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Aggregation chimeras demonstrate that the primary defect responsible for aganglionic megacolon in *lethal spotted* mice is not neuroblast autonomous

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SUMMARY

The *lethal spotted* (*ls*) mouse has been used as a model for the human disorder Hirschsprung's disease, because as in the latter condition, *ls/ls* homozygotes are born without ganglion cells in their terminal colons and, without surgical intervention, die early as a consequence of intestinal obstruction. Previous studies have led to the conclusion that hereditary aganglionosis in *ls/ls* mice occurs because neural crest-derived enteric neuroblasts fail to colonize the distal large intestine during embryogenesis, perhaps due to a primary defect in non-neuroblastic mesenchyme rather than migrating neuroblasts themselves. In this investigation, the latter issue was addressed directly, in vivo, by comparing the distributions of *ls/ls* and wild-type neurons in aggregation chimeras. Expression of a transgene, *DβH-nlacZ*, in enteric neurons derived from the vagal neural crest, was used as a marker for *ls/ls* enteric neurons in chimeric mice. In these animals, when greater than 20% of the cells were wild-type, the *ls/ls* phenotype was rescued; such mice were neither spotted nor aganglionic. In addition, these 'rescued' mice had mixtures of *ls/ls* and wild-type neurons throughout their gastrointestinal systems including distal rectum. In contrast, mice with smaller relative numbers of wild-type cells exhibited the classic *ls/ls* phenotype. The aganglionic terminal bowel of the latter mice contained neither *ls/ls* nor wild-type neurons. These results confirm that the primary defect in *ls/ls* embryos is not autonomous to enteric neuroblasts, but instead exists in the non-neuroblastic mesenchyme of the large intestine.

Key words: cell migration, piebaldism, neural crest, congenital defect, *lethal spotted* mouse, Hirschsprung's disease

INTRODUCTION

Hirschsprung's disease (HD), or congenital colonic aganglionosis, is a birth defect that affects an estimated 1 in 5000 liveborn humans (Passarge, 1973). HD is characterized by absent myenteric and submucosal ganglia in the distal gastrointestinal tract with consequent symptoms that range from chronic constipation to life threatening obstruction and megacolon (Meier-Ruge, 1974; Cass, 1986). The *lethal spotted (ls/ls)* mouse has been used as a model for HD since mice homozygous for the *ls* allele lack ganglion cells in their terminal bowel and exhibit similar pathological and clinical characteristics to humans with HD (Lane, 1966; Bolande, 1975).

Enteric neurons are derived from neural crest cells that populate the murine gut on embryonic day 9.5 (Rothman and Gershon, 1984). Enteric neural crest (ENC) cells originate from vagal and sacral segments of the neural tube (Le Douarin and Teillet, 1973; Cochard and Le Douarin, 1982; Pomeranz et al., 1991; Pomeranz and Gershon, 1990; Serbedzija et al., 1991). Vagal ENC cells proliferate and migrate caudally in the gut wall to give rise to neurons along its entire length. Prior to their overt morphological differention into neurons, mammalian vagal ENC cells coexpress various neuronal markers incuding neurofilament peptides and catecholaminergic markers such as the biosynthetic enzymes, tyrosine hydroxylase and dopamine-β-hydroxylase (DβH; Cochard et al., 1978; Gershon et al., 1984; Baetge and Gershon, 1988; Baetge et al., 1990a,b). The human DβH promoter has been used to direct expression of the reporter gene, *nlacZ*, to vagal ENC-derived neuroblasts and neurons, in order to study the distribution of these cells in transgenic mice at different stages of development (Mercer et al., 1991; Kapur et al., 1991, 1992).

