Increased Expression of Laminin-1 and Collagen (IV) Subunits in the Aganglionic Bowel of ls/ls, but Not c-ret --/-- Mice

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Extracellular matrix molecules, including laminin, affect the development of enteric neurons and accumulate in the aganglionic colon of ls/ls mice. Quantitative Northern analysis revealed that mRNAs encoding the β1 and γ1 subunits of laminin and collagens α1(IV) and α2(IV) are increased in the colons of ls/ls mice. Transcripts of laminin α1 were evaluated quantitatively with reverse transcription and the competitive polymerase chain reaction (RT-cPCR). The abundance of laminin α1 transcripts was developmentally regulated, but greater in the ls/ls than the wild-type colon at each age examined. In situ hybridization revealed that transcripts in the colon encoding laminin α1 and β1 and collagen α2(IV) were initially expressed in the endoderm, but by E15, expression shifted to cells of the colonic mesenchyme (ls/ls -> wild type) where crest-derived cells migrate. The expression of laminin α1 was examined in the totally aganglionic intestine of E15 and newborn c-ret --/-- mice, to determine whether an increase occurs when neurogenesis fails independently of the ls/ls defect. RT-cPCR revealed no difference from control in mRNA encoding laminin α1 in the c-ret --/-- colon in either E15 or newborn animals. The accumulation of immunohistochemically demonstrable laminin that is prominent in the newborn ls/ls colon could not be detected in that of c-ret --/-- animals. These observations suggest that transcripts encoding laminin-1 and collagen (IV) are increased in the colon and surrounding pelvic mesenchyme of ls/ls mice because of an intrinsic lesion, rather than a secondary consequence of aganglionosis. The data are compatible with the hypothesis that the increased expression of laminin-1 contributes to the failure of crest-derived cells to complete their colonization of the ls/ls colon.

INTRODUCTION

The neural crest is remarkable both for the phenotypic diversity of the terminally differentiated cells to which it gives rise and for the extensive distribution of its derivatives in the vertebrate body (Le Douarin, 1982, 1986). Considerable evidence suggests that the cells of the premigratory crest itself are committed either to a final phenotype (Baroffio et al., 1988; Duff et al., 1991; Ito et al., 1993; Le Douarin and Dupin, 1993; Sieber-Blum and Cohen, 1980; Sieber-Blum et al., 1993) or to a particular destination in the embryo (Bronner-Fraser and Fraser, 1988; Fraser and Bronner-Fraser, 1991). As a result, a variety of microenvironmental factors are important in determining the fate of cells from the neural crest as well as in defining the pathways along which they migrate (Le Douarin and Dupin, 1993).

One of the most complex neuronal derivatives of the neural crest is the enteric nervous system (ENS). This system, which is extraordinarily large, has as many neurons as the spinal cord (Furness and Costa, 1987; Gershon et al., 1994). It also contains a greater variety of neurons than any other peripheral ganglion and, unlike the ganglia of other organs, is able to mediate reflex activity in the absence of input from the central nervous system (CNS). In order to understand why the intrinsic innervation of the gut is different from that of any other organ it is necessary to know which molecules interact with the crest-derived precursors of the ENS to direct their migration to the bowel, to cause them to stop at appropriate sites, and to differentiate as enteric neurons and glia.

Insight into the factors that influence development can be gained from studies of mutant animals in which develop-
ment is abnormal. Intrinsic ganglia of the ENS fail to de-
velop in the terminal segment of hindgut in lethal spotted
(\textit{ls}/\textit{ls}), piebald-lethal (\textit{s}/\textit{s}) (Lane, 1966), and Dominant me-
colon (\textit{Dom} +/−) mutant mice (Kapur et al., 1996). These
murine conditions resemble Hirschsprung's disease of hu-
mans, in which the terminal colon is also agangionic (Bo-
lande, 1975). The absence of intrinsic enteric reflex activity in
the agangionic murine and human bowel causes the colon to dilate massively (megacolon) proximal to the le-

The development of aganglionosis in both mice and
humans has been associated with abnormalities of genes
encoding the endothelin-\textit{B} receptor (EDN3/R) or one of its
ligands, endothelin-\textit{B} (EDN3). Missense mutations in ednrb
(Hosoda et al., 1994) and EDNRB (Puffenberger et al., 1994),
which encode EDN3/R, occur, respectively, in \textit{s}/\textit{s} mice and
humans with Hirschprung's disease. In \textit{ls}/\textit{ls} mice, the edn3
gene is mutated so as to prevent the biosynthesis of endo-
thelin-\textit{B} (Baynash et al., 1994); therefore, although neither
the cells in the developing bowel that express EDN3 nor
those which express EDN3/R have been identified, both
molecules appear to play critical roles in the development of
the most distal region of the ENS. Still to be resolved,
however, is how the development of enteric neurons is af-
fected by the EDNRB and why its absence disrupts enteric
neurogenesis only in the colon.

A great deal of evidence indicates that aganglionosis oc-
curs in the \textit{ls}/\textit{ls} mouse because otherwise normal crest-
derived cells do not enter an intrinsically abnormal pre-
sumptive aganglionic gut. This evidence includes the fail-
ure of crest-derived cells to enter the terminal bowel of
\textit{ls}/\textit{ls} fetal mice, either in coculture experiments (Coulter
et al., 1988; Jacobs-Cohen et al., 1987) or after the backtrans-
plantation of the presumptive aganglionic gut to a neural
crest migration pathway in vivo (Rothman et al., 1993a,b).
In \textit{ls}/\textit{ls} mice carrying a transgene that enables vagal crest-
derived cells to be identified, the proximodistal progression of
nerve precursors becomes abnormal as soon as these
cells enter the developing colon and they stop migrating
short of the terminal bowel (Coventry et al., 1994; Kapur
et al., 1992). In addition, genomically \textit{ls}/\textit{ls} crest-derived

cells are able to colonize the terminal colon of aggregation
chimeric \textit{ls}/\textit{ls} ↔ wild-type mice and give rise to normal
enteric neurons (Kapur et al., 1993; Rothman et al., 1993a).

The evident inability of crest-derived cells to colonize the
presumptive aganglionic \textit{ls}/\textit{ls} gut could potentially be
explained by a localized abnormality of the extracellular
matrix of the fetal colon. In adult \textit{ls}/\textit{ls} mice, colonic smooth
muscle cells in the aganglionic zone are hypertrophic and
their surrounding basal laminae are reduplicated (Tennyson
et al., 1986). In the fetal \textit{ls}/\textit{ls} colon, furthermore, there is
an abnormal accumulation of several of the molecules that
are normally found in basal laminae, such as laminin, type
IV collagen, and glycosaminoglycans (Payette et al., 1988).

The normal pattern of chondroitin sulfate proteoglycan in the
mesenchyme is also disrupted (Tennyson et al., 1990).

