret, or p75 staining was observed in the hindgut prior to the arrival of vagal neural crest cells. When segments of hindgut were removed prior to the arrival of vagal neural crest cells, but after the reported arrival of sacral neural crest cells (Serbedzija *et al.*, 1991), and grown either in the renal subcapsular space or in organ culture, no enteric neurons were observed in the explants.

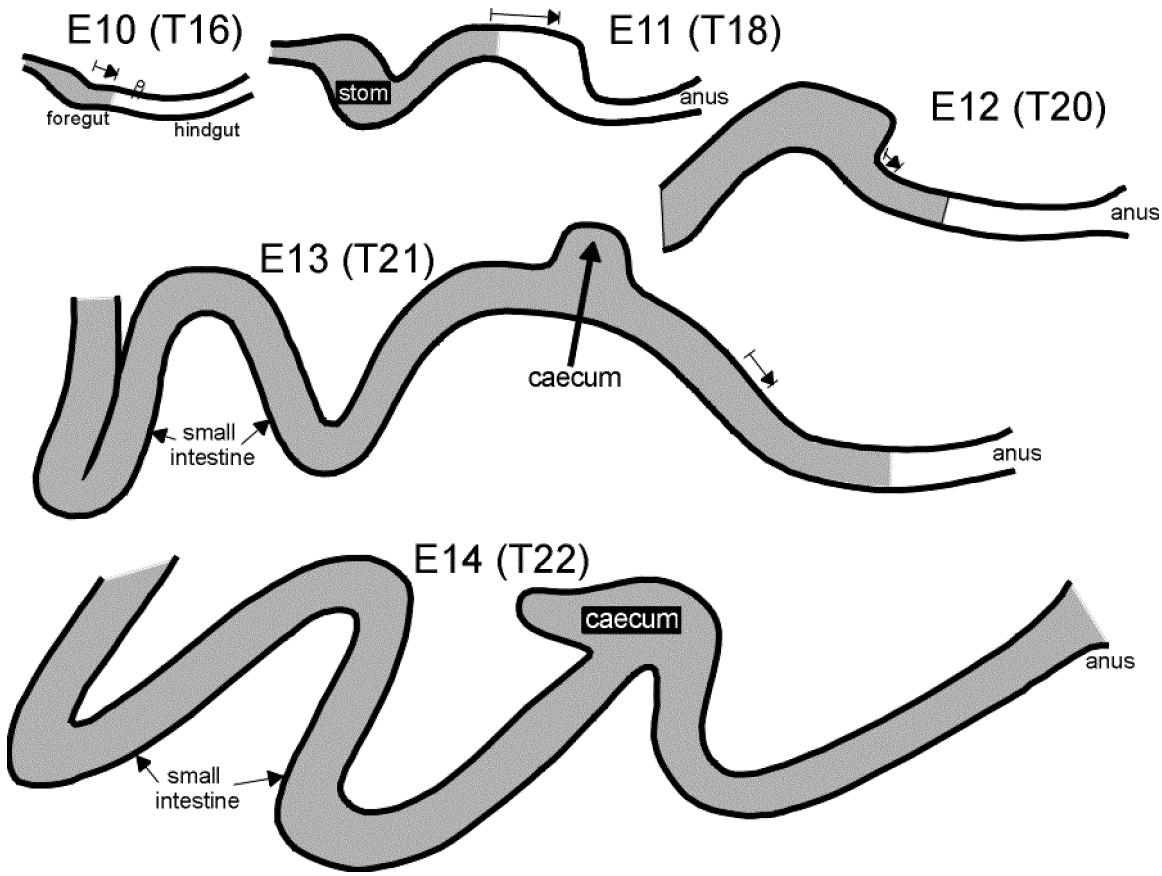

FIG. 8. Summary of location of Phox2b-stained cells (*grey*) and that of enteric neuron precursors deduced from renal subcapsular grafts (*arrows*). The left-hand limits of the arrows indicate regions where enteric neurons were always found in the explants, and the right-hand limits indicate regions where enteric neurons were never found. The wavefront is therefore within the limits of the arrows.

Phox2b as a Marker of Enteric Neurons and Their Precursors

This study has shown that an important feature of the homeodomain transcription factor, Phox2b, is that, unlike other markers of enteric neuron progenitors such as *DβH-nlacZ* in mice (Kapur *et al.*, 1992) and HNK-1 in rats (D. F. Newgreen, personal observations), Phox2b is expressed by essentially all differentiated enteric neurons and also a subpopulation of glial cells. The Phox2b antiserum appears to stain migratory-stage, or immediate post-migratory-stage, enteric neuron precursors, because Phox2b-stained cells were observed adjacent to the foregut in E9.5 mice. Furthermore, the location of neuron precursors in the embryonic mouse gut determined using Phox2b immunostaining on whole-mount preparations of gut was very similar to that observed using antisera to Ret and p75 and to that deduced from the kidney subcapsular graft (see Fig. 8) and organ culture experiments.

