Intercellular signals downstream of endothelin receptor-B mediate colonization of the large intestine by enteric neuroblasts

Raj P. Kapur1,*, David A. Sweetser2, Barbara Doggett1, Joseph R. Siebert1 and Richard D. Palmiter2

1 Departments of Pathology, CH-37, Children’s Hospital and Medical Center, and University of Washington, PO Box 5371, Seattle, Washington 98105 USA
2 Department of Biochemistry, Howard Hughes Medical Institute, University of Washington, SL-15, Seattle, Washington 98195, USA

*Author for correspondence

SUMMARY

Mice homozygous for the piebald lethal (s\(^l\)) mutation, which have a complete deletion of endothelin receptor-B, fail to form ganglion cells in the distal large intestine and are nearly devoid of cutaneous melanocytes. These phenotypic features stem from incomplete colonization of the hindgut and skin by neural crest-derived neuroblasts and melanoblasts, respectively. We have used expression of a transgene, dopamine-\(\beta\)-hydroxylase-nlacZ, to study colonization of the enteric nervous system in s\(^l/s\(^l\) embryos and s\(^l/s\(^+\)→wild-type chimeric mice. Enteric neuroblasts derived from the vagal neural crest colonize the developing foregut, midgut and distal small intestine of s\(^l/s\(^l\) embryos in a cranial-to-caudal manner indistinguishable from s\(^l/+\) or +/- embryos. However, colonization of the large intestine is retarded and the distal large intestine is never colonized, a developmental defect identical to that observed in lethal spotted (endothelin-3 deficient) embryos. The coat pigmentation and relative distributions of mutant and wild-type ganglion cells in s\(^l/s\(^l\)→wild-type chimeras indicate that the defect associated with endothelin receptor-B gene deletion is not strictly neuroblast autonomous (independent of environmental factors). Instead, intercellular interactions downstream of the endothelin receptor-B mediate complete colonization of the skin and gut by neural crest cells.

Key words: endothelin, enteric nervous system, piebald lethal, intestine, neuroblast, mouse

INTRODUCTION

In vertebrates, enteric neurons and melanocytes are derived from neural crest cells. Melanoblasts arise along the entire length of the cranial-caudal axis on either side of the neural tube and spread laterally through superficial mesenchyme under the surface ectoderm (Erickson and Loring, 1987; Erickson et al., 1992). Enteric neuroblasts are derived predominantly from the vagal neural crest, although sacral cells may contribute to neural or glial derivatives in the hindgut (Gershon et al., 1993). Vagal neural crest cells enter the foregut of the early embryo and colonize the entire length of the gut wall through a caudal progression. To completely colonize the skin and gut, melanoblast and enteric neuroblast populations must survive, multiply, negotiate relatively long distances, find sites of permanent residence with appropriate spacing and differentiate. These biological properties are undoubtedly influenced in both cell types by overlapping intercellular and intracellular molecular signals as evidenced by murine and human mutations which produce coincident failure of neural crest cells to colonize the entire gut (colonic aganglionosis) and coat (spotting) (Edery et al., 1994; Greenstein-Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994; Romeo et al., 1994; Schuchardt et al., 1994).

Mutant alleles at three distinct loci, piebald lethal (s\(^l\)), lethal spotted (ls) and Dominant megacolon (Dom), produce spotting and colonic aganglionosis in mice. All three of these mutations arose spontaneously and map to different loci in the murine genome (Lane, 1966; Lane and Liu, 1984). The s\(^l\) and ls mutations are autosomal recessive alleles, which produce no recognizable phenotype in heterozygotes, whereas a fully penetrant phenotype characterized by absence of ganglion cells in the distal large intestine and absence of melanocytes in portions of the coat has been described for both homozygotes. In both cases, the mutation has been maintained on a C57Bl/6J background and the majority of affected animals die prematurely, most prior to weaning, as a consequence of intestinal obstruction and secondary enterocolitis (Caniano et al., 1989). Although the extent of enteric aganglionosis is similar in s\(^l/s\(^l\) and ls/ls mice, the latter display a highly variable degree of spotting whereas the former are devoid of pigmentation except around the ears and rump. In addition, lethality prior to weaning is more common in s\(^l/s\(^l\) mice, such that establishment of s\(^l/s\(^l\) colonies is challenging.