These abnormalities are apparent at the time when crest-
derived cells should be migrating into the colon and they
are located in what should be the path of incoming crest-
derived cells. It has therefore been proposed that smooth
muscle cells and their precursors are abnormal in the devel-
oping colon of \textit{ls}/\textit{ls} mice and that the excess of components
of basal laminae, which these cells secrete, may be causally
related to the development of aganglionosis (Gershon et al.,
1993b; Payette et al., 1988). Because the EDNRB is ex-
pressed in the bowel by smooth muscle cells (Okabe et al.,
1995; Yoshinaga et al., 1992), it is possible that the malfunc-
tion of the EDN3/EDNRB system could cause an abnormal
secretion of components of basal laminae by smooth muscle
cells and their precursors in the developing \textit{ls}/\textit{ls} colon. Such
a defect could indirectly affect the colonization of the a-

The current experiments were undertaken to determine
whether the abnormal accumulation of laminin and type
IV collagen is that which occurs in the \textit{ls}/\textit{ls} terminal gut could be
due to an increase in transcripts encoding these proteins in
the \textit{ls}/\textit{ls} colon and, if so, to identify the cells in which this
abnormality occurs. Measurements were made of mRNA
encoding the \(α1\), \(β1\), and \(γ1\) chains (formerly \(Ae\), \(B1e\), and
\(B2e\)) (Burgeson et al., 1994) of laminin-1 and the \(α1\) and \(α2\)
chains of type IV collagen \([\alpha1(IV)\) and \(α2(IV)\)] in control
\textit{ls}/\textit{ls} mice. We also sought to determine whether the in-
crease in the concentration of laminin and type IV colla-
gen that occurs in the \textit{ls}/\textit{ls} colon is a primary defect or a
secondary result of aganglionosis. For this purpose, we
analyzed the expression of laminin in the bowel of fetal and
newborn mice homozygous for a targeted mutation in the
c-\textit{ret} gene, in which neuronal development fails because of
a mutation in a gene that is expressed by crest-derived cells
and evidently essential for their development below the
level of the esophagus and stomach (Durbec et al., 1996;
Pachnis et al., 1993; Schuchardt et al., 1994). Our data sug-

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colon and small intestine were either placed in a solution of 6 M guanidinium thiocyanate for extraction of RNA or fixed for 4 hr with 4% formaldehyde (from paraformaldehyde; buffered with PBS to pH 7.0) for in situ hybridization and/or immunocytochemistry. Fixed tissues were washed with PBS for 3 hr and cryoprotected by incubation in 30% sucrose for 24–48 hr. Tissues were embedded in OCT (Miles Diagnostics) and sectioned at 10 μm in a cryostat-microtome. Sections were stored at −20°C until used.

**Northern Analysis**

RNA was isolated from tissues by acid guanidinium (6.0 M) thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Samples of total RNA (25 μg) were subjected to electrophoresis through 0.9% agarose–formaldehyde gels and blotted onto nitrocellulose membranes. Northern analyses were carried out to assess the relative abundance of transcripts of mRNA encoding the β1 and γ1 chains of laminin as well as collagens α1(IV) and α2(IV) in control and aganglionic ILS/IS guts. cDNA probes encoding collagen α1(IV) and α2(IV) were obtained from Dr. M. Kurkinen (University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School) and probes encoding the β1 and γ1 chains of laminin were obtained from Dr. Y. Yamada (National Institute of Dental Research). cDNA was digested with appropriate restriction enzymes and the resulting fragments (β1, 4.6 kb; γ1, 1.7 kb; α1, 1.8 kb; α2, 1.1 kb) were labeled with [32P]dCTP by random priming. Hybridization was carried out with the [32P]-labeled cDNA probes (1 × 106 cpm/ml). Blots were also probed with cDNA encoding cyclophilin (2 × 105 cpm/ml) in order to correct for amounts of RNA loaded onto the gels. The radioactivity of hybridizing probes was estimated indirectly from the developed radioautograms by computer-assisted video densitometry (MCID 4; Imaging Research).

**RT - cPCR**

mRNA encoding laminin α1 was not present in sufficient abundance to be studied by Northern analysis; therefore, reverse transcription followed by the competitive polymerase chain reaction (RT - cPCR) was used to provide a quantitative evaluation of its presence in the bowel of control, ILS/IS, and c-ret −/− mice. RT-cPCR is far more sensitive than Northern analysis and thus is better suited for measurements of change in mRNAs of relatively low abundance in small fetal tissue samples. cPCR was employed in order to quantify data (Siebert and Larrick, 1992). For this purpose, a smaller mutant DNA fragment, was prepared, which contained the same primer template sequences as the target DNA (see below), to compete with the target for binding to the primers and thus for amplification. The PCR products of the target and the competitor were distinguished by their size on polyacrylamide gels. The use of the internal controls that contained the same primer template sequences as the target enabled the amount of target cDNA to be ascertained. Known and constant amounts of competitor RNA were added to compete with the target for binding to the primers during the amplification. Total RNA was incubated with Moloney murine leukemia virus reverse transcriptase (MMLV RT; Gibco BRL) with random hexamers. The resulting cDNAs were amplified by PCR using 21-bp 5′ primer, "w" (5′-GCAGATGTCGACACGAGAAGCC-3′) and a 21-bp 3′ primer "d" (5′-CTGATCAGGCAGTGGATCTCC-3′). Primer w corresponds to nucleotides 5644–5664 and primer d to nucleotides 6392–6372 of mouse laminin α1 (Sasaki et al., 1988). A 39-bp primer for producing the smaller

mutant competitor was constructed to include the sequence of primer w appended to the 5′ end of a sequence corresponding to bases 5820–5837 of the laminin α1 cDNA (Sasaki et al., 1988). PCR amplification using primer "m" as the 5′ primer and primer d as the 3′ primer amplified 572 bp of the laminin α1 sequence and resulted in a 939-bp DNA fragment containing the binding sequence for primer w at its 5′ end and the binding sequence for primer d at its 3′ end. The PCR product was cloned with a TA-cloning kit (Invitrogen) and the sense mRNA of the competitor was synthesized by using T7 RNA polymerase and quantified spectrophotometrically. The effectiveness of the RT - cPCR assay was evaluated by titration experiments in which doubling amounts of internal standard competitor RNA were added to 200 ng of RNA extracted from fetal bowel (see results).

