A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice

RAJ P. KAPUR1,*, CYNTHIA YOST2 and RICHARD D. PALMITER2

1Department of Pathology, CH-37, Children’s Hospital and Medical Center, PO Box 5371, Seattle, Washington 98105, USA
2Department of Biochemistry, Howard Hughes Medical Institute, University of Washington, SL15, Seattle, Washington 98195, USA

*Author for correspondence

Summary

The dopamine β-hydroxylase promoter has been shown to direct expression of the reporter gene product, β-galactosidase, to enteric neurons and putative embryonic neuroblasts in transgenic mice (Mercer et al., 1991; Kapur et al., 1991). In this paper, expression of the transgene, DβH-nlacZ, in the gastrointestinal tract is characterized in more detail in wild-type mice and mice which are also homozygous for the lethal spotted allele (ls). Expression of the transgene in wild-type embryos was first detected in scattered mesenchymal cells in the proximal foregut on embryonic day 9.5, and progressed distally until embryonic day 13.5 when the entire length of the gut was colonized by such cells. Several observations suggest that the mesenchymal cells which express the transgene (MCET) are, in fact, enteric neuroblasts, probably derived from the vagal neural crest. (1) The presence of MCET in progressively more caudal portions of the embryonic gut correlated with the neurogenic potential of isolated gastrointestinal segments grafted under the renal capsule. (2) Mitotic activity of MCET was demonstrated by incorporation of [3H]thymidine in utero. (3) The migratory behavior of MCET and/or their precursors was revealed in anastomotic subcapsular grafts of gut from transgenic and non-transgenic embryos; enteric ganglia of the latter were populated by MCET from the former. (4) Enteric expression of the transgene postnatally was restricted to intrinsic neurons that coexpressed other phenotypic markers of neuronal differentiation.

The pattern of transgene expression in ls/ls mice was identical to that seen in ls/+ and +/+ mice until embryonic day 12.5. At that stage, the cranial-to-caudal progression of gastrointestinal colonization by cells that expressed the transgene extended into the proximal colon of wild-type embryos. In dramatic contrast, caudal expression of the transgene in ls/ls embryos ended abruptly at the ileocecal valve. Colonization of the proximal and mid-colon proceeded during subsequent stages of development in ls/ls embryos, but at a retarded rate and in an irregular pattern. The terminal gut was never colonized in these mutant mice. These observations suggest that congenital aganglionosis coli which develops in ls/ls embryos results from a defect of non-neuroblastic mesenchyme that regionally impairs neuroblast migration.

Key words: neuroblast, neural crest, Hirschsprung disease, cell migration.

Introduction

The neural and glial cells which constitute the intrinsic portion of the enteric nervous system (ENS) are derived from neural crest cells. This lineage has been demonstrated in birds and amphibians by crest ablation (Yntema and Hammond, 1954, 1955), isotopic tissue transplantation (Le Douarin and Teillet, 1973; Cochard and Le Douarin, 1982; Epperlein et al., 1990), immunohistochemical studies (Tucker et al., 1986), and retroviral/fluorescent dye labeling of crest cells prior to their migration (Pomeranz and Gershon, 1990; Pomeranz et al., 1991). In both of these vertebrate classes, enteric ganglia are derived primarily from vagal crest populations. Vagal enteric neural crest (VENC) cells arise from the neural tube at the level of somites 1-7 and colonize the entire length of the gut in a cranial-to-caudal fashion. VENC cells migrate caudally through the mesenchyme of the bowel wall en route to populating enteric ganglia. In addition to colonization by the VENC, the postumbilical gut is also populated by sacral enteric neural crest (SENC) cells (Le Douarin and Teillet, 1973; Pomeranz et al., 1991; Serbedzija et al., 1991). In birds, SENC contributes to the formation of postumbilical myenteric and sub-mucosal ganglia and a specialized ganglion (of Remak) which is located in the mesorectum and is unique to birds (Le Douarin and Teillet, 1973).

Until recently, most of our accepted notions about the development of the mammalian ENS were based on morphological studies, principally of rodents, and inferences from avian biology. The development of molecular probes
and retroviral/fluorescent dye techniques for cell lineage analyses have improved our understanding greatly. Intrinsically ganglia are not recognizable morphologically in the murine gastrointestinal tract until embryonic day (E) 12.0 (Rothman and Gershon, 1982). Histologically, myenteric neurons are first evident in the foregut and appear at progressively more caudal levels until E14.5 when they are seen in the distal rectum (Webster, 1973). Various studies have demonstrated that prior to this cranial-to-caudal wave of overt neuronal differentiation the gut is populated by neuroblasts that are indistinguishable microscopically from surrounding mesenchymal cells (Rothman and Gershon, 1982; Baetge and Gershon, 1989; Nishijima et al., 1990; Serbedzija et al., 1991), although the embryonic timing of the initial colonization is controversial. Based on the avian studies described above, it is assumed that the origin of enteric neuroblasts is vagal and possibly sacral neural crest. Colonization of the murine hindgut has been demonstrated by fluorescence labeling of SENC, but neuronal differentiation of these cells has not been documented (Serbedzija et al., 1991).