Recently, s\(^l\) and ls were shown to be mutations in the genes that encode endothelin-receptor B (ET-B, also abbreviated EDNR-B) and its ligand, endothelin-3 (ET-3), respectively (Greenstein-Baynash et al., 1994; Hosoda et al., 1994). Endothelin peptides are a family with homologous members, endo-
MATERIALS AND METHODS

**MICE**

*DβH-nlacZ* constructs and several lines of transgenic mice that 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 *s/s* 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 *s/s* allele. Wild-type CeHeB/Fej males and females were obtained from the Jackson Laboratory (Bar Harbor, ME). Swiss-Webster females and vasectomized males were purchased from Simonson Laboratories (Gilroy, CA). All the animals were maintained with a 12 hour:12 hour light:dark regimen.

**Analysis of neuroblast/ganglion cell distributions at different stages of development**

Litters from *s/s* × *s/s* or *s/s* × *s/+* pairings were examined pre- or postnatally. For prenatal specimens, one hour timed-matings between 7:00 AM and 9:00 AM were conducted and copulation was verified immediately thereafter by the presence of a vaginal plug (embryonic day, E0). The pregnant females were killed at various stages of gestation. Embryos or intestinal tracts were isolated, fixed and stained with X-gal to visualize sites of transgene expression as described previously (Kapur et al., 1991). The stained tissues were photographed and portions were embedded in paraffin for histological evaluation.

**Preparation of aggregation chimeras**

Four-to-six-week virgin C3HeB/FeJ and transgenic *s/+* females were induced to superovulate by serial intraperitoneal injections of pregnant mare serum gonadotropin (5 IU, 48 hours prior to mating, Sigma, St Louis, MO) and human chorionic gonadotropin (5 IU, immediately prior to mating, Sigma, St Louis, MO). The wild-type and heterozygous female mice were mated overnight with non-transgenic *s/+* males, and each mating was followed by an injection of 10 minutes treatment with pronase (0.5% in M2 medium, Difco, Detroit, MI). The pairs were transferred to M2 with approximately equal affinities, whereas ET-A is relatively selective for ET-1. The *ls* allele is a point mutation that prevents conversion of the preproendothelin-3 peptide to its active form and thereby leads to undetectable levels of functional ET-3 (Greenstein-Baynash et al., 1994). The *ls* mutation represents a complete deletion of the *ET-B* gene (Hosoda et al., 1994). Details concerning expression patterns of ET-3 or ET-B in the embryonic gut have not been reported. However, endothelin immunoreactivity and endothelin binding sites have been identified in the ganglion cells of human fetal and postnatal intestines (Inagaki et al., 1991a,b; Escrig et al., 1992). In addition, ET-B receptors have been identified in the circular smooth muscle of guinea pig cecum (Okabe et al., 1995). Based on these observations, ET-3/ET-B-mediated intercellular signals are believed to regulate the behavior of enteric neuroblasts and melanoblasts in an autocrine or paracrine manner such that inactivity of either is incompatible with complete enteric or skin colonization.

Details about the cellular events affected by the *ls* or *s1* mutations are not understood, in part because convenient markers for studying the distribution of murine enteric neuroblasts or melanoblasts have only been developed recently. Our laboratory has taken advantage of the fact that enteric neuroblasts derived from the vagal crest express the catecholaminergic enzyme, dopamine-β hydroxylase (*DβH*), by making transgenic mice in which enteric neurons and their embryonic precursors express β-galactosidase driven by the human *DβH* promoter (Kapur et al., 1991; Mercer et al., 1991). Evaluation of transgenic *ls/ls* embryos and *ls/ls* (transgenic)-wild-type *s/+* embryos, as in *ls/ls* homoyzygotes, neuroblast colonization of the small intestine and portions of various organs were isolated for histological evaluation.

**Phenotypic analysis of chimeric mice**

2-3 weeks after birth, chimeric mice were killed, photographed and their coats examined for areas devoid of pigmentation. The viscera were removed and the gastrointestinal tracts were isolated. A segment of small intestine and portions of various organs were isolated for DNA analysis. The remainder of the gut and other tissues were fixed for 1 hour in 10% formalin, rinsed with 0.1 M phosphate buffer (pH 7.4) and then stained overnight at 37°C with buffer containing 1 mg/ml X-gal substrate (Boehringer Mannheim, W. Germany), 5 mM FeCN and 5 mM FeCN 2 . Stained gastrointestinal tracts were postfixed for 24 hours in 10% formalin and examined grossly and photographed with an Olympus SZ-PT dissecting microscope. 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 previously (Kapur, 1991).