**In Situ Hybridization**

Probes for in situ hybridization were subcloned into RNA expression vectors (pGEM 32 or 42; Promega Biotek). Probes supplied by Dr. Y. Yamada included p1235, which contains a cDNA spanning nucleotides 566–5126 of the laminin β1 coding region, an xba restriction fragment of laminin β1 spanning nucleotides 4729–5245, and p1238, a cDNA spanning nucleotides 5232–6640 of the coding sequence for laminin α1. An additional probe, pPE18, containing 1.1 kb of CDNA encoding collagen α2 (IV) was supplied by Dr. M. Kurkinen. Sense and antisense riboprobes were synthesized by using [35S]UTP-labeled nucleotides by runoff transcription according to the manufacturer’s directions (Promega Biotek). The quality of the probes was checked by polyacrylamide gel electrophoresis and radioautography; only probes that ran as a single band of the appropriate size were employed in these studies. Sense strand probes were used as controls to verify that hybridization obtained with antisense probes was specific. Data were considered valid only if a signal was obtained with the antisense and not the sense probes. With the exception of the xba restriction fragment, probes were hydrolyzed to reduce their size to 1-kb fragments. The xba fragment of laminin β1 was used for the investigation of adult tissue. Sections were removed from the freezer and postfixed with 4% formaldehyde (from paraformaldehyde). The tissue was acetylated with 0.2% acetic anhydride and washed in 0.2× SSC. Sections were prehybridized for 3 hr at room temperature in a solution containing 50% formamide, 600 mM NaCl, 10 mM Tris (pH 7.5), 1× Denhardt’s reagent, 1.0 mM EDTA, 0.05% sheared DNA, 0.05% yeast total RNA, and 0.005% yeast tRNA. Hybridization was carried out at 50°C for 16 hr in the presence of 600 mM NaCl, 10 mM Tris (pH 7.5), 1× Denhardt’s reagent, 0.5 mM EDTA, 0.01% sheared DNA, 0.05% yeast total RNA, 0.005% yeast tRNA, 10.0% dextran sulfate, 10.0 mM dithiothreitol, and 0.1% SDS. Heat-denatured probes were added to the hybridization buffer at 5 × 105 cpm per section. Following hybridization, the sections were washed in 5% formamide, 1× SSC, 10.0 mM dithiothreitol at 50°C for 30 min. The sections were again washed for 30 min with 0.5× SSC at room temperature and then treated with 0.1 mg/ml RNase at room temperature in order to hydrolyze single-stranded RNA. After washing with 500 mM NaCl, 10 mM Tris (pH 7.5), and 1.0 mM EDTA, the slides were washed for 3 hr at 55°C in 0.2× SSC. Sections were dehydrated in the presence of ammonium acetate and coated with liquid photographic emulsion (Kodak NTB-2 or Ilford, L2) for radioautography. Slides were exposed in dry light-tight boxes at 4°C and developed with Kodak D19. Processed sections were stained with hematoxylin and eosin and visualized by using indirect darkfield optics.

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amplify RNA in the developing or mature bowel. Amplification was detected by Northern analysis, RT-PCR was used to express the level of mRNA encoding the laminin chains of laminin and collagen. Because mRNA encoding the laminin chains of laminin and collagen 1(IV) and 2(IV) was not detected in the colon and small intestine of the same animals. Hybridization was carried out with 3P-labeled cDNA (~1 x 10^6 cpm/ml). The same blots were also probed with cDNA encoding cyclophilin in order to correct for different amounts of RNA loaded onto the gels. The radioactivity of hybridizing probes was estimated indirectly from the developed radioautograms by computer-assisted video densitometry. mRNA encoding the α1 chain of laminin could not be detected by Northern analysis in either the control or the ls/ls bowel. For the other transcripts, data were expressed as the ratio of the density of the signal obtained from the transcription level of the small intestine to that of the same blots probed simultaneously with cDNA encoding cyclophilin. In initial studies, the control terminal was analyzed separately from the proximal region of the organ because neurons are found in the proximal but not the distal colon of ls/ls mice. No difference between terminal and proximal colon was observed in the mRNA encoding any of the investigated proteins in either is/ls or control mice. As a result, the entire colon was investigated without distinction as to region. Similarly, differences between is/ls and control mice in the abundance of mRNA encoding these proteins in the small intestine were slight (Table 1). The relative abundance of mRNA in the colon was therefore normalized to the levels found in the small intestine of the same animals. This normalization reduced the variation between animals of the same type. The relative abundance of mRNA encoding laminin 1(IV) and 1(IV) and collagen 1(IV) and 2(IV) in the colon and small intestine is shown in Table 1 and the data for the control normalized to the small intestine are presented in Fig. 1. Comparison of the levels of mRNA normalized to those of the small intestine of the same animals revealed that the abundance of transcripts encoding the 1(IV) and 1(IV) chains of laminin and collagen 1(IV) and 2(IV) was increased in the colons of ls/ls mice (Fig. 1; P < 0.001).

Because mRNA encoding the laminin 1 chain could not be detected by Northern analysis, RT-PCR was used to amplify RNA in the developing or mature bowel. Amplimers corresponded to nucleotides 5644–5664 and 6392–6372 of the mouse laminin 1 chain (Sasaki et al., 1988).

### Immunocytochemistry

Sections were washed twice with PBS and then fixed to the slides with 4% formaldehyde (from paraformaldehyde) in PBS at pH 7.4. When laminin immunoreactivity was to be demonstrated, tissues were postfixed in 4% formaldehyde buffered with 0.1 M lysine (periodate). Tissues were permeabilized with 0.1% Triton X-100 (Sigma Chemical Co.) in PBS and then incubated in a blocking solution containing 0.1 M Tris–HCl, 1.5% NaN3, 0.1% Triton X-100, and 10% horse serum (GIBCO, Grand Island, NY) for 30 min at room temperature to reduce background staining. Sections were exposed overnight in a humidified chamber at 4°C to polyclonal rabbit primary antibodies. These included antilaminin (Sigma Chemical Co.; diluted 1:200), anticollagen (IV) (supplied by Dr. Hynda Kleinman; diluted 1:1000), or anti-PGP 9.5 (Biogenesis Inc.; diluted 1:400). After several washes to remove unbound primary antibodies, the tissues were subjected to affinity-purified goat anti-rabbit IgG (Kirkgaard and Perry Labs, Gaithersburg, MD) coupled to fluorescein isothiocyanate (FITC diluted 1:80) or to biotin (diluted 1:400) for 3 hr at room temperature. Those preparations that were exposed to a biotinylated secondary antibody were subsequently treated for 1 hr with streptavidin–Texas red (Immunoselect Gibco; diluted 1:400) or streptavidin–FITC (Vector Laboratories), to visualize sites of bound antibody. As an immunocytochemical control, nonimmune serum (1:100) was substituted for primary immune reagents. Specific immunostaining was defined as that not present in the controls. Specimens were mounted in 22.0 ml 1,4-diazabicyclo-(2,2,2) octane (Sigma) in 9 parts glycerol and 1 part PBS (final pH, 8.0) in order to retard bleaching of FITC. Sections were viewed with vertical illumination in a Leitz DMRD microscope. FITC fluorescence was visualized using an “L4” dichroic mirror/filter cube (exciting filter BP 450–490; dichroic mirror RKP 510; barrier filter BP 515–560). Texas red fluorescence was visualized with the aid of an “N2.1” dichroic mirror/filter cube (exciting filter BP 515–560; dichroic mirror RKP 580; barrier filter LP 590) that passed no FITC fluorescence. Tissues were photographed in color with Kodak Ektachrome film (ASA 400).