It has been known for some time that a subset of mesenchymal cells in the gastrointestinal tracts of embryonic mice and rats transiently exhibits catecholaminergic features, including catecholamine uptake and the expression of enzymes involved specifically in catecholamine biosynthesis, tyrosine hydroxylase and dopamine β-hydroxylase (Teitelman et al., 1978, 1981; Jonakait et al., 1984, 1985; Baetge and Gershon, 1989; Baetge et al., 1990a,b). The latter enzymes are expressed relatively early in gastrointestinal development in a cranial-to-caudal progression which extends at least as far as the developing midgut prior to the overt morphological differentiation of enteric neurons (Baetge and Gershon, 1989). These transiently catecholaminergic cells are thought to be enteric neuroblasts derived from the vagal neural crest (vagal enteric neuroblasts).

Recently, we reported stable lines of transgenic mice in which the human dopamine β-hydroxylase gene promoter was used to direct tissue-specific expression of the reporter gene product, β-galactosidase (Mercer et al., 1991; Kapur et al., 1991). Expression of the transgene was observed in many neuronal cell populations including enteric ganglia. In the gastrointestinal systems of transgenic embryos, expression was observed in mesenchymal cells presumed to be precursors of myenteric and submucosal ganglia. In this paper, the spatial and temporal patterns of expression of the DβH-nlacZ transgene are examined in greater detail. Evidence is provided that expression of the transgene is a reliable marker of cranial-to-caudal colonization of the gut by vagal enteric neuroblasts in wild-type and lethal spotted mice.

The lethal spotted allele (ls/ls) is an autosomal recessive mutation associated with congenital aganglionosis coli, i.e. complete absence of ganglion cells in the terminal rectum (Lane, 1966). ls/ls animals are considered to be a model for congenital aganglionosis coli in humans (Hirschsprung disease) (Bolande, 1975). Although the phenotype of ls/ls mice suggests a primary failure in neural crest colonization of the terminal hindgut, neither the molecular nor morphogenetic bases for aganglionosis coli have been characterized.

Methods and materials

Mice

DβH-nlacZ constructs and several lines of transgenic mice which were generated with these constructs have been described previously (Mercer et al., 1991; Kapur et al., 1991). The present study utilized transgenic mice from the line designated 2860-8 which originated from a hybrid cross of C57BL/6J and SJL inbred strains. Non-transgenic ls/+ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and selectively bred with wild-type transgenic animals to establish a line of DβH-nlacZ transgenic mice that bore the lethal spotted allele. Since 30-50% of ls/ls homozygotes live long enough to breed successfully, we were able to establish a line of mice homozygous at both the ls and transgene loci for use in some experiments.

Examination of transgene expression at different stages of development

Timed matings were performed overnight and females were inspected for vaginal plugs the next morning (E0.5). At timed intervals between E8.5 and birth, pregnant animals were killed and the embryonic sites were dissected in phosphate-buffered saline, pH 7.4 (PBS). Entire E8.5 embryos or isolated gastrointestinal tracts from later stages of development were fixed for 1 hour in 10% formalin, rinsed with 0.1 M phosphate buffer (pH 7.4), and then stained overnight with buffer containing 1 mg/ml X-gal substrate (Boehringer Mannheim, W. Germany) in 5 mM FeCN and 5 mM FeCN₂. Stained gastrointestinal tracts were postfixed for 24 hours in 10% formalin and examined grossly and photographed with an Olympus SZ-PT dissecting microscope. Postnatal gastrointestinal tracts were handled in the same manner, except that in some cases the mucosa was stripped from the overlying muscularis propria and whole mounts of the myenteric plexus were examined. Paraffin sections were prepared and examined as described (Kapur et al., 1991).

Renal subcapsular grafts of embryonic gastrointestinal tissue

A modification of the procedure of Nishijima et al. (1990) was used to transplant isolated gastrointestinal segments under the renal capsule. For single transplants, embryos were obtained from transgenic mice at different gestational ages from E11.5 to E14.5 after timed matings. At each stage, the caudal boundary of transgene expression was predicted based on the developmental survey performed initially. From E11.5 and E12.5 embryos, approximately 1 mm long segments of bowel from proximal and distal sides of the boundary were resected with forceps. The intervening segment was fixed and stained as described above to confirm the proximal and distal relationships of the grafts to the boundary of transgene expression. At the later developmental stages, the terminal 1 mm of gut was resected. Adult female C57BL/6J mice were anesthetized with a 90 mg/kg intraperitoneal injection of pentobarbital prior to use as graft recipients. The isolated proximal and distal segments of bowel were grafted surgically under the renal capsule and allowed to develop in that environment until they reached an age of E18.5.