HD is generally assumed to be due to failure of enteric neuroblasts to colonize the distal colon during embryogenesis. This hypothesis has been supported by studies of *ls/ls* embryos. In contrast to wild-type or *ls/+* hindgut, the ter-

minal hindgut from an *ls/ls* embryo never contains cells with neurogenic potential (Rothman and Gershon, 1984; Nishijima et al., 1990). Similarly, use of *DβH-nlacZ* transgene expression to visualize enteric neuroblasts has shown that the cranial-to-caudal progression of enteric neuroblasts in *ls/ls* embryos never reaches the distal colon (Kapur et al., 1992). In the latter embryos, retarded neuroblast colonization is first evident at the junction of small and large intestine, suggesting that the underlying defect in *ls/ls* mice is pancolonic.

A fundamental question regarding the pathogenesis of HD is whether failed colonization is due to intrinsic defects in migrating neuroblasts (neuroblast autonomous) or extrinsic defects in the hindgut mesenchyme. In vitro studies suggest that, in *ls/ls* embryos, the latter is the case (Jacobs-Cohen et al., 1987). Neurons failed to develop in segments of *ls/ls* hindgut that were cocultured with sources of wild-type murine or chick ENC. However, neuroblasts derived from *ls/ls* foregut emigrated into cocultured chick hindgut and exhibited neuronal differentiation. Although the latter studies suggest that the mesenchyme of *ls/ls* hindgut may not permit neuroblast colonization, it is not clear how reliably in vitro colonization of explanted murine or chick hindgut recapitulates events which occur in vivo.

Analysis of aggregation chimeras is a traditional method for distinguishing cell autonomous from environmental defects in mutant mice (Rossant, 1990). Such experiments require some type of marker, which distinguishes mutant from non-mutant cells in chimeric tissues of interest. In this study, expression of the *DβH-nlacZ* transgene was used to identify enteric neurons that where derived from *ls/ls* mice in *ls/ls* ↔ wild-type chimeras. In support of the in vitro studies described above, we found that, in chimeric mice composed of greater than 20% wild-type cells, *ls/ls* (transgenic) neuroblasts colonize the terminal colon equally as well as wild-type (non-transgenic) neuroblasts.

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 *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. The pattern of transgene expression in these mutant animals has been described (Kapur et al., 1992). Wild-type C57BL/6J 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 light:12 hour dark regimen.

Preparation of aggregation chimeras

4-6 week virgin C57BL/6J and transgenic *ls/ls* 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 and human chorionic gonadotropin (5 IU, immediately prior to mating, Sigma, St. Louis, MO). The female mice were mated overnight with genetically identical males and inspected for vaginal plugs the next morning, embryonic day (E) 0.5. Pregnant mice were killed on E2.5 and pre-compaction 8-cell embryos were flushed from their oviducts into M2 medium as described by Hogan et al. (1986). Zonae pellucidae were removed by 5-10 minutes treatment with pronase (0.5% in M2**,** Sigma, St. Louis, MO), the embryos rinsed briefly in M2, and then incubated as *ls/ls* ↔ wild-type pairs in drops of M2 supplemented with 5 μl/ml phytohemagglutinin (10 minutes, 37°C, Difco, Detroit, MI). The pairs were transferred to M16 medium in individual wells of a microtiter plate and cultured at 37°C in an atmosphere of 5% CO_2. After 48 hours, the chimeric embryos had developed into expanded blastocysts and they were transferred to the uteri of E2.5 pseudopregnant Swiss-Webster female mice as described by Hogan et al. (1986).

Examination of transgene expression at different stages of development

At varying stages after birth, chimeric mice were sacrificed and their coats carefully examined for areas devoid of pigmentation. The internal 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.l 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 $FeCN_2$. Stained gastrointestinal tracts were post-fixed for 24 hours in 10% formalin and examined grossly and photographed using 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 (Kapur et al., 1991).