**Genotype analysis of chimeras by amplification of DNA polymorphisms**

A polymorphic microsatellite sequence amplified using the polymerase chain reaction (PCR) with the D14MIT7 *f* and *r* primers.
DNA analysis of s/l/s l—wild-type chimeric tissues for tissue mosaicism

The relative amount of mosaicism in each tissue was determined in one of two ways. For some samples, relative hybridizations to DNA dot-blot with probes specific for the DβH-nlacZ transgene found in wild-type cells and a constitutive gene present in all cells (Hox 1.4) was assessed as described previously (Kapur et al., 1993). Quantitative Southern blot analysis with probes for the ET-B gene (found only in mutant cells) and metallothionein gene (found in all cells) was conducted for other samples as follows. A phosphomager (Molecular Dynamics 400S, Sunnyville, CA) was used to quantify ET-B-specific 4.8 and 8.2 kb EcoRI bands detected by a radiolabelled 0.7 kb EcoRI/BamHI fragment of the mouse ET-B cDNA (Sakurai et al., 1990). The relative intensities of these bands were compared with a unique 4.0 kb EcoRI fragment detected using a radiolabelled 0.75 kb Stul/BglII fragment from the mouse metallothionein promoter (Searle, 1984). The ratio of these values was then compared to similar values obtained from a standard series of mixtures of C3HeB/FeJ and s/l/s l DNAs to estimate percent chimerism in each specimen.

Preparation of figures

Gross and microscopic slides were digitized (Nikon Coolscan) and brightness and contrast were optimized (Adobe Photoshop 3.0, Adobe Systems Incorporated, Mountainview, CA) for entire images. Neither the distribution of neuroblasts nor coat pigmentation were altered with respect to the original transparencies.

RESULTS

Characteristics of s/l/s l mice carrying the DβH-nlacZ transgene

Non-transgenic mice carrying the s' allele were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred with DβH-nlacZ-transgenic animals in our colony to establish a line of mice that carried the s' mutation and were homozygous for the transgene. Presence of the latter appeared to have no effect on the phenotypes of s/l/s l, s/l/+ or +/+ mice in this colony. Both s/l/+ and +/+ individuals were phenotypically identical with solid black coats and ganglion cells throughout the lengths of their intestinal tracts (Fig. 1). Occasionally, a very thin, linear midline streak of white pigmentation was observed on the ventral surface of some s/l/+ animals. In contrast, s/l/s l mice had primarily white coats except around the base of their ears and/or tails where a small patch of black pigmentation usually appeared. Crosses between s/l/+ parents yielded average litters of 6.3±2.4 pups, approximately one-fourth (32/120) of which exhibited the s/l/s l phenotype. Mortality of the homozygotes was high, 75% dying 2-4 weeks after birth. Those s/l/s l animals that survived weaning died by 4 months of age, but it was possible to successfully mate some s/l/s l × s/l/s l pairs to obtain litters composed entirely of affected individuals. The average litter sizes from such matings (5.8±2.8) were not significantly different from those of heterozygote pairings.

The distribution of neuroblasts derived from the vagal neural crest was visualized in intact gastrointestinal tracts from s/l/s l, s/l/+ and +/+ littersmates by histochemical staining at various stages of pre- and postnatal development. The histochemical procedure yielded a blue insoluble reaction product in the nuclei of ganglion cells in the terminal colons of s/l/s l (top) and s/l/+ (bottom) mice that carry the DβH-nlacZ transgene. Nuclei of ganglion cells nuclei that appear blue after X-gal staining are absent in the terminal large intestine of s/l/s l animals, but are present throughout the length of the entire intestinal tract in s/l/+ heterozygotes.
of neurons or their precursors which expressed the \(\text{D}\beta\text{H-nlacZ}\) transgene. As expected, histochemical reactivity in the guts of postnatal \(s^l/s^l\), \(s^l/+\) and \(+/+\) mice correlated with the distribution of ganglion cells associated with each genotype. Ganglion cells with blue nuclear staining were observed throughout the gastrointestinal tracts of \(s^l/+\) and \(+/+\) mice, but not in the distal colons of \(s^l/s^l\) animals (Fig. 1). The distal 3 mm of rectum was always devoid of ganglion cells, but the proximal extent of aganglionosis varied. At most, the terminal 25 mm of large intestine lacked ganglion cells. The aganglionic segment was invariably preceded by a hypoganglionic region (transitional zone), which ranged between 5 and 15 mm in length.