Appositional grafts of proximal segments from transgenic embryos with distal segments from non-transgenic embryos were prepared by manually apposing the isolated portions of gut under the renal capsule. An effort was made to place grafts end-to-end. However, the proximal/distal orientation of the individual seg-
ments was randomized such that in some cases the proximal end of proximal grafts was apposed with the distal end of distal grafts.

**Immunohistochemical studies of adult and embryonic gut**

Four antibodies were obtained commercially for use in colocalization studies of histological sections from transgenic gastrointestinal tracts: polyclonal antisera specific for neuron-specific enolase (NSE, rat, Polysciences Inc., Warrington, PA), dopamine β-hydroxylation (DβH, rabbit, Eugene Biotech., Eugene, OR) and vasoactive intestinal peptide (VIP, rabbit, Incstar, Stillwater, MN), and a monoclonal antibody directed against both phosphorylated and non-phosphorylated neurofilaments H and M (SMI-33, Sternberger Monoclonals, Baltimore, MD). A murine monoclonal antibody specific for muscle actin (HHF-35) was generously provided by Dr. Allen Gown (University of Washington, Washington). Paraaffin-embedded sections of tissues previously stained with X-gal were probed with all of the antibodies except anti-DβH. In the latter case, 10 micron thick cryostat sections of snap frozen tissue were utilized. Both paraaffin and frozen sections were collected on glass slides coated with 0.01% polylysine (Sigma, St. Louis, MO) and preincubated for 10 minutes in an aqueous solution of 0.1% sodium azide, 0.3% hydrogen peroxide prior to immunolabelling. The antibodies were applied overnight at the following dilutions in PBS with 1% BSA: VIP 1/250, NSE 1/1000, SMI-33 1/1000, DβH 1/100, and HHF-35 1/4000. After several rinses with PBS, appropriate biotinylated secondary antibodies (1/500, Amersham International, Arlington Hts., IL) were applied for 1 hour. After several rinses with PBS, the “ABC-immunodetection kit” (Vector Laboratories, Burlingame, CA) was used to visualize sites of indirect immunoreactivity. The sections were rinsed again with PBS, dehydrated and mounted with Histomount (National Diagnostics, Burlingame, CA). Frozen sections were mounted with Aquamount (Lerner Lab., Pittsburg, PA) immediately after rinsing.

[^3H]thymidine incorporation

[^3H]thymidine (500 µCi, Amersham International, Arlington Hts., IL) was injected intraperitoneally into pregnant mice on gestational day 13.5. One hour later, the mice were killed and their embryos collected. Embryonic gastrointestinal tracts were dissected, fixed and stained with X-gal as described above and then embedded in paraaffin. Serial sections of the gastrointestinal tract were prepared at 4 µm intervals and every third section was coated with radiographic emulsion (NTB-2, Eastman-Kodak, Rochester, NY) and exposed for 10 days. The slides were developed and then counterstained with eosin. All histological sections were examined and photographed with an Olympus microscope.

**Results**

**DβH-nlacZ expression in the developing enteric nervous system of transgenic mice**

Expression of the DβH-nlacZ transgene in adult mice was restricted to several discrete cell populations including myenteric and submucosal ganglia throughout the length of the adult gastrointestinal tract (Mercer et al., 1991). In the embryo, the earliest expression of the transgene in the gut was at E9.5 and consisted of scattered mesenchymal cells in the rostral foregut. At subsequent developmental stages, similar cells were distributed in a continuous pattern that extended from the rostral foregut to progressively more distal portions of the gastrointestinal tract (Fig. 1). Expression along the entire length of the gastrointestinal tract was not evident until E13.5.

In embryos at E9.5, the gut is a relatively simple tube with a small ventral loop in the midgut region. The gastrointestinal tract continues to elongate, rotate and differentiate into regionally distinct segments between E9.5 to E13.5 in a stereotyped manner which correlates with gestational age. By E10.5, the ventral umbilical loop has lengthened considerably and the caudal limit of transgene expression in the gut wall on E10.5 was in the proximal limb of the umbilical loop (Fig. 1a,b). In E11.5 embryos, an obvious gastric bulge and rudimentary ceaccum/appendix are recognizable. At this stage, mesenchymal cells that express the transgene (MCET) were distributed as far caudally as the distal portion of the developing small intestine near the apex of the umbilical loop. During the next 24 hours, the “leading edge” of transgene expression progressed through the cecum. In E12.5 embryos, the cecum is an easily recognized structure with a well-developed sacculated diverticulum (Fig. 1c,d); the midgut is a convoluted tube partially enclosed in the umbilical hernia. MCET were present in the proximal third of the colon at this stage, cranial to its retroperitoneal reflection. Beginning on E13.5, and at all later stages of development, MCET were present in the gut wall from esophagus to anus (Fig. 1c,g).