Quantitative DNA analysis of chimeric tissues for tissue mosaicism

Pieces of brain, skin, intestine, lung, and kidney were obtained from each chimeric mouse and solubilized overnight at 37°C in 1× SET buffer (1% SDS, 10 mM Tris-HCl, 5 mM EDTA, pH 8) containing 20 μg/ml proteinase K. NaCl and KCl were added to 1.4 M and 70 mM, respectively, and the sample was centrifuged at 1500 ***g*** for 5 minutes. A 400 μl aliquot of the supernatant was mixed with 800 μl ethanol in a 1.5 ml Eppendorf tube and the precipitate was dissolved in 12 μl of 2 M NaCl and 0.1 M NaOH by boiling for 1 minute. Aliquots (5 μl) were spotted directly onto duplicate sheets of BA 85 nitrocellulose (Schleicher and Schuell). The nitrocellulose sheets were rinsed in 2× SCC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), baked for 2 hours at 80°C, and then prehybridized and hybridized with nick-translated probes as described (Palmiter et al., 1982). One sheet was hybridized with a probe made from the *lacZ* gene and the other was hybridized with a unique probe from the region between the murine *Hoxa-3* and *Hoxa-4* genes. After washing the filters first in 2× SCC plus 0.5% SDS at 68°C and then in 0.5× SET at 68°C, the filters were exposed to X-ray film overnight and squares containing each dot were cut from the filters and counted in 2 ml Ecolume in a Packard scintillation counter. About 5000 counts/minute were obtained with the *Hox* probe; background values were about 70 counts/minute. The relative number of transgenic cells (%T) was calculated by the following formula

$$\%T = \frac{(\text{LacZ cpm}_s - \text{LacZ bck}) \div (\text{Hox cpm}_s - \text{Hox bck})}{(\text{LacZ cpm}_{ref} - \text{LacZ bck}) \div (\text{Hox cpm}_{ref} - \text{Hox bck})} \times 100$$

Table 1. Phenotypes of *ls/ls* ↔ wild-type chimeric mice

Mouse	Coat spots[+]	Age (wks)	Sex/weight (g)	Caudal extent of transgenic neurons	% of cells derived from *ls/ls* embryo*
S1	40-50%	2	F/4.7	5 mm from anus	82 (78-86)
S2	10-20%	2	M/6.7	3 mm from anus	92 (81-103)
NS1	none	3	M/11.2	To anus	84 (69-105)
NS2	none	2	F/n.d.	To anus	73 (57-91)
NS3	none	3	F/8.8	To anus	64 (53-80)
NS4	none	3	F/10.8	To anus	59 (43-72)
NS5	none	3	M/7.3	To anus	53 (42-65)
NS6	none	2	F/7.6	To anus	51 (35-74)
NS7	none	3	M/10.0	To anus	36 (27-45)
NS8	none	2	F/8.0	To anus	35 (29-44)
NS9	none	3	M/11.1	To anus	27 (16-38)

[+]Percentage of animal's coat that lacked pigmentation.
n.d., not done.
*Values given are mean percentages calculated from all organs, including intestine, followed by range of percentages observed in different organs from each animal.

where LacZ and Hox refer to the two probes, bck refers to background counts associated with a piece of nitrocellulose paper on which no DNA was dotted, and cpm_s and cpm_{ref} refer respectively to the counts per minute for a given sample and reference tissue from a non-chimeric mouse homozygous at the transgene locus. The reference tissue is 100% transgenic by definition.

RESULTS

External phenotype of chimeric mice

A total of 11 chimeric mice were produced (Table 1). Two of these mice resembled *ls/ls* mice in that they had non pigmented 'spots' (Fig. 1). The remaining nine mice had no spots and resembled wild-type or *ls/+* animals (Fig. 2). The amount of cutaneous depigmentation in the spotted chimeras varied as is the case with non-chimeric *ls/ls* mice. The spotted mice were smaller than the non-spotted chimeras and the former had colonic aganglionosis and incipient megacolon when they were killed at 2 weeks of age. All of the spotted animals had estimated transgenic compositions in excess of 80% and showed transgene expression in the proximal ganglionated portions of their intestinal tracts (Table 1). Ganglion cells, most of which expressed the transgene, were evident microscopically in all sections from the proximal colon (Fig. 1C). However, no ganglion cells could be identified histologically in any of 10 representative sections taken from their terminal colons (Fig. 1F).