### RESULTS

**mRNA Encoding Chains of Laminin-1 and Type IV Collagen Is More Abundant in Is/ls Than Control Colon**

Northern analyses were carried out to assess the relative abundance of transcripts of mRNA encoding the three chains of laminin-1 as well as collagen α1(IV) and α2(IV) in control and aganglionic ls/ls gut. Tissue was obtained from mature animals. Hybridization was carried out with 3P-labeled cDNA (~1 x 10^6 cpm/ml). The same blots were also probed with cDNA encoding cyclophilin in order to correct for different amounts of RNA loaded onto the gels. The radioactivity of hybridizing probes was estimated indirectly from the developed radioautograms by computer-assisted video densitometry. mRNA encoding the α1 chain of laminin could not be detected by Northern analysis in either the control or the ls/ls bowel. For the other transcripts, data were expressed as the ratio of the density of the signal obtained from the transcription level of the small intestine to that of the same blots probed simultaneously with cDNA encoding cyclophilin. In initial studies, the control terminal was analyzed separately from the proximal region of the organ because neurons are found in the proximal but not the distal colon of ls/ls mice. No difference between terminal and proximal colon was observed in the mRNA encoding any of the investigated proteins in either ls/ls or control mice. As a result, the entire colon was investigated without distinction as to region. Similarly, differences between ls/ls and control mice in the abundance of mRNA encoding these proteins in the small intestine were slight (Table 1). The relative abundance of mRNA in the colon was therefore normalized to the levels found in the small intestine of the same animals. This normalization reduced the variation between animals of the same type. The relative abundance of mRNA encoding laminin 1(IV) and 1(IV) and collagen α1(IV) and α2(IV) in the colon and small intestine is shown in Table 1 and the data for the control normalized to the small intestine are presented in Fig. 1. Comparison of the levels of mRNA normalized to those of the small intestine of the same animals revealed that the abundance of transcripts encoding the 1(IV) and 1(IV) chains of laminin and collagen α1(IV) and α2(IV) was increased in the colons of ls/ls mice (Fig. 1; P < 0.001).

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### Table 1

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<th>Control</th>
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<td>Laminin β1</td>
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<td>Laminin γ1</td>
<td>44.0 ± 16.0</td>
<td>20.0 ± 1.2</td>
<td>8.0 ± 2.5</td>
<td>8.0 ± 4.0</td>
</tr>
<tr>
<td>Collagen α1(IV)</td>
<td>458.0 ± 230.0</td>
<td>24.0 ± 8</td>
<td>8.0 ± 1.3</td>
<td>12.0 ± 4.0</td>
</tr>
<tr>
<td>Collagen α2(IV)</td>
<td>442.0 ± 6.0</td>
<td>28.0 ± 2</td>
<td>4.0 ± 0.3</td>
<td>2.0 ± 1.4</td>
</tr>
</tbody>
</table>

Note. Densitometric measurements are shown relative to the mean of those obtained for laminin β1, which was assigned a value of 1.

Data are expressed as means ± SE, n = 4–5 for each probe.

**ECM Expression in Aganglionic Bowel**

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Data are expressed as means ± SE, n = 4–5 for each probe.
When tested with the plasmids in which the cDNAs were cloned, these primers were found to amplify only cDNA encoding $\alpha_1$ and not cDNA encoding $\beta_1$ or $\gamma_1$. In order to obtain a quantitative estimate of the change in mRNA encoding $\alpha_1$ as a function of age, cPCR was employed (Siebert and Larrick, 1992). For cPCR, a smaller cDNA fragment containing the same primer template sequences as the target was added as an internal standard. The final products were separated on polyacrylamide gels and distinguished by size (Figs. 2A and 2B). When RT-cPCR was used, mRNA encoding laminin $\alpha_1$ was found in both the fetal terminal colon and small intestine (Figs. 3A and 3B). Transcripts encoding laminin $\alpha_1$ could also be detected in the fetal and adult kidneys, which were investigated as positive controls. When material that was not exposed to reverse transcriptase was subjected to PCR, no amplified products were detected; therefore, the appearance of laminin $\alpha_1$ PCR reaction products was not due to contamination of samples by genomic DNA. In contrast to the fetal bowel, cPCR revealed that there was very little mRNA encoding $\alpha_1$ in the postnatal control or Is/Is gut (Figs. 3A and 3B). The relative expression of mRNA encoding $\beta$-actin in the same material was unchanged (data not illustrated). Transcripts encoding laminin $\alpha_1$ were detected in both the fetal Is/Is and wild-type bowel as early as E11. These transcripts were quantified, as a function of region and fetal age, by cPCR (Fig. 3B). Expression of laminin $\alpha_1$ was found to be developmentally regulated in both the wild-type and Is/Is colon. Expression of laminin $\alpha_1$ declined as a function of age so that it was greatest during fetal life when ganglia are formed and very low in the mature gut. mRNA encoding laminin $\alpha_1$ was greatly increased in the Is/Is relative to the wild-type colon at each age examined. In contrast, mRNA encoding laminin $\alpha_1$ in the terminal colon and the small intestine in ret $-/-$ mice was not significantly different from that in the terminal colon and small intestine of ret $+/-$ animals either at E15 or in newborn animals (Fig. 4). At both of these ages, expression of mRNA encoding laminin $\alpha_1$ was significantly greater in Is/Is mice than in control or ret $-/-$ animals (Fig. 4).

The Distribution of mRNA Encoding Chains of Laminin-1 and Type IV Collagen Is Qualitatively Similar in Is/Is and Control Mice

In situ hybridization was employed to study the distribution of mRNA encoding the $\alpha_1$ and $\beta_1$ chains of laminin.
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and \( \alpha2(IV) \), so that the pattern of expression in the bowel was not readily demarcated from that of the surrounding mesenchyme. This phenomenon was especially pronounced in the \( \text{ls/ls} \) fetuses (Fig. 5A).

By Day E16, the qualitative distribution of cells containing mRNA encoding laminin \( \beta1 \) and \( \alpha2(IV) \) (Figs. 6A–6D) was different from that found at E12 (Figs. 5A and 5B). At this time, mRNA was more abundant in the enteric mesenchyme than in the epithelium lining the lumen of the hindgut, from which it had largely disappeared. Within the mesenchyme, both of these transcripts were predominantly concentrated in two zones. One region of transcript abundance, which was more intense, was beneath the mucosa; the other was in the outer region of the gut, where the longitudinal smooth muscle ultimately forms and the crest-derived precursors of enteric neurons and glia migrate (Pomeranz et al., 1991b; Tucker et al., 1986). In addition, especially in \( \text{ls/ls} \) animals, a sling of cells rich in transcripts passed around the lateral and dorsal borders of the hindgut (Fig. 6A). The density of labeling was estimated by determining the number of silver grains crossed by a 2.0-mm line superimposed on the tissue. The intensity of labeling

FIG. 3. mRNA encoding laminin \( \alpha1 \) is developmentally regulated and increased, relative to control, in the colon of \( \text{ls/ls} \) mice. (A) RT-cPCR. A constant amount of competitor was coamplified with target cDNA. The PCR products, corresponding to the target (TAR) and the competitor (COM) were separated on a 3.6% polyacrylamide gel and visualized with ethidium bromide as in Fig. 2. RNA was extracted from the colon (c) and small intestine (s) of fetal (at E11, E13, E15, and E17) and adult (Ad) control and \( \text{ls/ls} \) mice. (B) The relative abundance of mRNA encoding laminin \( \alpha1 \) is plotted as a function of age. The quantity of mRNA encoding laminin \( \alpha1 \) was normalized to that found in control samples at E15. Note that mRNA encoding laminin \( \alpha1 \) is more abundant in \( \text{ls/ls} \) (■) than control (○) colon at each age examined.

FIG. 4. There is no significant difference in the abundance of mRNA encoding laminin \( \alpha1 \) in the colons of \( \text{c-ret}^{+/+} \) and \( \text{c-ret}^{0/0} \) mice, but the abundance in each is significantly less than that found in \( \text{ls/ls} \) mice.