### Defective colonization of the gut by vagal neural crest cells in \(s^l/s^l\) embryos

In \(s^l/s^l\), \(s^l/+\) and \(+/+\) embryos, colonization proceeded in a cranial-to-caudal manner beginning with the appearance of neuroblasts in the rostral foregut on day 9. At successively later stages, the distribution of neuroblasts progressed to encompass the entire esophageal, gastric and small intestinal portions of the gut at equivalent rates such that, by E11.5, neuroblasts had reached the ileocecal junction in all embryos of the three genotypes. However, a dramatic and reproducible difference in the colonization process was observed in some of the embryos from this developmental stage onward. Neuroblasts in \(s^l/+\) and \(+/+\) embryos (three-fourths of all embryos derived from \(s^l/+ \times s^l/+\) matings) continued a steady colonization of the large intestine at a pace identical to that described in the past for wild-type transgenic embryos (Kapur et al., 1992), such that the cecum was completely colonized by E12.5 and the entire large intestine by E13.5 (Fig. 2A). In contrast, neuroblasts in \(s^l/s^l\) embryos (50% of embryos derived from \(s^l/+ \times s^l/s^l\) and 100% of embryos from \(s^l/s^l \times s^l/s^l\) matings) transiently arrested cranial-to-caudal colonization at the ileocecal junction (Fig. 2B,C). The period of arrest at the ileocecal junction was characterized by spread of neuroblasts along the mesenteric border of the proximal large intestine without invasion of the cecum or proximal colon, analogous to that observed in \(ls/ls\) embryos (Coventry et al., 1994). Failure to colonize even the cecum in \(s^l/s^l\) embryos until after E12.5, when neuroblasts gradually appeared in the proximal colon. By E13.5, neuroblasts in \(s^l/s^l\) embryos were present in the proximal colon, but had not yet colonized the cecal tip or the distal large intestine (Fig. 2C). Eventually, the cecum was completely colonized. However, the distal colon was never colonized in \(s^l/s^l\) postnatal mice.

### Intestinal colonization in \(s^l/s^l (\text{D}\beta\text{H-nlacZ})\leftrightarrow+/+\) chimeric mice

To determine whether the \(s^l/s^l\) genotype caused an autonomous defect in neuroblasts, which rendered them incapable of colonizing the distal large intestine, aggregation chimeras were produced and analyzed. The mutant embryos which contributed to these chimeras also carried the \(\text{D}\beta\text{H-nlacZ}\) transgene, expression of which served as a marker for mutant neurons in the chimera. As a source of mutant embryos, it was necessary to use litters from \(s^l/+ \times s^l/s^l\) and \(s^l/+ \times s^l/+\) matings because so few \(s^l/s^l\) animals survived to reproduce. Wild-type embryos derived from the inbred strain C3HeB/FeJ were aggregated with embryos derived from the mutant crosses. The wild-type allele at the piebald locus in C3HeB/FeJ embryos was linked to the same 95 bp microsatellite polymorphism (D14Mit7) as linked to the \(s^l\) allele and either could be easily distinguished electrophoretically from the 107 base pair polymorphism linked to the \(+\) allele derived from an \(s^l/+\) parent (Fig. 3). Amplification of this polymorphic microsatellite DNA sequence, which is closely linked to the \(s^l\) locus, produced a sensitive assay for the \(+\) allele derived from either \(s^l/+\) parent and thereby allowed rapid identification of chimeras derived in part from a \(s^l/s^l\) embryo.