The phenotypes of the non-spotted chimeric mice were similar to each other. All of these were robust animals with no evidence of colonic distension. Transgene expression was evident in a subset of ganglion cells through the length of the entire gastrointestinal tract in all of the mice. In these non-spotted chimeras, transgenic neurons derived from the *ls/ls* donor were present in the distal rectum which is normally aganglionic in *ls/ls* mice (Fig. 2). This phenotype was seen in all of the non-spotted mice despite the fact that the average percentage of trangenic cells in their tissues varied considerably (Table 1).

The fraction of transgenic cells was calculated as described in the Materials and Methods in order to estimate the relative contribution of transgenic (*ls/ls*) and non-transgenic (wild-type) cells to various organs in the chimeric embryos (Table 1). Different organs from the same animal generally had similar transgenic compositions, although some organs within a given mouse varied as much as two-fold in their relative compositions of transgenic cells. A general correlation existed between the percentage of transgenic (mutant) cells in a chimera and its phenotype, such that tissues from all of the spotted animals were composed of more than 80% transgenic cells on average, in contrast to non-spotted mice, the majority of which were composed of less than 80% transgenic cells. This correlation was not absolute since one non-spotted chimera did have some tissues with more than 80% transgenic cells, and yet had a normal phenotype.

Distribution of *ls/ls* and wild-type enteric neurons in chimeric mice

There were no differences between the distributions of *ls/ls* and non-mutant neurons in the chimeras. In both spotted and non-spotted mice, histological sections from ganglionated intestine contained a mixture of transgenic (X-gal positive) and non-transgenic (X-gal negative) neurons (Figs 1, 2). In those mice with higher percentages of transgenic cells as evidenced by DNA analysis, X-gal positive and negative ganglion cells were scattered rather uniformly in the myenteric and submucosal plexi of the gut wall. However, in some mice, particularly those with lesser transgenic cell compositions, patches devoid of transgenic enteric neurons were frequently evident in the myenteric plexus. Histologically, individual ganglia were composed solely of either X-gal positive neurons, X-gal negative neurons, or a mixture of the two. A similar mixture of transgenic and non-transgenic neurons was seen in sympathetic ganglia (Fig. 3C).

DISCUSSION

Lethal spotted mice are models for human Hirschsprung's disease because they lack ganglion cells in the terminal segments of their large intestines. In the animal model and the