Since Phox2b is expressed by all differentiated enteric neurons, it would therefore seem possible that Phox2b is expressed by all enteric neuron precursors. It appeared that there were considerably more Phox2b-labeled cells than Ret-labeled cells in the gut mesenchyme of embryonic mice. Thus, like *DβH-nlacZ* and HNK-1, Ret appears to be a marker of only a subpopulation of neural crest cells and their progenitors in the embryonic gut (Lo and Anderson, 1995). We were unable to determine whether the p75 antiserum stains as many enteric nervous system precursors as the Phox2b antiserum, or if all differentiated enteric neurons express p75.

Elsewhere in the nervous system, Phox2b is also expressed by early neuron precursors (Pattyn *et al.*, 1997). For example, in the rhombencephalon, Phox2b is expressed in the proliferative neuroepithelium (Pattyn *et al.*, 1997). The expression of Phox2b by both differentiated neurons and a subpopulation of glial cells suggests that the onset of Phox2b expression by neural crest cells is prior to the formation of separate enteric neuron and glial cell lineages. Studies using postmigratory neural crest cells isolated from the gut of E14.5 rat embryos have shown that although some neural crest cells at this stage

TABLE 3

Presence of Neurons in Segments of Mid- or Hindgut from Embryonic Mice Grown in Organ Culture for 10 Days

Neuronal marker	Embryonic stage	No. of explants with stained neurons/total No. of explants examined	
		Midgut	Hindgut
NOS	E10.5 (T17)	4/4	0/4
	E11 (T18)	11/11	0/11
	E11.5 (T19)	21/21	1[a]/23
	Late E11.5	10/10	3/10
NSE	E10.5 (T17)	4/4	0/4
	E11 (T18)	11/11	0/11
	E11.5 (T19)	18/18	1[a]/19
	Late E11.5	9/9	3/9

[a] Neurons were present only along 25% of the length of the explant.

are committed to becoming either neurons or glial cells, others are multipotent and have the potential to give rise to both neurons and glial cells (Lo and Anderson, 1995). Thus, for different neural crest cells, the formation of distinct neuron and glial cell lineages appears to occur at different developmental stages.

The spatiotemporal colonization of the rat gut by enteric neuron precursors has been examined previously using an antiserum to the cell surface antigen, HNK-1, which appears to be expressed by enteric neuron precursors, but not glial cell precursors (Newgreen and Hartley, 1995). Using Phox2b immunostaining, we observed rostrocaudal colonization of the embryonic rat gut similar to that reported previously. However, Phox2b appears to be expressed earlier than HNK-1, because the most caudal HNK-1-stained cell was rostral to the most caudal Phox2b-stained cell. Thus, while Phox2b appears to be expressed by migrating, undifferentiated enteric neuron precursors, HNK-1 is a marker of a subpopulation of early differentiating neurons in the rat gut.

Characteristics of Sacral Neural Crest Cells That Migrate into the Hindgut

Studies in mice and avian embryos using a variety of labeling techniques have shown that neural crest cells arising from sacral levels of the neural tube give rise to cells in the hindgut. When the neural axis posterior to somite 28 of quails is grafted into chicken embryos, quail cells are subsequently found within the enteric plexuses of the hindgut of the chimeras (Le Douarin and Teillet, 1973, 1974). Similarly, when a vital dye or a retrovirus is introduced into or near the premigratory sacral-level neural crest cells (Pomeranz *et al.*, 1991; Serbedzija *et al.*, 1991), labeled cells are observed later in the gut mesenchyme and within the gut epithelium. The fate of the sacral-level neural crest cells in the hindgut has never been established. As no enteric neurons develop in the hindgut following removal of the vagal neural crest (Yntema and Hammond, 1954), transection of the midgut prior to the arrival of vagal neural crest cells (Meijers *et al.*, 1989), or removal and culture of the hindgut prior to the arrival of vagal neural crest cells (Allan and Newgreen, 1980; Nishijima *et al.*, 1990; Kapur *et al.*, 1992; Young *et al.*, 1996), it appears either that sacral neural crest cells do not give rise to enteric neurons or that they only differentiate into enteric neurons in the presence of the vagal-level neural crest (see Allan and Newgreen, 1980; Gershon *et al.*, 1992; Kapur, 1993).