A total of 11 \(s^l/s^l\leftrightarrow+/+\) chimeras were produced (Table 1). The phenotypic manifestations varied greatly in severity and correlated roughly with the relative contribution of mutant cells to the chimeric animal. All exhibited the \(s^l/s^l\) phenotype to some degree. Five of the \(s^l/s^l\leftrightarrow+/+\) chimeras resembled non-chimeric \(s^l/s^l\) mice, in that their coats were virtually devoid of pigment except for small amounts of black around the ears and/or base of the tail and they lacked both mutant (lacZ-positive) and wild-type ganglion cells in their terminal colons (Fig. 4A). The proportion of mutant cells in various tissues in each of these animals was greater than 70%. The remaining six \(s^l/s^l\leftrightarrow+/+\) chimeras had black and agouti mosaic coats with one or two white spots devoid of melanoblasts (Fig. 4A-C). The sizes of the spots ranged from small patches in the mid-abdomen, legs, or dorsal snout to a broad abdominal belt which

### Table 1. Phenotypes of \(s^l/s^l\leftrightarrow\)wild-type chimeras

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Chimerism* ((s^l/s^l) as % of total)</th>
<th>Coat pigmentation(^b)</th>
<th>Enteric colonization(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-1</td>
<td>91-96(^a)</td>
<td>White with black near ears/tail</td>
<td>Aganglionosis coli</td>
</tr>
<tr>
<td>15-2</td>
<td>88-95(^a)</td>
<td>White with black near ears/tail</td>
<td>Aganglionosis coli</td>
</tr>
<tr>
<td>1-1</td>
<td>70-100(^a)</td>
<td>White with black near ears/tail</td>
<td>Aganglionosis coli</td>
</tr>
<tr>
<td>1-4</td>
<td>63-75(^a)</td>
<td>White with black near ears/tail</td>
<td>Aganglionosis coli</td>
</tr>
<tr>
<td>20-2</td>
<td>78-93(^a)</td>
<td>White with black near ears/tail</td>
<td>Aganglionosis coli</td>
</tr>
<tr>
<td>1-2</td>
<td>77-87(^a)</td>
<td>Black=agouti with ventral spot</td>
<td>Agangionosis coli</td>
</tr>
<tr>
<td>20-4</td>
<td>72-88(^a)</td>
<td>Black=agouti with 2 large spots</td>
<td>Agangionosis coli</td>
</tr>
<tr>
<td>1-3</td>
<td>58-66(^a)</td>
<td>Black=agouti with ventral spot</td>
<td>Agangionosis coli</td>
</tr>
<tr>
<td>20-3</td>
<td>48-61(^a)</td>
<td>Black=agouti with small spots</td>
<td>Hypoganglionic</td>
</tr>
<tr>
<td>15-3</td>
<td>0-56(^a)</td>
<td>Agouti=black with ventral spot</td>
<td>Normoganglionic</td>
</tr>
<tr>
<td>20-1</td>
<td>25-55(^a)</td>
<td>Agouti=black with white paw</td>
<td>Normoganglionic</td>
</tr>
</tbody>
</table>

\(^a\)Range of chimerism was calculated in 3 different tissues including gut either by dot blot or Southern blot analyses as described in Methods and is expressed as the relative number of \(s^l/s^l\) cells.

\(^b\)Spots refer to white areas devoid of melanoblasts.

\(^d\)A mixture of mutant ganglion cells, which expressed the transgene, and presumed wild-type ganglion cells, which did not express the transgene, were observed in every colonized region. “Aganglionosis” refers to absence of both mutant (x-gal positive) and wild-type (x-gal negative) neurons.
encompassed almost one-third of the abdomen and flanks of one mouse. Two of the animals with spots had aganglionosis of the terminal colon and one had a markedly hypoganglionic terminal colon. The other three animals had normal numbers of ganglion cells throughout the length of their intestinal tracts, including many mutant ganglion cells which expressed the DβH-macZ transgene in their distal large intestines. Neither white spots nor colonic aganglionosis were observed in sI/+ or +/+ mice.

DISCUSSION

ET-3/ET-B-mediated intercellular signals and colonization of the murine large intestine

The distributions of neuroblasts observed in sI/sI embryos were identical temporally and spatially to those described previously for ls/ls embryos (Kapur et al., 1992). As in ls/ls embryos, colonization of the small intestine proceeded normally but colonization of the entire length of the large intestine was impaired. These results concur with the observations of Webster (1973) who used acetylcholinesterase histochemistry to study the distribution of neuroblasts in sI/sI, sI/+ and +/+ embryos. Webster also reported normal colonization of sI/sI intestinal tracts until E12.5 with subsequent impaired colonization of the large intestine. However, he concluded that ‘neuroblasts migrate at a slower rate than normal’ in sI/sI embryos and did not discuss the fact that colonization was retarded exclusively throughout the length of the large intestine (Webster, 1973).