The proximodistal wave of transgene expression in the enteric mesenchyme between E9.5 and E13.5 was followed by a similar wave of morphological differentiation in the wall of the gastrointestinal tract (Fig. 1d,f,h). The latter phenomenon, characterized initially by circumferential orientation of mesenchymal cells destined to give rise to the inner layer of the muscularis propria, was evident in the foregut and proximal midgut on E12.5 and extended distally thereafter (Fig. 1f,h). Concomitant with these changes in the bowel wall, cells expressing the transgene became organized into small clusters adjacent and external to the circumferential smooth muscle layer. This distribution was maintained and corresponds with transgene expression postnatally in the neurons of myenteric ganglia. At E18.5, transgene expression was also observed in submucosal ganglia; postnatal transgene expression in this site has been documented previously (Mercer et al., 1991). The neuronal fate of at least some of the cells which express the transgene was established by immunocytochemical studies of postnatal bowel. Nuclear staining with X-gal in enteric ganglia colocalized with cytoplasmic immunoreactivity of antibodies specifically directed against neurofilament peptides, neuron-specific enolase, and vasoactive intestinal polypeptide (Fig. 2a-c). Vasoactive intestinal polypeptide was identified in many but not all of the cells expressing the transgene, in contrast with the other neural markers which were expressed in all of the cells expressing the transgene in a given section. Immunoreactivity with antibodies specific for DβH was inconsistently observed in adult enteric neurons (not shown). Appropriate control sections from which primary antibody was eliminated showed no staining of these neurons (Fig. 2d).

**Mitotic activity in enteric mesenchymal cells which express the transgene**

Tritiated thymidine incorporation in the embryonic gastrointestinal mesenchyme of embryos exposed to the isotope in utero was used to assess the replicative potential of
Fig. 1. Histochemical staining of nuclei with X-gal reveals a cranial-to-caudal progression of transgene expression in the developing gut which is described in the text; arrows in a-e indicate the caudal-most sites of expression. (a) The excised gut from E10.5 embryo shows transgene expression in foregut (fg) and proximal midgut (mg), but not hindgut (hg). (d) A histologic section through the ventral midgut loop at E10.5 shows expression in scattered mesenchymal cells in the proximal (p′), but not distal (d′) segments (L, liver; fb, forebrain).

(b,e) The entire gastrointestinal tract (b) and resected distal small intestine (si) plus large intestine (e) from embryos at E12.5 show expression throughout the foregut (s, stomach) and midgut, including the cecum (c′), and proximal colon. (c) The distal gastrointestinal tract from an embryo at E13.5 shows colonization of the entire length of the gut down to the anus (an) by cells which express the transgene. (f) A histologic section of the midgut at E13.5 shows organization of cells which express the transgene into primitive myenteric ganglia. (g) The external surface of adult large intestine and (h) a corresponding histologic section show transgene expression in neurons of myenteric (large arrows) and submucosal (small arrows) ganglia. Bars, a, 2.1 mm; b, 3.1 mm; c, 2.1 mm; d, 19 µm; e, 2.1 mm; f, 19 µm; g, 1.4 mm; h, 78 µm.
MCET. Although a detailed ontogenic survey was not performed, isotope incorporation was evident in MCET in all colonized portions of the gut between E9.5 and E14.5 (later developmental stages were not examined). Fig. 3 shows a representative section of gastrointestinal tracts from an E13.5 embryo. At the stages examined, 20-30% of MCET incorporated [3H]thymidine during a one-hour pulse and this percentage was the same in different portions of the colonized gut. In comparison, 25-40% of the mesenchymal cells which did not express the transgene incorporated isotope in E13.5 embryos. Thus, expression of the transgene occurs prior to postmitotic differentiation.

Transgene expression and neurogenic potential of isolated gastrointestinal segments

The proximodistal progression of transgene expression in the bowel mesenchyme might be explained as either migration of vagal neural crest cells destined to form enteric ganglia, or orderly differentiation of neuroblasts that colonized the gut earlier in development. To address this question, the neurogenic potential of “proximal” and “distal” segments of gut from transgenic embryos was examined after transplantation under the renal capsule. Proximal segments were lengths of developing small intestine that contained mesenchymal cells which expressed the transgene prior to transplantation. Distal segments represented the terminal hindgut which prior to E13.5 did not include MCET. The terminal hindgut alone was transplanted from embryos E13.5 or older since MCET were present throughout the length of the gut at these stages. The grafts were allowed to develop in this environment for a time period equivalent to E18.5.