RT-cPCR. As in Fig. 3, a constant amount of competitor was coamplified with target cDNA and the PCR products were separated on a polyacrylamide gel. The relative abundance of mRNA encoding laminin \( \alpha1 \) in the E15 and newborn colons of \( \text{c-ret}^{+/+} \) and \( \text{c-ret}^{-/-} \), \( \text{ls/ls} \), and control mice are compared. The quantity of mRNA encoding laminin \( \alpha1 \) is normalized to that found in control samples at E15. Differences between \( \text{c-ret}^{-/-} \) and \( \text{c-ret}^{+/+} \) mice are not significant at either age, although the abundance of mRNA encoding laminin \( \alpha1 \) is greater in the colon at E15 than at birth. At each age the relative abundance of mRNA encoding laminin \( \alpha1 \) in the \( \text{c-ret}^{-/-} \) and \( \text{c-ret}^{+/+} \) animals is about the same as that found in control mice and significantly less than that observed in \( \text{ls/ls} \) animals.
of the subepithelial mesenchyme of the Is/Is bowel was significantly greater than that of control (P < 0.0001) for both the laminin β1 (Is/Is = 10.7 ± 0.5; control = 2.8 ± 0.4) and the α2(IV) probes (Is/Is = 14.6 ± 1.4; control = 5.5 ± 0.5). The distribution of cells containing mRNA encoding laminin β1 in the adult terminal colon was qualitatively similar to that at E16. In the postnatal Is/Is animals, these cells were most abundant under the mucosal epithelium in the lamina propria (Fig. 6E). Laminin β1 transcripts, however, were far more abundant in the postnatal Is/Is than in postnatal control mouse colon, in which the radioautographic signal was almost too low to detect (data not illustrated). There were also localized regions in the submucosa and muscularis externa of the Is/Is, but not the control colon, in which cells containing mRNA encoding laminin β1 were highly concentrated.

At E13 and E15, mRNA encoding laminin α1 was extremely abundant in developing renal tubules, which are known to express laminin α1 (Klein et al., 1990) and were examined as a positive control (Fig. 7A). Within the developing Is/Is and control colon, at E13, transcripts of laminin α1 were expressed in the luminal epithelium to a greater extent than in the underlying enteric mesenchyme (Figs. 7B and 7C). Similarly, epithelial labeling was greater than mesenchymal labeling in the small intestines of the same animals (Fig. 7C). The level of mesenchymal labeling by the antisense probe was low (3.0 ± 0.2 times background [measured outside the tissue]), but consistently higher than that seen when sections were hybridized with the sense probe (1.2 ± 0.1 times background; P < 0.0001). There was no qualitative difference between Is/Is and control mice in the pattern of labeling in either the colon or small intestine.

By E15, however, the pattern changed in the colon and mRNA encoding α1 was more heavily expressed by the mesenchyme than by the epithelium (Fig. 7E). As in the cases of β1 laminin and α2(IV), the difference in expression of laminin α1 transcripts between Is/Is and control mice was more quantitative than qualitative. mRNA was far more abundant in the Is/Is animals than in their control counterparts (Fig. 7F); however, the distribution of mRNA hybridizing with the laminin α1 probe was similar in both types of animal. Again, in the Is/Is animals, mRNA encoding laminin α1 was extremely concentrated in the entire medial region of the distal embryo; thus, the mesenchyme of the hindgut, as well as that of its surroundings, contained a greater abundance of laminin α1 transcripts in Is/Is than in control mice.

The Pattern of Laminin and Type IV Collagen Deposition in the Gut Is Not Changed by the Absence of Enteric Neurons in ret−/− Mice

Immunocytochemistry was used to compare the patterns of laminin and type IV collagen immunoreactivities in the colons and small intestines of newborn ret−/− and control (ret+/− or ret+/+) mice. Southern analysis of tissue from the tails was used to distinguish the genotypes of newborn pups. Preparations were immunostained with antibodies to PGP 9.5, a cytosolic protein that is related to ubiquitin (Wilkinson et al., 1989) and is expressed in the vast majority of enteric neurons (Eaker and Sallustio, 1994). As has previously been reported (Schuchardt et al., 1994), intrinsic neurons were entirely missing from both the colon and small intestine in the ret−/− animals (compare Fig. 8A with 8B). Neurons were present and appeared normally distributed in both myenteric and submucosal ganglia in the ret+/− and ret+/+ controls (Fig. 8A). The smooth muscle layers of the gut and the luminal epithelia, however, were comparable in ret−/− mice and control animals. The mean thickness of the muscularis externa of the newborn ret+/− bowel (83.4 ± 3.9 μm) was not significantly different from that of ret−/− mice (96.1 ± 6.4 μm). In preparations of control tissue, in which laminin and type IV collagen immunoreactivities were demonstrated (Figs. 8C and 8E),
FIG. 6. At E16 expression of mRNA encoding laminin β1 and collagen α2 (IV) is found by in situ hybridization to be more intense in the mesenchyme than in the epithelium of the ls/ls and control colon. (A) ls/ls colon hybridized with a cRNA probe encoding laminin β1. The epithelium (e) of the terminal colon is no longer labeled. Cells in the surrounding mesenchyme (arrow), however, contain hybridizing mRNA. One ring of intensely labeled mesenchymal cells can be distinguished just below the epithelium. Others are found in the outer gut mesenchyme (m). The boundary between the mesenchyme of the gut and that of the surrounding pelvis is indistinct. (B) Control colon hybridized with a cRNA probe encoding laminin β1. Again, the epithelium (e) of the terminal colon is no longer labeled; however, relatively little hybridizing mRNA is apparent in the mesenchyme (m) of the bowel or that of the surrounding pelvis. (C) ls/ls colon hybridized with a cRNA probe encoding collagen α2 (IV). The epithelium (e) no longer contains hybridizing mRNA. The pattern of labeling in the mesenchyme resembles that found for laminin β1 in the ls/ls colon; however, the restriction of hybridizing mRNA in the colon to inner and outer concentric rings (arrows) is more apparent. The amount of hybridizing mRNA in the surrounding pelvic mesenchyme is equal to that of the colon. (D) Control colon hybridized with a cRNA probe encoding collagen α2 (IV). The epithelium (e) no longer contains hybridizing mRNA. The pattern of labeling is somewhat different from that found in the ls/ls terminal colon in that only one ring of cells containing hybridizing mRNA, that just beneath the epithelium (arrows), is evident. The amount of signal in the enteric and pelvic mesenchyme are each much less than in the equivalent regions of the ls/ls animals (compare with C). (E) Adult colon of ls/ls mice. Cells that contain mRNA encoding laminin β1 are most concentrated in the lamina propria (arrows); however, additional concentrations can be seen in the muscularis mucosa (arrowhead), submucosa (s), and muscularis externa (M). Scale bars, 50 μm.