Using DiI to label premigratory neural crest cells in embryonic mice, Serbedzija *et al.* (1991) reported that the sacral neural crest cells leave the neural tube between E9 and E9.5 and arrive in the hindgut approximately 12 h later; the fate of the DiI-labeled cells could not be determined. However, in the current study, Phox2b-, Ret-, or p75-positive-cells were not observed in the hindgut until the rostrocaudal wave at E12–E14, which represented the migration of vagal neural crest cells. Double-label studies in mature mice also showed that all neurons, including those in the large intestine, that could be labeled with antisera to neuron-specific markers also showed Phox2b staining; thus, the sacral neural crest cells do not differentiate into Phox2b-negative enteric neurons. There are two possible explanations for the absence of Phox2b-labeled cells in the embryonic hindgut prior to the vagal wave. First, the sacral-level neural crest cells that colonize the gut do not normally become enteric neurons. It is already apparent that not all of the cells that populate the hindgut from the sacral level of the neural axis are destined to become neurons, as following the labeling of premigratory cells with DiI, some of the labeled cells are found in the epithelium lining the gut (Serbedzija *et al.*, 1991). The fate of these cells is unknown. Likewise, it is feasible that the cells in the mesenchyme of the mouse hindgut that arise from the sacral neural crest are not normally enteric neuron precursors. In the present study, only a subpopulation of the enteric glial cells showed Phox2b staining, and it is conceivable that the sacral neural crest cells give rise to the Phox2b-negative enteric glial cells in the hindgut. The second possible explanation for the absence of Phox2b-stained cells in the hindgut prior to the arrival of vagal neural crest cells is that the sacral neural crest cells that colonize the hindgut show a delayed expression of Phox2b. This would seem unlikely because, like the enteric neuron precursors of vagal neural crest origin, the precursors of sympathetic ganglion cells in embryonic mice express Phox2b at E9.5 (Pattyn *et al.*, 1997). Moreover, in the current study, groups of Phox2b- and p75-stained cells were observed in close association with, but clearly outside of, the hindgut of embryonic mice prior to the arrival of vagal enteric neuron progenitors. These groups of Phox2b- and p75-stained cells are probably the primordia of the pelvic plexus, which arises from the sacral neural crest (Yntema and Hammond, 1955). Hence, if the sacral neural crest cells