The mature large and small intestine differ in many respects including gross anatomy, microanatomy and gene expression (Bloom and Polak, 1978; Christensen, 1991; Dolle et al., 1991; Kikuta et al., 1991). Regional differentiation of these two segments of the alimentary tract is initiated prior to colonization by neuroblasts, as evidenced by gross anatomical differences (i.e. formation of the cecum), although microscopic distinctions between small and large intestinal wall are inconspicuous at this stage. Neither the cellular nor molecular events which underly pattern formation in the vertebrate intestinal tract are understood. Possibly, expression of similar genes to those involved in pattern determination elsewhere in the embryo are involved. For example, a variety of Hox genes are those involved in pattern determination elsewhere in the embryo and affect its competence as a substrate for neuroblast colonization and/or differentiation. Unfortunately the sites of ET-3 or ET-B expression in the embryonic intestinal tract have not been reported. Endothelin peptides have been detected immunohistochemically and chromatographically in the large intestine and other portions of pre- and human postnatal gut (Inagaki et al., 1991a,b; Escrig et al., 1992). ET-1 was the major endothelin peptide detected at each time point, but ET-3 was also present and was relatively greater than in other parts of the intestinal tract.

Alternatively, ET-3 and ET-B may play an essential role in neuroblast colonization only in the large intestine. The results of in vitro studies by Jacobs-Cohen et al. (1987) suggest that defects in ET-3/ET-B-mediated signals function at least partially in the non-neuroblastic mesenchyme of the hindgut. They showed that primary cultures of hindgut from ls/ls embryos would not support colonization and differentiation by wild-type or mutant neuroblasts, in contrast with similar cultures of wild-type hindgut which were conducive to neuroblasts of either genotype. Based on these results, ET-3, ET-B or both must be expressed by the non-neuroblastic mesenchyme of the embryonic hindgut and affect its competence as a substrate for neuroblast colonization and/or differentiation. Unfortunately the sites of ET-3 or ET-B expression in the embryonic intestinal tract have not been reported. Endothelin peptides have been detected immunohistochemically and chromatographically in the large intestine and other portions of pre- and human postnatal gut (Inagaki et al., 1991a,b; Escrig et al., 1992). ET-1 was the major endothelin peptide detected at each time point, but ET-3 was also present and was relatively increased in intestinal tissue from a 12-week fetus (colonization of the large intestine is completed by 10-12 weeks in human embryos (Okamoto and Ueda, 1967)). Immunohistochemical studies designed to analyze the distribution of ET-3 in the gut have not been reported, but ET-1-immunoreactivity was observed in enteric ganglion cells pre- and postnatally. ET-A and ET-B receptors have also been observed in postnatal ganglion cells (Inagaki et al., 1991a,b). The patterns of ET-3 and ET-B mRNA expression observed in murine embryos by in situ hybridization also suggest expression of both in enteric neuroblasts (M. Yanagisawa, personal communication). These indirect data suggest that ET-3 and ET-B may both be expressed by enteric ganglion cell precursors such that autocrine or paracrine signalling between neural crest cells may regulate their behavior in the embryonic gut. However, ET-B and ET-A receptor activities have also been detected in enteric smooth muscle cells from postnatal guinea pig cecum which are derived from non-neural crest mesenchymal cells in the embryonic gut wall (Okabe et al., 1995). Clearly, more data...
are needed regarding the specific cell types which produce various endothelin peptides in the embryonic gut.

The \( s^l \) mutation does not act in a neuroblast autonomous manner

The observed phenotypes of \( s^l/s^l \rightarrow \) wild-type chimeras in this study were unexpected, based on the knowledge that the \( s^l \) mutation is a deletion of the ET-B receptor which probably is expressed on enteric neurons and presumably their neural crest precursors. Hosoda et al. (1994) proposed that lack of the ET-B receptor in \( s^l/s^l \) neural crest cells causes neural crest cell autonomous defects, which should be characterized in chimeric embryos by failure of \( s^l/s^l \) neural crest cells to colonize the skin or terminal gut independent of the influence of surrounding non-mutant cells. Similarly, the presence of \( s^l/s^l \) cells would not be expected to influence migration of wild-type neural crest cells, if lack of ET-B exerted a truly cell autonomous effect. To the contrary, the distribution of mutant versus non-mutant neurons in our chimeric mice indicates that intercellular signals ‘downstream’ of ET-B activation influence neural crest cell colonization and that absence of ET-B has non-cell autonomous effects on these two neural crest populations.