the ganglia appeared as unstained islands, since laminin and type IV collagen are excluded from the enteric plexuses (Mawe and Gershon, 1989). These islands were not visible in similarly immunostained material from retn-/- animals (Figs. 8D and 8F). The patterns of type IV collagen (compare Fig. 8C with 8D) and laminin (compare Fig. 8E with 8F; and
Fig. 7. Expression of mRNA encoding laminin $\alpha_1$ is found by in situ hybridization to switch from the epithelium at E13 to the mesenchyme at E15 in the ls/ls colon. (A) The presumptive kidney of an ls/ls mouse examined as a positive control. At E13, the epithelium of the developing tubules is heavily labeled by a cRNA encoding laminin $\alpha_1$. (B) Colon of an ls/ls mouse at E13. The epithelial cells (arrow) are more heavily labeled than the cells of the enteric mesenchyme (m). (C and D) Small intestine of an ls/ls mouse at E13 (near serial sections). C, antisense probe; D, sense probe. Note that the antisense probe (C) labels the intestinal epithelium (arrow), but that the sense probe (D) does not. (E) Fetal colon from an E15 ls/ls mouse. The distal bowel was hybridized with a cRNA probe encoding laminin $\alpha_1$. The epithelium (e) no longer contains hybridizing mRNA. Labeled cells, however, are found in the enteric mesenchyme (m) and in the surrounding pelvic mesenchyme. The boundary of the colon is indistinct. (F) Fetal colon from an E15 control mouse. There is a low level of mesenchymal labeling that is very much less than that found in the ls/ls animals. Scale bars: A–D, 30 $\mu$m; E and F, 50 $\mu$m.

Fig. 8G with 8H) immunoreactivities in the ret $^{--}$ colon and small intestine (not illustrated) appeared identical to those of the ret $^{+/-}$ and ret $^{+-}$ control colon and small intestine. The intensity of laminin and type IV collagen immunoreactivities was not distinguishable from control in the ret $^{--}$ bowel. Laminin and type IV collagen immunoreactivities were also similarly distributed, except for the presence of nonimmunostained ganglia in the ret $^{+/-}$ and ret $^{+-}$ animals. This equivalent immunostaining of laminin in the newborn ret $^{--}$ and control bowel contrasts
FIG. 8. Aganglionosis of the bowel in mice homozygous for a targeted mutation in c-ret is not associated with increased expression of laminin or collagen (IV). (A and B) PGP 9.5 immunoreactivity of colon. Note that the newborn control gut (A) contains well-developed myenteric (large arrow) and submucosal (small arrow) plexuses. Nerves are also immunostained in the mesentery (me). There are no ganglia in the ret+/− bowel (B), although the muscularis externa (m) is as well developed as in the control. There are PGP 9.5-immunoreactive nerves (n), which can be seen in connective tissue outside the gut. (C and D) Collagen (IV) immunoreactivity in colon. The intensity
markedly with the increased immunostaining of laminin in the newborn Ia/Ia colon (Figs. 8I and 8J). In the Ia/Ia bowel, laminin immunoreactivity is strikingly increased in the muscle layers; moreover, the muscle of the Ia/Ia gut is hypertrophic. The mean thickness of the muscularis externa of newborn Ia/Ia colon (146.9 ± 8.8 μm) is thus significantly (P < 0.001) greater than that of either the ret -/+ or the ret -/- animals. The distribution of nerve fibers, revealed by their PGP 9.5 immunoreactivity, in the newborn Ia/Ia (Figs. 8K and 8L) and ret -/-/ terminal colon (Fig. 8B) was not identical. Neither preparation contained ganglia or nerve cell bodies; however, the number of axons appeared to be greater in the Ia/Ia than the ret -/-/ colon. Coarse nerve bundles, furthermore, were common in the Ia/Ia gut and were never seen in the ret -/-/ bowel.

DISCUSSION

The lesion that leads to the development of congenital megacolon in Ia/Ia mice is associated with a highly localized failure of enteric neuron development. Only the most terminal region of the bowel becomes completely aganglionic (Lane, 1966; Rothman and Gershon, 1984). Enteric neurons are found in the remainder of the colon, albeit in reduced numbers and in an abnormal pattern (Payette et al., 1987). The submucosal plexus is more severely affected than the myenteric and is aganglionic through a greater length of the colon. In contrast to the colon, no defect at all has been detected in the development of enteric neurons in the Ia/Ia esophagus, stomach, or small intestine. Any mechanistic explanation of the pathogenesis of congenital megacolon, therefore, must account for the regional specificity of the defective development of enteric neurons. The restriction of the Ia/Ia lesion to the colon could be explained by a localized abnormality in the microenvironment of the colonic wall that disturbs either the migration of crest-derived cells or their differentiation into neurons. Alternatively, the precursors of enteric neurons in Ia/Ia mice may themselves be abnormal, such that they can develop in all regions of the bowel except the colon. In this case, the microenvironment provided by the Ia/Ia colon could be intrinsically normal, but still unsuitable for the formation of defective neural precursors.

The accumulations of laminin, type IV collagen, and other components of basal laminae, that arise in the colon of developing and adult Ia/Ia mice (Payette et al., 1988), like the lesion in enteric neuron development, are highly localized abnormalities. Similar abnormalities have been described in the colon of human patients with Hirschsprung's disease (Parikh et al., 1992). The location of the defects in the matrix components in Ia/Ia mice coincides with the region of the bowel in which neuronal development fails; moreover, the matrix is demonstrably abnormal before neurons develop in the terminal colon (Payette et al., 1988; Rothman and Gershon, 1982, 1984). These observations are consistent with the possibilities that the extracellular matrix abnormalities in the colon of Ia/Ia mice interfere with the colonization of the defective region by crest-derived cells and/or with their ability to form ganglia. It is thus important to determine whether the accumulation of components of basal laminae in the Ia/Ia colon occurs as a result of a primary defect, in the expression of mRNA encoding these molecules, for example, or as a result of the absence of enteric neurons.

In the present study, transcripts encoding chains of laminin and collagen (IV) were found to be increased in the colon of fetal Ia/Ia mice. Transcripts encoding laminin α1, β1, and γ1, as well as collagen α1(IV) and α2(IV), were all significantly more abundant in the colon of Ia/Ia than in control mice. Interestingly, while laminin α1 expression appeared to be elevated in both the colon and small intestine of Ia/Ia mice, β1 and γ1 were elevated only in the Ia/Ia colon; moreover, previous studies have failed to detect any overabundance of laminin protein in the Ia/Ia small intestine (Payette et al., 1988). The β1 and γ1 chains are found in several isoforms of laminin, including laminin-1 (EHS laminin; α1(1)β1γ1), laminin-2 (merosin; α2(1)γ1), and laminin-6 (K-laminin; α4β1γ1) (Burgeson et al., 1994). The α1 chain is present in laminin-3 (S-laminin; α1α2γ1) as well as laminin-1; however, the observation that transcripts encoding α1, β1, and γ1 are all increased implies that laminin-1 is overexpressed in the Ia/Ia colon, although the possibility that other laminin isoforms might also be upregulated has been considered.