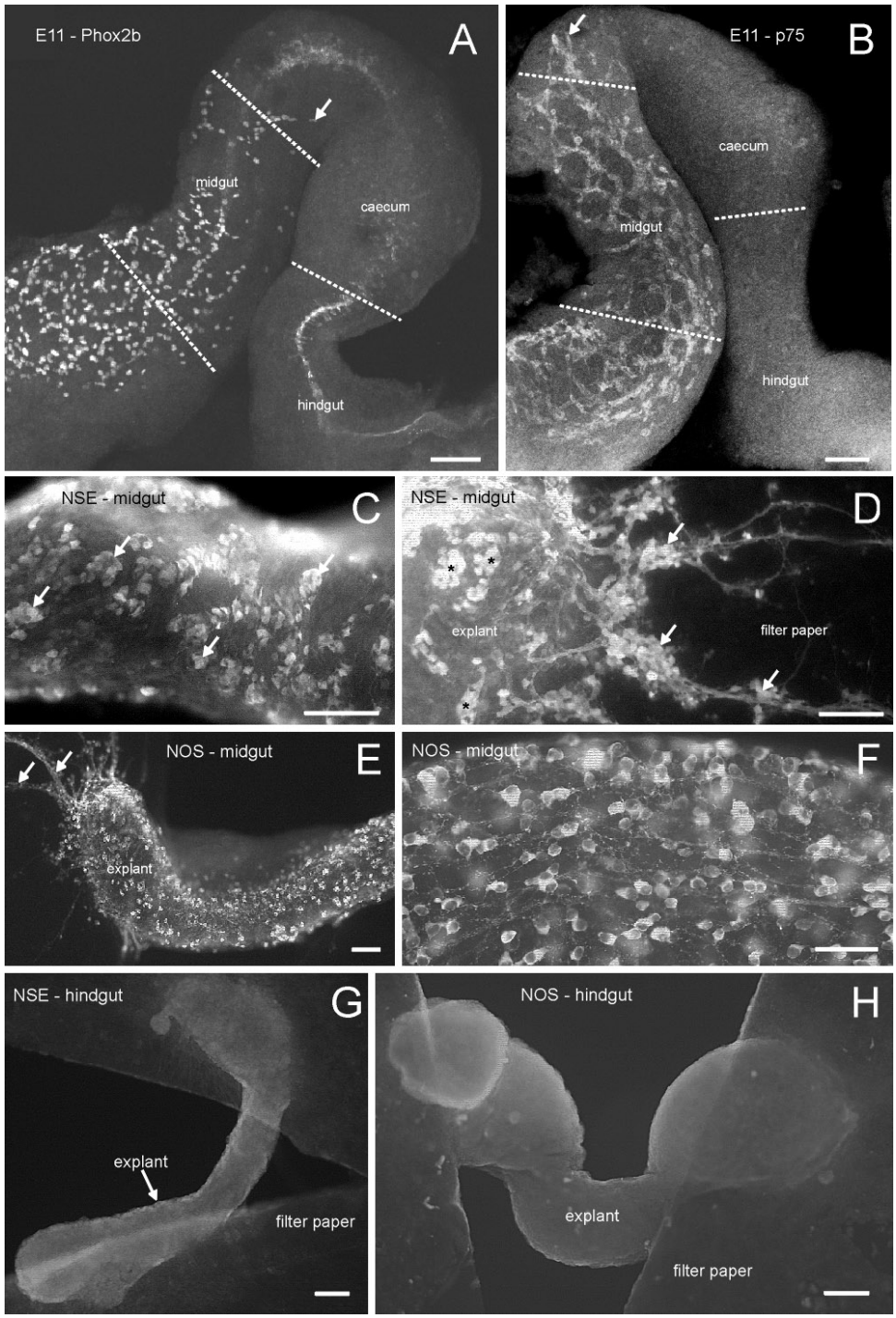

FIG. 9. (A and B) Mid- and hindgut from E11 mice that had been processed for Phox2b (A) or p75 (B) immunohistochemistry. The most caudal stained cells (*arrows*) are located rostral to the cecum. The midgut (between the stippled lines) and hindgut (caudal to the cecum) were the regions that were used for the organ culture experiments. The line of fluorescence down the middle of the preparation stained with the Phox2b antiserum is nonspecific staining of the lumenal contents. Scale bars, 100 μm. (C–H) Explants of midgut (C–F) and hindgut (G and H) from E11.5 mice following growth in organ culture for 10 days and processing for NSE (C, D, and G) or NOS (E, F, and H) immunohistochemistry. (C) Groups of NSE-immunostained nerve cell bodies (*arrows*) within an explant of midgut. Scale bar, 100 μm. (D) The edge of a midgut explant showing NSE-positive neurons within the explant (*asterisks*) and NSE-positive cell bodies (*arrows*) and nerve fibers outside of the explant. Scale bar, 100 μm. (E) Low-power micrograph of an explant of midgut processed for NOS immunohistochem-

that colonize the hindgut do show a delayed expression of Phox2b or p75, they differ not only from the vagal-level neural crest cells that give rise to enteric neurons and the trunk neural crest cells that give rise to sympathetic ganglia and enteric ganglia in the esophagus but also from the sacral-level neural crest cells that give rise to the pelvic plexus, all of which show Phox2b and p75 staining from very early stages.

The sacral-level neural crest cells that colonize the gut either do not express, or show a delayed expression, of many other markers expressed by the vagal- and anterior trunk-level enteric neuron progenitors. Studies using markers of vagal-level enteric neuron progenitors such as c-*ret* (Pachnis *et al.*, 1993, Tsuzuki *et al.*, 1995, Watanabe *et al.*, 1997; current study), *DβH-nlacZ* (Kapur *et al.*, 1992), HNK-1 (Newgreen and Hartley, 1995), p75 and Phox2b (current study) during mouse and rat embryonic development have all shown a progressive, unidirectional, rostrocaudal expression of these markers along the gut, with no reports of expression of the markers in the hindgut prior to the arrival of vagal-level neural crest cells. Unfortunately, the spatiotemporal pattern of expression of the transcription factor, *MASH1*, which is also expressed by enteric neuron precursors (Lo *et al.*, 1991, 1997; Lo and Anderson, 1995), has not been examined in the gastrointestinal tract of any species. Anterior trunk-level neural crest cells that give rise to enteric neurons in the esophagus (Durbec *et al.*, 1996) also express c-*ret* (Tsuzuki *et al.*, 1995) and Phox2b (current study). Thus, the sacral-level neural crest cells that migrate into the hindgut differ from both vagal and anterior trunk enteric neuron precursors in that they either do not express c-*ret*, *DβH-nlacZ*, HNK-1, p75, or Phox2b at all, or they do not express them until 3–4 days after arriving within the gut mesenchyme.