In the subcapsular environment, gastrointestinal segments from embryos ranging from E11.5 to E14.5 showed considerable growth and differentiation (Fig. 4a-d). Segments obtained cranial to the cecum developed a primitive villous architecture with columnar epithelium comprised predominantly of absorptive cells with occasional goblet cells (Fig. 4b). In contrast, segments of distal hindgut developed relatively flat mucosa with small crypt-like indentations reminiscent of rectal mucosa (Fig. 4d). Mucous-containing goblet cells made up the majority of epithelial cells in the latter grafts. In both proximal and distal grafts, an easily recognized muscularis propria was formed. The smooth muscle fibers existed primarily as a circumferential layer of spindled cells that were immunoreactive with a monoclonal antibody specific for muscle actin (not shown). In these respects, the grafted tissues resembled corresponding regions of normal gut at E18.5.

In all cases, gastrointestinal segments that expressed the transgene prior to transplantation (proximal segments from E11.5 and E12.5 embryos, terminal hindgut from later stages) gave rise to myenteric and submucosal ganglia in subcapsular grafts (Table 1). The neurons in these ganglia expressed the transgene. In contrast, distal segments from E11.5 or E12.5 embryos failed to develop ganglia and did not show evidence of transgene expression. Thus, there was a direct correlation between colonization of the gut wall by MCET and the neurogenic potential of isolated intestinal segments when transplanted under the renal capsule.

These data suggest that MCET are enteric neuroblasts which migrated caudally in the gastrointestinal mesenchyme and successively colonized more distal portions of the gastrointestinal tract. To assess the migratory potential of these cells, proximal segments from transgenic, E12.5-stage embryos were cocultured under the renal capsule in direct apposition with distal segments of bowel from non-transgenic embryos at the same gestational age. After 6 days under the renal capsule, the proximal and distal segments differentiated into small and large intestine, respectively. More importantly, neurons derived from the proximal (transgenic) graft colonized the distal (non-transgenic) bowel and formed ganglia similar in location and histological appearance to those seen in distal grafts from E13.5 or older transgenic embryos (Fig. 5a,b). In most cases, many transgenic neurons were present near sites of apposition of transgenic and non-transgenic gut and progressively fewer were seen at greater distances from the transgenic bowel. However, in some cotransplants, transgene expression was evident even in the most distant portions of the non-transgenic gut. Although it seemed to be important that the one end of the proximal segment be in contact with the distal segment for neuroblast migration, the orientation of the distal segment was not critical since neuroblast migration succeeded after end-to-end and end-to-side appositions.

The pattern of transgene expression in lethal-spotted mice

By selective breeding, a colony of mice that express the transgene and carry the lethal spotted mutation was established. Is/I$^s$ mice from this colony displayed the characteristic phenotype, abnormal pigmentation and aganglionosis coli (Lane, 1966). As expected, postnatal homozygous mutant animals lacked ganglia and did not express the transgene in their caudal rectum and a variable length of contiguous large intestine (Fig. 6a,b). Heterozygous (Is/+), and wild type (+/+), siblings, in contrast, showed expression of the transgene in enteric ganglia through the entire length of the gastrointestinal tract. The aganglionic segment ranged from 0.5 to 2.0 cm in length and was invariably preceded by a proximal segment of hypoganglionic colon which sometimes extended into the ascending third of the large intestine (Fig. 6b). More proximally, the number and distribution of enteric neurons which expressed the transgene was similar in spotted mice and their litter mates.

<table>
<thead>
<tr>
<th>Stage of donor</th>
<th>Site of origin</th>
<th>Number of grafts</th>
<th>Neuronal differentiation</th>
<th>Transgene expression</th>
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<tbody>
<tr>
<td>E11.5</td>
<td>Proximal</td>
<td>3</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Distal</td>
<td>3</td>
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<td>E12.5</td>
<td>Proximal</td>
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<td></td>
<td>Distal</td>
<td>5</td>
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</tr>
<tr>
<td>E13.5</td>
<td>Terminal hindgut</td>
<td>5</td>
<td>+ (4/5)</td>
<td>+ (4/5)</td>
</tr>
<tr>
<td>E14.5</td>
<td>Terminal hindgut</td>
<td>3</td>
<td>+</td>
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*Proximal” and “Distal” refer to the relationship of the donor segment to the “leading edge” of mesenchymal colonization by cells that express the transgene in the donor intestinal tract. “Terminal hindgut” consisted of the most caudal 1 mm segment of intestinal tract.

As assessed histologically and immunohistochemically with anti-NSE antibodies.

As assessed by X-gal staining.
Examination of transgene expression prenatally in the mutant line revealed striking differences between homozygous mutants and wild-type or heterozygous embryos. Prior to E11.5, no differences could be discerned between these genotypes. However, a difference in the pattern of transgene expression emerged on E12.5. As noted above, transgene expression in +/+ (and \(ls/+\)) embryos at this stage extended caudally in the gastrointestinal tract past the ileocecal junction and into the proximal large intestine (Fig. 7a). However, in \(ls/ls\) embryos, cells that expressed the transgene were present only as far as the ileocecal valve, where their density increased slightly as though caudal migration was abruptly halted at the junction of small and large intestine (Fig. 7b). At later stages expression progressed irregularly beyond this interface in a retarded manner, and the distal colon was never completely colonized (Fig. 7c,d). These are reproducible, easily recognized abnormalities in the transgenic embryos.