and distribution of collagen (IV) immunoreactivity in the colons of newborn control (C) and ret -/-/ mice (D). The negative image of the myenteric ganglia (arrow), which lack internal collagen (IV), is seen in the control, but not in the ret -/-/ tissue, which is aganglionic. (E-H) Laminin immunoreactivity in colon. The intensity and distribution of laminin immunostaining are not different in newborn control (heterozygous littermates) (E, G) and ret -/-/ mice (F, H). The negative image of the myenteric ganglia (arrow), which lack laminin in the control, but not in the ret -/-/ tissue, which is aganglionic. The epithelium (e) is not immunostained in either preparation. Note the extensive distribution of laminin immunoreactivity in the connective tissue of both preparations. (E) and (G) are transverse sections, (F) and (H) are longitudinal sections. (I and J) Laminin immunoreactivity in newborn Ia/Ia terminal colon. The intensity of laminin immunoreactivity in the muscularis externa (compare the region between arrows in the two preparations) is greater in the Ia/Ia (J) than in the normal (E, G) colon at the same age. Note that the laminin immunoreactivity around individual muscle fibers is also thicker in Ia/Ia than in control. At the electron microscope level, reduplicated basal laminae have been observed in the Ia/Ia colon (Tennison et al., 1986). (K and L) PPG 9.5 immunoreactivity in newborn Ia/Ia terminal colon. The Ia/Ia gut (K) contains no myenteric ganglia, but many nerve fibers (small arrows) in the muscularis externa and mucosa. Coarse nerve bundles (large arrow), which are characteristic of the Ia/Ia bowel, penetrate as far as the mucosa. Scale bars, 50 μm.
not been eliminated. The increased abundance of mRNA encoding laminin-α1 in the Is/Is animals, moreover, was found in the colon and was detected as early as E11. Crest-derived cells are colonizing the terminal bowel at this age, which precedes by about 4 days the differentiation of recognizable neurons, which is detected at approx E15 (Rothman and Gershon, 1982, 1984). The timing of the overexpression of laminin-1 and collagen (IV) in the Is/Is colon, therefore, is consistent with the ideas that these molecules affect either the migration of neural precursors or the ability of these cells to give rise to enteric neurons.

mRNA encoding the α1 chain of laminin was not very abundant at any age and required RT-PCR for its detection in both the Is/Is and the control gut. This observation, which is consistent with previous immunohistochemical studies (Simo et al., 1991, 1992), suggests that α1 expression may be rate-limiting in the assembly of laminin-1. The expression of α1, moreover, was developmentally regulated; thus, mRNA encoding α1 was present after E11 in both the control and Is/Is fetal colon and small intestine, but declined through E17, and very little could be detected after birth. The expression of α1, therefore, appears to be a characteristic of the fetal bowel that coincides with the period of ganglion formation (Payette et al., 1988; Rothman and Gershon, 1982, 1984; Rothman et al., 1986). This coincidence is potentially important because there is evidence that the α1-containing laminin-1 affects the development of enteric neurons (Pomeranz et al., 1993).

Crest-derived cells migrating through the enteric mesenchyme have been shown to make contact with diffuse tufts of laminin that have not been incorporated into recognizable basal laminae (Pomeranz et al., 1991b). At sites where these contacts with laminin occur, a laminin binding protein, LBP110, is present in the plasma membranes of the crest-derived cells (Pomeranz et al., 1991b). LBP110 is a member of the β-amyloid precursor family of proteins that binds specifically to a domain of α1 that mediates laminin-induced neurite extension (Kibbey et al., 1993) and is also responsible for the promotion by laminin of enteric neuronal development in vitro (Chalazonitis et al., 1992; Gershon et al., 1993b; Pomeranz et al., 1993). Whether the α1-containing laminin-1 promotes the differentiation of enteric neurons in vivo, as it does in vitro, remains to be demonstrated; nevertheless, the possibility that laminin-1 is important in the development of enteric neurons in situ is supported by the current observation that the expression of mRNA encoding laminin α1 is high in both colon and small intestine during the period of enteric neurogenesis. The timing of expression of LBP110 by the crest-derived precursors of enteric neurons is also of interest in this regard. LBP110 is not expressed by crest-derived cells while they migrate to the gut (Pomeranz et al., 1993). It is acquired by these cells only after they have colonized the bowel. Within the gut, moreover, LBP110 is expressed asynchronously and its expression is followed closely by the appearance of neural markers. Before they colonize the bowel, therefore, crest-derived cells can adhere to laminin-1 and even migrate on it, but they cannot interact with laminin via LBP110 until after their arrival in the gut. The timing of its expression thus prevents the premature occurrence of LBP110-mediated laminin α1-induced neurogenesis.

Transcripts encoding the α1 and β1 chains of laminin, as well as collagen α2(IV), were found, by in situ hybridization, both in cells of the enteric mesenchyme and in the intestinal epithelium. Epithelial expression of the collagen α1 transcripts was most pronounced early in development (E12), but at later ages (E15–E16) expression in the outer gut mesenchyme became more dominant. Previous studies of laminin biosynthesis using chain-specific antibodies have also suggested that laminins are synthesized by both endodermal and mesenchymal cells (Simo et al., 1991, 1992). These earlier immunocytochemical experiments, moreover, demonstrated that laminin α1 is highly concentrated in the basal lamina underlying the luminal epithelium early in development (prior to E13), but at later ages (after E16) becomes prominent in the muscularis externa. In fact, at E16, laminin α1 immunoreactivity is strikingly concentrated in the basal lamina that surrounds developing ganglia (Simo et al., 1991). The location of cells that contain mRNA encoding laminin α1 and β1 and collagen α2(IV) found in the present study was qualitatively similar in Is/Is and control colon; however, all of these transcripts appeared to be more abundant in the colons of Is/Is animals. The increased abundance of laminin α1 and β1 and collagen α2(IV) mRNA in the Is/Is mice was found in the pelvic tissue that surrounded the distal colon as well as in the colon itself. These observations suggest that the Is/Is defect leads to an upregulation of the expression of laminin-1 and collagen IV in a caudal region of the mutant fetuses that includes the colon. Some of the cells in which this upregulation occurs are located in the enteric mesenchyme. The extracellular matrix molecules that these cells secrete, therefore, would appear to be positioned to interact both with migrating vagal and sacral crest-derived cells attempting to colonize the bowel. The distribution of mesenchymal cells that synthesize laminin thus corresponds well with the distribution of the diffuse tufts of laminin in the enteric mesenchyme that come into contact with migrating crest-derived cells (Pomeranz et al., 1991b).