The importance of intercellular signals that operate distal to ET-B activation is evident from the fact that the presence of wild-type cells ‘rescued’ mutant neural crest cell migration in some chimeric embryos and vice-versa. Hence, some chimeras showed complete colonization of their alimentary tracts by ganglion cells of both genotypes. In the latter group were mice with large numbers of mutant ganglion cells that reached the distal large intestine which is invariably aganglionic in non-chimeric \( s^l/s^l \) mice. In other chimeras, the gut was incompletely colonized. In these animals, wild-type ganglion cells failed to populate the rectum, a finding never observed in non-chimeric mice of the wild-type strain. The inhibitory effect of \( s^l/s^l \) cells on colonization by wild-type cells is presumably due to lack of the ET-B receptor in the mutant cells and must be mediated directly or indirectly by intercellular signals from these cells. This concept is reinforced by the ability of wild-type cells to rescue \( s^l/s^l \) neural crest cells indicating that lack of ET-B in neural crest cells can be overcome by signals from wild-type cells that express ET-B.

The mixture of black and agouti coat pigmentation in many of our chimeras is a good indication of chimerism since the \( s^l/s^l \) genetic background is non-agouti (black fur) and the wild-type C3HeB/FeJ mice are agouti. However, it gives no indication of the relative distribution of \( s^l/s^l \) and wild-type melanocytes, since the agouti allele acts within follicular epithelial cells to influence melanoblast differentiation in a non-autonomous manner (Silvers, 1979). In any case, the presence of white spots in our chimeric mice indicates areas that were not colonized by wild-type or mutant melanoblasts, analogous to the terminal colon of aganglionic chimeric animals.

A simple model to account for these results is based on the concept that neuroblast behavior is regulated indirectly by environmental cues that are affected by ET-B activation (Fig. 5). According to this model, autocrine or paracrine stimulation of cells that express ET-B (by ET-3) initiate downstream events that alter the extracellular environment encountered by enteric neuroblasts and thereby modulate their behavior. The

Fig. 2. Impaired colonization of the large intestine in \( s^l/s^l \) embryos. Neuroblast colonization extends into the mid-colon in a \( s^l/+ \) embryo on E12.0 (A), but is arrested at the ileocecal junction in a \( s^l/s^l \) littermate (B). At E13, when the entire length of the large intestine has been colonized in \( s^l/+ \) embryos (not shown), neuroblasts have only colonized a portion of the proximal large intestine of \( s^l/s^l \) (C) embryos. Colonization of the large intestine is preceded by spread of neuroblasts along the mesenteric border (arrow in C).

Fig. 3. Use of microsatellite polymorphisms to establish genotypes of chimeric mice. The \( s^l \) locus (ET-B gene) is closely linked to an 107 bp the D14Mit7 microsatellite polymorphism (Metallinos, 1994). In our colony, the polymorphic sequence linked to the \( s^l \) gene measured 95 bp which is the same length as the inbred strain C3HeB/FeJ, but differs from the 107 bp polymorphism found in the inbred strain C57Bl/6J. By crossing \( s^l/s^l \) mice with inbred C57Bl/6J mice, \( s^l/+ \) mice were obtained in which the \( s^l \) and + alleles were linked with the 95 and 107 bp polymorphisms, respectively. Crosses between such heterozygotes were used as donors for embryos to aggregate with C3HeB/FeJ embryos. Any \( s^l/s^l \rightarrow \) wild-type embryos that resulted could be distinguished from \( s^l/+ \rightarrow \) wild-type or \(+/+ \rightarrow \) wild-type chimeras by PCR analysis, since only \( s^l/s^l \rightarrow \) wild-type mice would lack the 107 bp polymorphism.
intercellular signal(s) provided by ET-B-bearing cells in response to ET-3 might prevent differentiation of neural crest cells and/or stimulate their proliferation, migration and/or survival.