\(^{3}H\)thymidine incorporation in gastrointestinal MCET of E12.5 and E13.5 \(ls/ls\) embryos was also examined and compared with \(ls/+\) and wild-type controls. As with control embryos, 20-30% of MCET incorporated the isotope in \(ls/ls\) gut. No dramatic differences in the percentage of mitotically active MCET cells were apparent in any region, including the ileocecal junction.

**Discussion**

We believe that the most straightforward interpretations for the observations presented lead to the following conclusions. (1) Expression of the transgene initiates in enteric neuroblasts derived from the vagal neural crest (vagal enteric neuroblasts) prior to their morphological differen-
Enteric expression of the transgene indicates proximodistal colonization of the gut between E9.5 and E11.5 by vagal enteric neuroblasts capable of replication, migration and neuronal differentiation in enteric segments transplanted under the renal capsule. (3) Aganglionsis coli in ls/ls embryos is due, at least in part, to defective proximodistal colonization of the gastrointestinal tract by vagal enteric neuroblasts, and this defect is first evident in the ileocecal junction of transgenic (ls/ls) embryos at E12.5.

The conclusion that transgene expression occurs in vagal enteric neuroblasts destined to differentiate into enteric neurons is consistent with hypotheses concerning “transient catecholaminergic” (TC) cells which have been observed in the developing gastrointestinal systems of rats, mice and humans (Cochard et al., 1978, 1979; Teitelman et al., 1978, 1979, 1981; Jonakait et al., 1984, 1985; Vaos and Lister, 1988; Baetge and Gershon, 1989; Baetge et al., 1990a,b). These cells appear in the gastrointestinal wall prior to overt morphological differentiation of enteric neurons. They are defined by their expression of one or more phenotypic markers of catecholaminergic differentiation such as aldehyde-induced catecholamine histofluorescence (Cochard et al., 1978; Gershon et al., 1984), amine uptake (Jonakait et al., 1979) and immunoreactivity with antisera specific for tyrosine hydroxylase (TH) or DβH (Baetge and Gershon, 1989; Baetge et al., 1990a,b; Jonakait et al., 1979; Teitelman et al., 1979). They have been termed “transient” because in many instances these phenotypic features are lost later in development and the neurons of mature enteric ganglia do not contain detectable catecholamines.

Baetge and his colleagues have begun to characterize enteric TC cells in mice and rats (Baetge and Gershon, 1989; Baetge et al., 1990a,b). In their studies, antibodies specific for either TH and DβH identify a subset of mesenchymal cells in the developing gut. Although the cells they have identified by this technique share many similarities with MCET, there are some differences between the two. The temporal and spatial characteristics of the TC cells they identified immunocytochemically closely parallel the pattern of DβH-nlacZ transgene expression that is observed between E9.5 and E11.5. However, the progressive proximodistal progression of immunocytochemically defined TC cells reported by Baetge and Gershon (1989) only extends as far caudally as the midgut. In contrast, caudal progression of enteric cells expressing the transgene continues to the terminal hindgut. In addition, they reported that TC cells can no longer be detected in the murine gut after E12.5, although anti-DβH immunoreactivity persists throughout life in neurons of rat enteric ganglia (Baetge et al., 1990b). We believe that cells in the murine embryonic gut which express the transgene are, at least in part, the same as immunocytochemically-defined TC cells. The apparent discrepancies in the distribution and persistence of the latter cells may reflect relative insensitivity of the immunocytochemical probes or unresponsiveness of the human promoter in the transgene to ontogenic transcriptional inactivation.

These observations add to contradictory data concerning the gastrointestinal colonization by enteric neuroblasts. Historically, two approaches have been used to study gut colonization by enteric neuroblasts, immunocytochemistry and tissue explantation. Antibodies have been used to identify histologically undifferentiated neuroblasts. Although many antigens have been localized immunocytochemically in embryonic enteric mesenchyme, two classes of antigens have been particularly valuable, i.e. general proteins expressed in most mature neurons (e.g. neurofilament peptides) (Webster, 1973; Nishijima et al., 1990; Baetge and Gershon, 1989; Baetge et al., 1990a,b) and catecholaminergic markers (discussed above). In rodents, progressive cranial-to-caudal expression of these antigens has generally been observed and precedes morphological differentiation of enteric neurons.