The relationship of migrating crest-derived cells to laminin-1 is likely to be disturbed by the upregulation of laminin-1 that occurs in the Is/Is colon and surrounding mesenchyme. Because laminin-1 promotes neuronal development (Chalazonitis et al., 1992; Gershon et al., 1993b; Pomeranz et al., 1993), it is possible that the excess of laminin-1 in the Is/Is animals induces crest-derived cells to differentiate as neurons or glia before their migration is complete. Such an effect of laminin-1 would have to be exerted by the entire molecule. Peptides containing the active region of laminin α1 have been found to be unable to stimulate neural development and, in fact, antagonize the differentiation-promoting action of trimeric laminin (Gershon et al., 1993a). Although mRNA encoding laminin α1 may be increased in the small intestine of Is/Is mice, that encoding β1 and γ1.
is not; furthermore, no increase in trimeric laminin has been detected in the I\(\text{l}\)/l small intestine (Payette et al., 1988); the I\(\text{l}\)/l-related increase in trimeric laminin is restricted, in the gut, to the colon. Vagal crest-derived cells migrate down the length of the gut to reach the colon (Kapur et al., 1992; Le Douarin and Telfit, 1973; Pomeranz et al., 1991a; Tucker et al., 1986), while sacral crest-derived cells approach the hindgut from its dorsolateral surface, near the junction with the allantois (Pomeranz and Gershon, 1990). Migrating vagal crest-derived cells would thus be expected to be affected by the excess of laminin-1 within the bowel, while the sacral crest-derived cells would encounter an excess of laminin-1 in the pelvic mesenchyme, even before they reach the bowel. If vagal crest-derived cells were to differentiate prematurely, they would be expected to withdraw from the cell cycle, as well as stop migrating too soon. Such an effect would lead to a decrease in the total number of enteric neurons and to aganglionosis in the regions of the colon that are colonized last. Each of these predictions have been verified in the I\(\text{l}\)/l colon. The I\(\text{l}\)/l colon contains fewer neurons than does the control colon and the neurons it does contain diminish in numbers proximodistally (Payette et al., 1987). The submucosal plexus, furthermore, which forms late, as a result of a secondary migration of precursors from the myenteric plexus (Gershon et al., 1980; Pomeranz and Gershon, 1990), is more severely affected than the myenteric plexus (Payette et al., 1987). Ectopic ganglia have also been found in the pelvic tissue outside the I\(\text{l}\)/l colon, which do not exist in control mice (Payette et al., 1987; Rothman and Gershon, 1984). These ganglia have been proposed to arise from sacral crest-derived cells that differentiate prematurely and stop migrating before they colonize the bowel.

The idea that attempt to relate the upregulation of laminin-1 and the ability of its \(\alpha_1\) chain to promote development of enteric neurons to the development of aganglionosis in I\(\text{l}\)/l mice assume that the upregulation of components of basal laminae that is manifest in the colon and surrounding pelvic mesenchyme precedes the failure of enteric neurogenesis and is not secondary to it. This assumption was tested in the current study by immunocytochemically comparing the distributions of laminin and collagen (IV) in the aganglionic colon and small intestine of c-ret \(-/-\) mice with those in the normally ganglionated gut of their c-ret \(+/-\) and c-ret \(+/+\) litter mates. The immunoreactivities of laminin and type IV collagen in both the colon and small intestine in the c-ret \(-/-\) mice could not be distinguished from those of the controls, except that regions occupied by ganglia in the control tissue were not immunostained. These unstrained islands were not present in the c-ret \(-/-\) bowel, although, as in the aganglionic I\(\text{l}\)/l colon, immunostaining of PGP 9.5 revealed the presence of nerve fibers in the tissue. In contrast, as previously demonstrated in the fetal colon (Payette et al., 1988), laminin immunoreactivity was considerably more intense in the muscularis of the newborn I\(\text{l}\)/l colon than that of controls. Transcripts of laminin \(\alpha_1\), moreover, which were significantly increased in the I\(\text{l}\)/l colon relative to its control were not similarly increased over control in c-ret \(-/-\) animals. Neuronal development falls in the c-ret \(-/-\) mice (Schuchardt et al., 1994); however, it fails because c-ret expression, which occurs in enteric neuronal precursors (Pachnis et al., 1993), is essential for the development of enteric neurons (Schuchardt et al., 1994). The pathogenesis of the intestinal aganglionosis that arises in c-ret \(-/-\) mice is thus independent of the I\(\text{l}\)/l locus. These observations, therefore, suggest that an absence of neurons from a segment of gut or a failure or neurogenesis are not, by themselves, sufficient to cause an increase in the concentrations of components of basal laminae. The structure of the smooth muscle layers in the c-ret \(-/-\) mice was also relatively normal. Specifically, the hypertrophy that is characteristic of the smooth muscle in the aganglionic region of the gut in I\(\text{l}\)/l mice was not seen in the c-ret \(-/-\) animals. Again, these data are not compatible with the idea that the absence of neurons, by itself, causes smooth muscle hypertrophy. These observations, in c-ret \(-/-\) mice, imply that the aganglionosis that occurs in the bowel of I\(\text{l}\)/l mice does not explain the abnormal accumulation of laminin and type IV collagen that is found in the same segments of gut, nor does it explain the hypertrophy of smooth muscle that also distinguishes the defective region. It seems more likely, therefore, that the abnormalities of smooth muscle and its precursors in the mesenchyme of the fetal bowel are related to the genetic lesion in I\(\text{l}\)/l mice and not nonspecific secondary effects that arise because enteric neurons are absent.

The mutation in I\(\text{l}\)/l mice that gives rise to megalocolon prevents the proteolytic activation of big EDN3 by the metalloprotease, endothelin-converting enzyme 1 (ECE-1) (Baynash et al., 1994). As a result of this defect, the I\(\text{l}\)/l mice are unable to produce active EDN3. An interaction of EDN3 with the EDNRB is essential for the development of enteric neurons from crest-derived precursors in the colon. This interaction, however, does not necessarily have to occur on the surfaces of the crest-derived cells themselves. Neither the sites of expression of the EDNRB nor those of expression of EDN3 have yet been reported; therefore, it is not yet clear how the interaction of EDN3 with the EDNRB affects enteric neuronal development. It has been postulated that both EDN 3 and the EDNRB are expressed by crest-derived melanoblasts and function in these cells as an autocrine signal that is needed to maintain migration of the cells and their ability to colonize the skin (Baynash et al., 1994; Hosoda et al., 1994). An analogous system could be invoked to explain the failure of enteric neurogenesis in the I\(\text{l}\)/l colon. This explanation, however, would not account for the successful colonization of the remainder of the bowel and the evidently normal formation of enteric neurons in all regions of the gut except the colon. Alternatively, the interaction of EDN3 with the EDNRB could be essential for the normal development of enteric smooth muscle cells or interstitial cells of Cajal (ICCs). ICCs are specialized relatives of smooth muscle cells that express the c-kit proto-oncogene and act as pacemakers for myogenic intestinal contracting.
slow waves (Huizinga et al., 1995; Ward et al., 1994). The precursors of one or both of these cells in the enteric mesenchyme express the \( \alpha 1 \) chain of laminin-1 and the degree to which they do so diminishes as a function of fetal age. Conceivably, a retardation of smooth muscle and/or ICC development, secondary to a failure of stimulation of the EDN RB, could account for the upregulation of laminin \( \alpha 1 \) (and other components of basal laminae) that persists throughout the period of neurogenesis in the Is/Is colon. In favor of this hypothesis are the facts that the EDN RB is actually known to be expressed by the smooth muscle cells of the muscularis externa of both the large (Okabe et al., 1995) and small intestines (Yoshinaga et al., 1992) and that intestinal smooth muscle responds directly to EDN3. Clearly, it remains to be demonstrated that the development of smooth muscle is affected by EDN3 and that smooth muscle or ICC precursors, like mature smooth muscle, express the EDN RB; moreover, the proposed regulation by EDN3 of laminin expression must still be shown to occur. The current data, however, demonstrate that the defect in Is/Is mice does lead to overproduction of at least one component of the extracellular matrix, laminin \( \alpha 1 \), that has been demonstrated to promote enteric neurogenesis. This effect, by causing premature development of neurons from crest-derived precursor cells, could be the cause of aganglionosis. Further studies are needed to test this hypothesis and to analyze the relationship of EDN3 and the EDN RB to smooth muscle development and the expression of its secretory products.

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