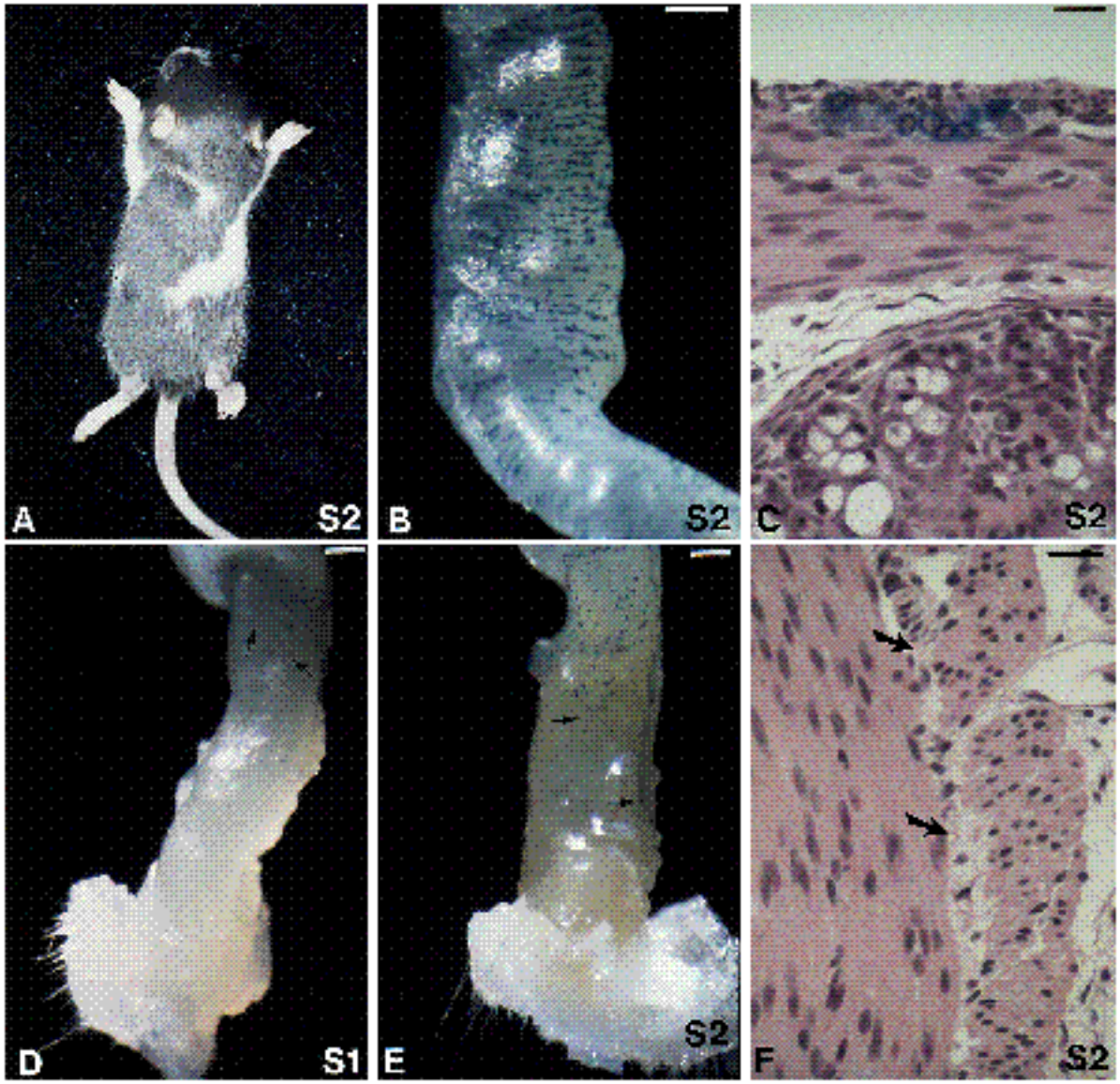

Fig. 1. Phenotype of chimeric mice with spotted coat pigmentation. (A) A photograph of a representative chimeric mouse demonstrates irregularly shaped zones of depigmentation due to regional absence of melanocytes. (B) X-gal staining shows transgene expression in ganglia in the midcolon (B,C) and proximal rectum (arrows in D,E) of spotted chimeras. However, the distal rectum of these mice showed no histochemical evidence of any transgene expression (D,E). (F) Histological sections of the distal colons from these mice reveal aganglionic myenteric plexi (arrows) identical to that evident in non-chimeric ls/ls mice. S1 and S2 refer to animal identifications given in Table 1. Bars (B,D,E) 0.5 mm; (C,F) 20 μm.

human disorder, colonic aganglionosis might be produced by either intrinsic defects in enteric neuroblasts, which impair their ability to colonize the large intestine, or extrinsic defects in colonic mesenchyme, which have a similar effect. In chimeric mice, colonization of the terminal hindgut by both *ls/ls* and wild-type neuroblasts occurred even in animals where mutant cells predominated. This result indicates that *ls/ls* neuroblasts are capable of colonizing the distal large intestine provided some wild-type cells are present. Apparently, only a relatively small contribution of wild-type cells is required to facilitate colonization of the distal hindgut by *ls/ls* neuroblasts, because even those mice with a 4:1 predominance of *ls/ls* cells showed a mixture of *ls/ls* and wild-type neurons throughout the gut, including the anorectal junction. Thus, these results indicate that when greater than 20% of the mesenchymal cells are wild-type, neuroblasts will colonize the terminal hindgut, although we cannot exclude the possibility that cooperative interactions between wild-type and mutant neuroblasts were involved. This interpretation, supports the conclusion of Jacobs-Cohen et al. (1987) which was based on in vitro data. Members of that same group have reported preliminary data from *ls/ls* ↔ wild-type aggregation chimeras which concur with the results reported here (Gershon and Tennyson, 1991).