At least two groups have explanted segments of gastrointestinal tract dissected from murine embryos at different stages of development and examined the neurogenic potential of such isolates in vitro (Rothman and Gershon, 1982; Nishijima et al., 1990). Unfortunately, these studies yielded conflicting results. Rothman and Gershon (1982) reported neural differentiation in both proximal and distal GI tracts obtained from embryos as early as E9.0 and cultured organotypically in a complex medium for 2 weeks. They concluded that the entire length of the gut is colonized by enteric neuroblasts as early as day 9.0. Nishijima et al. (1990) repeated a similar series of experiments, although they employed a different culture medium. The latter authors also transplanted proximal and distal segments of gut either onto chick chorioallantoic membranes or under the renal capsules of adult mice. One benefit of cultivating isolated gastrointestinal segments under the renal capsule is that growth and differentiation of the entire organ occurs, suggesting that at least in some respects the subcapsular environment is conducive to “normal” development. Neurons that arise in these transplants express neuronal antigens and demonstrate appropriate microanatomic relationships for myenteric and submucosal ganglia. In all three environments, neural differentiation failed to occur in explants of hindgut removed prior to E13.5; and this stage was proposed as the earliest time at which the terminal hindgut is colonized by enteric neuroblasts.

Our findings with renal subcapsular transplants of gut from DβH-nlacZ transgenic embryos are similar to the findings and interpretation of Nishijima et al. (1990). They strongly suggest that colonization of the developing gut by vagal enteric neuroblasts proceeds in a cranial-to-caudal manner and that these neuroenteric precursors can be distinguished from other mesenchymal cells because they express catecholaminergic markers like the DβH-nlacZ transgene. The colonization process probably involves migration and proliferation of vagal neural crest cells. Replication of these cells was demonstrated independently by Baetge and Gershon (1989) who showed bromodeoxyuridine colocalization with DβH in the developing foregut, and here, by [3H]thymidine incorporation in cells which expressed the transgene. The migratory properties of these cells is indicated by their ability to colonize segments of non-transgenic gut when cocultured under the renal capsule. The latter process probably involves cues provided by the mesenchymal environment since neuroblasts do not radiate from the subcapsular graft into the adjacent host renal tissue.
In the past, avian embryologists have used neural crest ablation and isotopic chimeric grafts to demonstrate a dual origin for enteric neuroblasts from the vagal and sacral neural crest (Yntema and Hammond, 1954, 1955; Le Douarin and Teillet, 1973; Cochard and Le Douarin, 1982; Epperlein et al., 1990). The latter populate the postumbilical gut and the ganglion of Remak (a ganglion unique to birds). The vagal neural crest has been accepted universally as a source of enteric neuroblasts; however, contribution by the sacral neural crest has been challenged (Allan and Newgreen, 1980). Recently, compelling evidence for sacral neural crest colonization of the hindgut in both avian and murine embryos has been reported (Pomeranz and Gershon, 1990; Pomeranz et al., 1991; Serbedzija et al., 1991). In the latter studies, fluorescent dyes and/or retroviral tracers were used to label directly sacral neural crest cell precursors prior to their migration into the gut. In avian embryos, these sacral crest cells clearly give rise to some of the neurons in postumbilical ganglia within the gut wall and in the ganglion of Remak.

Although expression of the transgene seems to be a reliable marker for at least a large subset of vagal neural crest cells that are destined to form enteric neurons, sacral enteric neural crest cells apparently do not express this transgene. In mice, sacral neural crest cells colonize the hindgut as early as E9.5, prior to colonization of this region by vagal neural crest cells (Serbedzija et al., 1991). However, the developmental fates of sacral neural crest cells in mice have not been established. The observation by Rothman and Gershon (1984) that explants of E9.5 hindgut will give rise to cells with neural characteristics after 10 days in tissue culture suggests that sacral neural crest cells in these explants may differentiate into neurons in vivo. However, under other culture conditions which permit differentiation of vagal enteric neuroblasts (i.e. subcapsular transplantation), neural differentiation is not observed in hindgut explants until E13.5 (Nishijima et al., 1990 and this paper). It is not clear whether the culture conditions used by Rothman and Gershon (1984) were permissive to differentiation events that occur normally in intact embryos, or induced neural differentiation in sacral neural crest cells which normally are not neurogenic. In any case, the subcapsular transplants suggest that sacral neural crest cells which colonize the hindgut are different from vagal enteric neuroblasts, perhaps in their respective environmental requirements to induce/permit neural differentiation.

Prior to these studies there was evidence to suggest that aganglionosis in ls/ls mice results from a primary failure in neuroblast colonization. Rothman and Gershon (1984) and Nishijima et al. (1990) examined the neurogenic potential of explanted segments of GI tracts from ls/ls embryos. Both concluded that the terminal bowel is never colonized in mutant embryos and that this defect is recognizable by E13.5. However, the two groups disagree as to when an abnormality is first recognizable, because of conflicting data regarding the stage at which the terminal bowel is colonized by neurogenic precursors in non-mutant embryos (discussed above).