The notion that the \( s^l \) mutation might not act in a strictly cell autonomous manner (i.e. independent of environmental factors) within neural crest cells was proposed previously by Mayer (1977). He suggested that defects in both neural crest cells and surrounding mesenchyme underly the phenotype of \( s/s \) (\( s \) is allelic to \( s^l \), but causes only spotting). He compared the abilities of neural crest cells from wild-type and \( s/s \) neural tube to colonize skin from wild-type or \( s/s \) embryos when cocultured in the coelom of embryonic chicks. Although \( s/s \) melanoblasts were less efficient than wild-type cells at colonizing hair follicles in skin from E11.0 embryos, this impairment could be eliminated by using skin from older wild-type embryos as a template. He offered the possibility that \( s^l \) acts in both the neural crest and skin as an explanation for his data. Our data are consistent with this hypothesis, if both neural crest cells and non-neural crest cells in the gut and skin function as ET-B-responsive as described below. Neuroblasts or adjacent non-neural crest cells in the embryonic gut may be the source and/or target of ET-3, as insufficient data exist at present to assign specific cell types.

According to our model, failed neuroblast/melanoblast col-

---

**Fig. 4.** Phenotypes of \( s^l/s^l \rightarrow \) wild-type chimeras. Gross photographs (A,E,I) of chimeric mice and X-gal-stained segments of their small (B,F,J) and large (C,G,K) intestines are shown along with microscopic sections (D,H,L) of their terminal colons. The chimeras included mice with almost complete coat pigmentation and full-length intestinal colonization by ganglion cells (A-D). In these animals, ganglia in the terminal colon contained a mixture of \( s^l/s^l \) neurons which expressed the \( DBH\text{-}\text{lacZ} \) transgene (short arrow in D) and non-transgenic neurons (long arrow in D). Other chimeras evidenced the \( s/s \) phenotype with almost completely white coats and colonic aganglionosis (E-H) or an intermediate phenotype characterized by relatively large white spots and colonic aganglionosis. Chimeric mice with aganglionic terminal large intestines lacked both mutant and wild-type ganglion cells in the affected gut (H,L).
signals affect laminin production in the gut. However, in diverse cell types, production of a variety of extracellular matrix components, including laminin, is influenced by a variety of intercellular signalling pathways, including endothelin stimulation (Vasios et al., 1989; Maheshwari et al., 1991; Huang and Chakrabarty, 1994; Ruiz-Ortega et al., 1994).

Modulation of signals mediated by the receptor tyrosine kinase, ret, is another potential mechanism through which ET-3-responsive cells might affect colonization of the gut by enteric neuroblasts. The ret receptor is expressed by enteric neural crest cells from the earliest stages of colonization (Pachnis et al., 1993) and inactivating mutations of the ret gene cause enteric aganglionosis in mice and humans (Edery et al., 1994; Romeo et al., 1994; Schuchardt et al., 1994). Mice homozygous for such mutations lack ganglion cells throughout their gastrointestinal tracts, suggesting that ret plays a critical role early in enteric colonization. However, humans heterozygous for ret mutations have partial or complete colonic aganglionosis, indicating that altered ret expression/activity may cause an enteric phenotype analogous to the ls/ls and s/s mutations. The ligand for ret is an obvious candidate for a secondary signal produced by ET-3-responsive cells.

The authors would like to thank Dr Masashi Yanigisawa for sharing the ET-B cDNA and his unpublished in situ hybridization data. This work was supported by NIH DK46277-02 (RKP) and the Howard Hughes Medical Institute (D. A. S. and R. D. P.)

REFERENCES


Fig. 5. Model to explain phenotypes of aggregation chimeras. The results of these studies suggest that paracrine communication between enteric neuroblasts and surrounding cells in the developing gut influence the ability of neural crest cells to completely colonize the large intestine. ET-3, which is probably produced by enteric neural crest cells (M. Yanasigawa, personal communication), binds to endothelin receptors on the same cell (autocrine loop) or its neighbors. Activation of ET-B leads to production of a secondary intercellular signal which facilitates complete colonization of the gut by wild-type or s/s (ET-B null) neuroblasts. At present, it is not clear whether the ET-B is expressed on neuroblasts, mesenchymal cells, or both.


(Accepted 6 August 1995)