The nature of the mesenchymal defect in *ls/ls* mice is unknown. Comparisons of neuroblast distributions in *ls/ls*, ls/+, and wild-type *DβH-nlacZ* embryos show that a defect

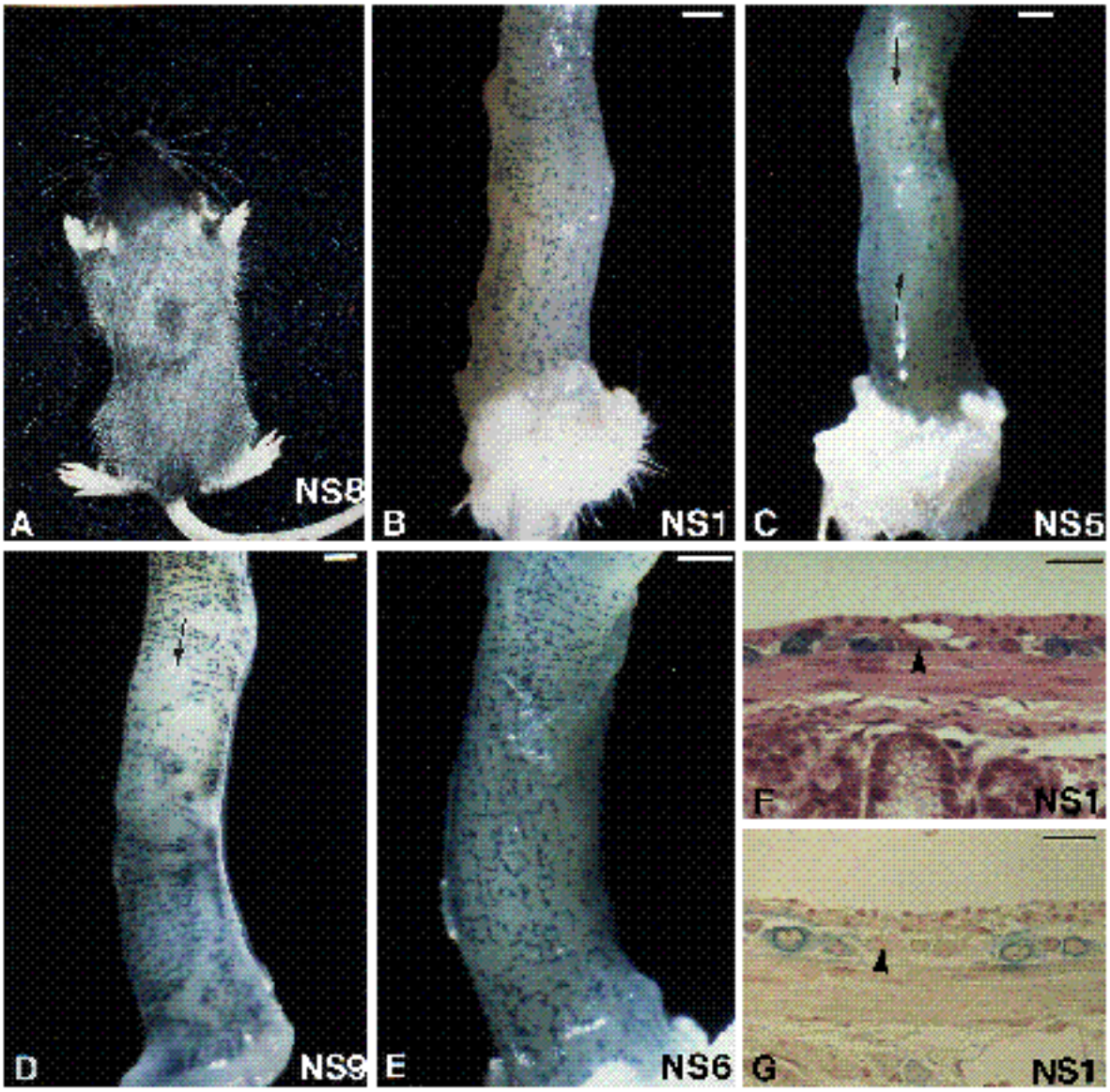

Fig. 2. Phenotype of chimeric mice with wild-type pattern of coat pigmentation. (A) A photograph of a representative chimeric mouse, which lacked cutaneous spots, is shown for comparison with Fig. 1A. In this group of chimeric animals, X-gal staining revealed ganglion cells throughout the entire length of the gastrointestinal tract, including terminal rectum (B-E). Patchy zones devoid of X-gal staining were present in some cases (arrows). (F,G) Histological sections demonstrate mixtures of *ls/ls* transgenic (X-gal positive) and wild-type non-transgenic (arrowheads, X-gal negative) neurons in enteric ganglia of chimeric mice. NS1,5,6,8,9 - refer to animal identifications given in Table 1. Bars: (B-E) 0.5 mm; (F,G) 20 μm.

is first evident when vagal enteric neuroblasts attempt to migrate from the small to large intestine. This finding, in conjunction with postnatal abnormalities identified in proximal 'euganglionic' colon from *ls/ls* mice (Payette et al., 1988) and humans (Kaplan and de Chaderevian, 1988), suggests that the underlying problem in some instances of aganglionosis may be pancolonic, rather than restricted to the terminal hindgut. In chick embryos, antigenic differences between small and large intestinal mesenchyme are evident prior to neuroblast colonization and disappear after neuroblasts colonize the latter (Luider et al., 1992). Others have shown pre- and postnatal excesses of laminin and type IV collagen exist in the extracellular matrix (ECM) of *ls/ls* hindgut (Tennyson et al., 1986; Payette et al., 1988); similar excesses have been noted in the colons of humans with HD (Parikh et al., 1992). ECM components, including laminin, have been shown to influence the migration and differentiation of neural precursors in a variety of systems (reviewed by Hynes and Lander, 1992). Alterations in ECM are, therefore, an attractive mechanism by which mesenchymal defects might impair neuroblast colonization.

Several other observations are consistent with the concept that primary disorders in the non-neuroblastic mesenchyme may lead to HD. *Hoxa-4* is expressed in mesenchyme of the gastrointestinal tract during the period when colonization by neuroblasts occurs (Galliot et al., 1989). Over expression of this gene causes lethal megacolon and hypoganglionosis of the terminal colon (Woglemuth et al.,