Comparison of the patterns of transgene expression in the developing gastrointestinal tracts of ls/ls embryos and their litter mates supports these earlier observations, and suggests that the defect manifests itself when vagal neural crest cells begin to colonize the large intestine. The abnormal distribution of MCET around the cecum of homozygous embryos suggests that migration and/or proliferation of the cells is retarded as they encounter the mesenchyme of the large intestine. Gershon and his colleagues have proposed that the mechanism underlying abnormal hindgut colonization in ls/ls embryos is a primary defect in mesenchyme of the terminal hindgut that results in a non-permissive environment for neural crest migration (Rothman and Gershon, 1984; Tennyson et al., 1986; Jacobs-Cohen et al., 1987; Payette et al., 1988); however, the molecular basis for this disorder is unknown. Our observations suggest that if the primary defect exists in the uncolonized gut wall as opposed to the migrating neuroblasts, then the mesenchyme of the developing colon may provide different molecular cues from the more proximal gut. There is sharp contrast in epithelial cytology, gland architecture and enteroendocrine secretory products in gut proximal versus distal to the ileocecal valve. Similarly, the shape and biochemical phenotypes of motile enteric neurons appear to be determined regionally with respect to a discrete boundary between foregut and midgut in the gastrointestinal tract (Copenhaver and Taghert, 1989a,b). The fact that the distribution of MCET in ls/ls embryos only becomes abnormal as colonization begins in the large intestine suggests that an essential migratory or trophic influence from colonic mesenchyme may be missing or defective. Such a pan-colonic embryological defect might have been predicted based on abnormalities observed previously in the enteric plexi, particularly the submucosal plexus, along the majority of the length of the colon of ls/ls mice (Payette et al., 1987).

We have established the value of DβH-nlacZ transgene expression as a marker for enteric neuroblasts in the normal and abnormal murine gut. The β-galactosidase reporter gene may have greater value in the future since it can be used selectively to isolate neuroblasts from mutant and non-mutant mice and permitting examination of their phenotypes in greater detail. In addition, the DβH promoter may be used to target expression of other genes to enteric neuroblasts in order to examine the effects of such gene products on the development and function of the enteric nervous system.

The authors would like to thank Drs Ralph Brinster, Gary Hoyle and Gary Cadd for their thoughtful comments about the manuscript. This work was supported in part by grant number HD09172.

References


(Accepted 10 June 1992)
Fig. 2. Postnatal coexpression of transgene and other markers of neural phenotype. (a-c) Sections of adult myenteric ganglia showing immunohistochemical localization (brown reaction product) of vasoactive intestinal peptide (VIP), neuron-specific enolase (NSE), and neurofilament peptides (NF), with transgene expression (blue nuclear staining) in mature enteric neurons. Immunohistochemical labelling of VIP and, to a lesser degree, NSE is also seen in intramuscular neuronal processes (arrows). (d) A control section in which primary antibody was omitted shows minimal non-specific staining. Bars, a, 10 \( \mu \text{m} \); b, 12 \( \mu \text{m} \); c, 6 \( \mu \text{m} \); d, 12 \( \mu \text{m} \).

Fig. 3. \( [3^\text{H}] \) thymidine incorporation in cells that express the transgene. In this section of gut from an E13.5 embryo, DNA replication is demonstrated autoradiographically in a subset of mesenchymal cells that express the transgene (arrows). Replication is evident in other cell types in the gastrointestinal tract including the luminal epithelium. L, lumen. Bar, 16 \( \mu \text{m} \).

Fig. 4. Neurogenic potential of isolated intestinal segments transplanted under the renal capsule. Gross (a,c) and histological (b,d) photomicrographs of intestinal segments from a transgenic, E12.5 donor that were grafted under the renal capsule of a non-transgenic adult mouse for 6 days are shown. Myenteric (long arrows) and submucosal neurons (short arrow) which express the transgene develop in segments taken proximal (PROX) to the “leading edge” of MCET colonization (a,b). In contrast, donor segments taken distal to this boundary (DIST) show no evidence of either neuronal differentiation or transgene expression (c,d). k, host kidney. Bars, a, 5.3 mm; b, 62 \( \mu \text{m} \); c, 3.2 mm; d, 25 \( \mu \text{m} \).

Fig. 5. Migration of MCET from transgenic to non-transgenic bowel under the renal capsule. Gross (a) and histological examples (b) of segments of transgenic (T) and non-transgenic (NT) gut from E12.5 donors which were placed in apposition under the renal capsule for 6 days. The transgenic gut was taken from developing small intestine at a site already colonized by mesenchymal cells that expressed the transgene. The non-transgenic segment was taken from developing large intestine. The dashed line indicates the interface between the two segments. The small intestinal versus colonic fates of the two segments are reflected in their differing histological architectures. Cells which express the transgene (arrows in b) have migrated from the transgenic segment across this boundary to colonized myenteric ganglia in the non-transgenic gut. Bars, a, 3.2 mm; b, 31 \( \mu \text{m} \).

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