A number of studies have shown that when segments of hindgut are removed from E10–E11 mice and grown in the kidney subcapsular space, neurons do not appear in the explants (Nishijima *et al.*, 1990; Kapur *et al.*, 1992; Young *et al.*, 1996; current study). Similarly, in the current study we also showed that neurons were not found in explants of hindgut from E10.5–E11 mice grown in organ culture for 10 days. Neurons were observed in 1 (of 23) of the E11.5 hindgut explants and in 3 of 10 of the hindgut explants taken from late E11.5 mice, and it is likely that these neurons arose from the most rostral of the vagally derived neural crest cells. In the E11.5 hindgut explant that possessed neurons, the neurons were only present at one end of the explant. Given that neural crest cell migration through the gastrointestinal tract appears to be driven by cell numbers (Yntema and Hammond, 1954; Newgreen *et al.*, 1996), it is likely that, when removed from the embryo, the explant contained only a very small number of enteric neuron precursors which were insufficient in number to populate the entire explant. In contrast to the results of the current study, Rothman and Gershon (1982) found neurons in explants of caudal foregut and hindgut from E9 and E10 mice that had been grown *in vitro* for 2 weeks. The differences between the results from the two *in vitro* studies may be due to different organ culture techniques, and it is possible that, under some environmental conditions, the sacral neural crest cells will differentiate into enteric neurons. There is no doubt that sacral neural crest cells normally provide nonenteric neurons, including those of the pelvic plexus, adjacent to the distal hindgut (Yntema and Hammond, 1955). The results of the current study indicate that either the sacral-level neural crest cells are not normally enteric neuron precursors, or if they are enteric neuron precursors, they are very unusual in that not only do they not express, or show delayed expression of, many of the markers shown by other autonomic neuron precursors, but they also require the presence of vagal level neural crest cells to differentiate into neurons.

Role of Phox2b in Enteric Neuron Precursors

The role of Phox2b in many early neural precursor cells is unknown. Phox2b is a closely related protein to Phox2a, and they show similar distribution patterns in the central and peripheral nervous systems (Tiveron *et al.*, 1996; Pattyn *et al.*, 1997). Although the role of Phox2b is still unknown, recent studies have started to reveal the role of Phox2a. Mice in which the *Phox2a* gene is knocked out die at birth (Morin *et al.*, 1997). They show abnormalities in some regions of the CNS and in the most rostral sympathetic and parasympathetic ganglia, but there are no obvious abnormalities in the enteric nervous system. There is also a reduced expression of Ret in cranial ganglia in the *Phox2a*−/− mice, indicating that Phox2a regulates Ret expression in these ganglia (Morin *et al.*, 1977). Subsequent studies have shown that the expression of Phox2a is itself regulated by another transcription factor, MASH1, and in *Mash1*−/− mice there is little or no expression of Phox2a (Hirsch *et al.*, 1998; Lo *et al.*, 1998). Thus it has been postulated that there is a cascade in which MASH1 induces Phox2a, which in turn activates Ret (Lo *et al.*, 1998). Phox2b is expressed normally in *Mash1*−/− mice (Hirsch *et al.*, 1998), and the genes regulating its expression and which are regulated by Phox2b have yet to be identified.

istry. Stained cells are present within the explant, and fibers have grown from the explant onto the filter paper (*arrows*). Scale bar, 100 μm. (F) Higher power micrograph showing NOS-immunoreactive nerve cell bodies in an explant of midgut. Scale bar, 50 μm. (G and H) No immunoreactive structures were present in explants of hindgut grown in organ culture and then processed for NSE (G) or NOS (H) immunohistochemistry. Scale bars, 100 μm.

CONCLUSIONS

The nuclear regulatory protein, Phox2b, was shown to be the most useful marker to date with which to study the colonization of the developing gut by enteric neuron precursors because it is expressed by all differentiated enteric neurons and because the onset of its expression is very early, in migratory-stage neural crest cells. The colonization of the gastrointestinal tract of embryonic mice and rats by Phox2b-stained cells occurred as a progressive, unidirectional, rostrocaudal wave representing the migration of vagal neural crest cells. A similar colonization of the gut was observed using antisera to Ret and p75 to label migrating neural crest cells and by examining explants grown in the renal subcapsular space or in organ culture. Enteric neurons did not develop in segments of embryonic mouse hindgut that were removed after the arrival of sacral neural crest cells, but prior to the arrival of vagal neural crest cells, and transplanted to the kidney subcapsular space or grown in organ culture. However, despite Phox2b, Ret, and p75 being expressed by migrating, undifferentiated enteric neuron precursors from the vagal neural crest and by the precursors of the pelvic plexus that arise from the sacral neural crest, the cells that populate the hindgut from the sacral level neural axis do not express Phox2b, Ret, or p75.

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