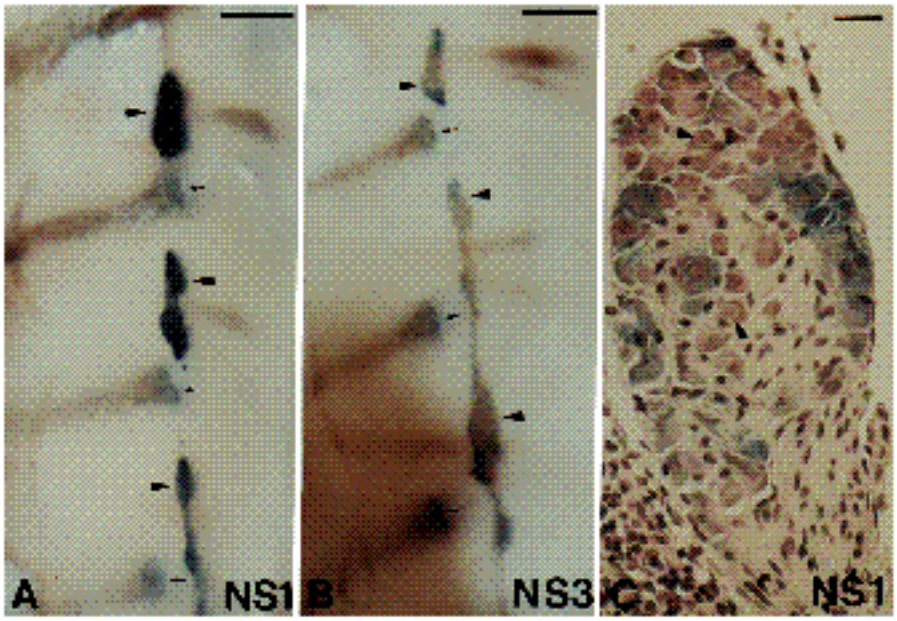

Fig. 3. Wild type and *ls/ls* chimerism in sympathetic ganglia. (A,B) Different intensities of X-gal staining in paravertebral ganglia (arrowheads) indicate expression of the transgene in a variable percentage of neurons. Endogenous galactosidase activity in osteoclasts of growing bones accounts for the background staining present in the ribs (small arrows). (C) A histological section showing a mixture of *ls/ls* transgenic (X-gal positive) and wild-type non-transgenic (arrowheads, X-gal negative) neurons in a prevertebral ganglion. Bars (A,B) 0.5 mm; (C) 20 μm.

1989). Human piebald trait, a hereditary disorder characterized by patchy absence of cutaneous melanocytes and a high incidence of colonic aganglionosis, has recently been attributed to mutations in c-*kit*, a receptor tyrosine kinase (Fleischman et al., 1991; Spritz et al., 1991). In mice, c-*kit* is a receptor for stem cell factor (SCF) and in situ hybridization studies of the expression patterns suggest that the receptor and its ligand are expressed in the enteric neuroblasts and adjacent mesenchyme respectively (Motro et al., 1991; Keshet et al., 1991). Although enteric neural disorders have not been reported in mice with mutations at these loci, the association of HD with human piebald trait and the expression patterns noted above suggest that growth factors produced by non-neuroblastic enteric mesenchyme may affect enteric neurodevelopment.

Many, but not all, individual enteric ganglia contained both *ls/ls* and wild-type neurons. Therefore, the neurons of each enteric ganglion are not derived from a single progenitor neuroblast. Similarly, prevertebral and paravertebral sympathetic ganglia in these mice contained a mixture of transgenic and non-transgenic neurons as evidence of their polyclonal origin. These observations are consistent with data from avian chimeras (Le Douarin and Teillet, 1973).

This study indicates the potential value of using transgenic mice with *lacZ*, or other reporter gene constructs, to address specific questions using aggregation chimeras. Aggregation chimeras provide a powerful technique with which issues of cell autonomy can be addressed in vivo. A potential difficulty in designing experiments that involve murine chimeras is the limited availability of appropriate markers to distinguish cells derived from each donor. The *DβH-nlacZ* transgenic model that we have developed targets expression of a sensitive histochemical marker to the nuclear membrane of only those cells that express the transgene. Other groups have used expression of different transgenes for a similar purposes (Katoh et al., 1988; Tam and Tan, 1992). In this case, *DβH-nlacZ* expression in enteric neurons was particularly important, and it provided an unambiguous marker for those ganglion cells derived from the transgenic donor. In some cases, it is possible to take advantage of differences in enzyme activity, repetitive DNA sequences, or antigens expressed in cells of various murine strains to design experiments with aggregation chimeras (Rossant, 1990; Gershon and Tennyson, 1991). Interpretation of histochemical assays based on strain differences indicating varying enzyme activity can be difficult since relative, rather than absolute, staining is used to distinguish cells derived from each donor embryo. Another potential problem with 'interstrain' chimeras is that significant differences in the genetic backgrounds of the two strains, aside from a locus of interest, may affect the phenotypic outcome. In our experiments, a common background was sought such that the genetic differences between the two donor embryos would be minimized to the transgene and *ls* loci.

The combination of this study with the in vitro coculture and immunocytochemical data discussed above provides compelling evidence that a primary defect in non-neuroblastic colonic mesenchyme is responsible for aganglionosis coli in the *lethal spotted* model for Hirschsprung's disease.

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