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Null mutation of endothelin receptor type B gene in spotting lethal rats causes aganglionic megacolon and white coat color

(Hirschsprung disease/enteric neurons/melanocytes/neural crest/Shah–Waardenburg syndrome)

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ABSTRACT **Mutations in the gene encoding the endothelin receptor type B (*EDNRB*) produce congenital aganglionic megacolon and pigment abnormalities in mice and humans. Here we report a naturally occurring null mutation of the *EDNRB* gene in spotting lethal (*sl*) rats, which exhibit aganglionic megacolon associated with white coat color. We found a 301-bp deletion spanning the exon 1–intron 1 junction of the *EDNRB* gene in *sl* rats. A restriction fragment length polymorphism caused by this deletion perfectly cosegregates with the *sl* phenotype. The deletion leads to production of an aberrantly spliced *EDNRB* mRNA that lacks the coding sequence for the first and second putative transmembrane domains of the G-protein-coupled receptor. Radioligand binding assays revealed undetectable levels of functional EDNRB in tissues from homozygous *sl/sl* rats. We conclude that EDNRB plays an essential role in the normal development of two neural crest-derived cell lineages, epidermal melanocytes and enteric neurons, in three mammalian species—humans, mice, and rats. The EDNRB-deficient rat may also prove valuable in defining the postnatal physiologic role of this receptor.**

Neural crest cells arise from the dorsal neural tube and migrate to form diverse tissues in the developing embryo. They differentiate into a wide variety of tissues including neuroendocrine cells, neurons and glia of the peripheral nervous system, pharyngeal arch tissues, and epidermal and choroidal melanocytes (1). A number of natural and targeted mutations have been reported to produce developmental defects in neural crest cell migration, differentiation, or survival (2). Study of these mutations continues to provide new insights into this complex system. Precursors of myenteric ganglion neurons originate in the vagal neural crest and migrate in a rostral-to-caudal direction along the developing gastrointestinal tract (3). Failure of these cells to reach the most distal intestine results in functional obstruction in the aganglionic intestine. Subsequent dilatation of the normal proximal intestine leads to the term "aganglionic megacolon." Precursors of epidermal melanocytes migrate dorsally from the neural crest and then through the mesenchymal layer beneath the ectoderm ventrally until they eventually enter the epidermis (4). Hereditary defects in development of myenteric ganglion cells and epidermal melanocytes often appear together as localized pigment abnormalities associated with aganglionic megacolon (5–9). This suggests a common mechanism involved in development of these two cell lineages.

Recent studies have established that cellular communication mediated by endothelins plays an essential role in the development of neural crest-derived melanoblasts and the enteric nervous system. Endothelins are a family of 21-amino acid peptides that act on G-protein-coupled heptahelical receptors (10, 11). Endothelin 1 (EDN1) was originally identified as a potent vasopressor derived from vascular endothelial cells (12). Three separate genes encode the known mammalian endothelins, *EDN1, EDN2*, and *EDN3*, which are expressed in various vascular and nonvascular tissues (13). Two endothelin receptor subtypes, endothelin receptor type A (EDNRA) and endothelin receptor type B (EDNRB), are also expressed in a variety of vascular and nonvascular tissues with partially overlapping distributions (14–16). EDNRA exhibits an affinity rank order toward endothelin isopeptide ligands: EDN1 ≥ EDN2 ≫ EDN3. EDNRB accepts all three isopeptides equally.

Mice with the naturally occurring recessive mutations piebald-lethal (s^l) and lethal spotting (*ls*) exhibit coat color spotting and aganglionic megacolon (5, 17). We previously found that the entire *EDNRB* gene is deleted in the s^l chromosome 14 (18). A mild mutant allele of the same locus, piebald (*s*), expresses reduced levels of structurally normal *EDNRB* mRNA. We also found that *ls* mice harbor a point mutation in the gene that encodes EDN3, a ligand for EDNRB (19). The mutation interferes with processing of the peptide ligand from its biologically inactive precursor big EDN3. Mice with a targeted null disruption of either the *EDNRB* or *EDN3* gene exhibit an identical phenotype, indicating that the interaction of EDN3 with EDNRB is crucial for normal development of these neural crest-derived tissues. In humans, a missense mutation of the *EDNRB* gene also produces congenital aganglionic megacolon, or Hirschsprung disease, associated with pigment abnormalities (Shah–Waardenburg syndrome; MIM 277580) (20). A similar combination of phenotypes occurs in horses with overo lethal white foal syndrome (8) and rats with the spotting lethal (*sl*) mutation (7), suggesting the possibility that these mutations may also involve either the *EDNRB* or *EDN3* gene.

Spotting lethal is a natural mutation described in 1979 in the progeny of a Wistar–Imamichi female and a wild male rat (7). Congenic wild-type and heterozygous animals have pigmented heads, backs, and tails. They appear healthy and normally fertile. Homozygous animals have coat pigment only in small spots on their heads or hips and die within 35 days after birth of intestinal obstruction. They have distal intestinal aganglionosis involving all or most of the large intestine. A minority of animals also have involvement of the distal small intestine. Here we report that the *sl* rat harbors a small deletion in the *EDNRB* gene. This results in aberrant splicing of the *EDNRB* mRNA that abrogates expression of functional receptor protein.

MATERIALS AND METHODS

Mutant Rats. The original inbred *sl*/+ rats (7) were provided by the Institute for Animal Reproduction (Omiya,

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Abbreviations: EDNRB, endothelin receptor type B; EDN3, endothelin 3; RT, reverse transcription; RFLP, restriction fragment length polymorphism.

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Japan), and maintained at the Westmead Hospital by brother–sister mating for at least 20 (males) to 40 (females) generations. Homozygous *sl/sl* rats showed an obvious phenotype of nearly completely white coat. Heterozygous and wild-type rats were initially genotyped by test mating, and congenic wild-type (+/+) rats were maintained by sibling intercross. Animal procedures used in this study were approved by the Animal Ethics Committee of the Westmead Hospital.

Radioligand Binding Assay. Membrane fractions were prepared from frozen rat tissues as described (18), and binding assays were carried out in triplicate with 10^{-11} M [^{125}I-Tyr^{13}]EDN1 (Amersham) as tracer, using 90 μg of membrane protein per reaction. Nonspecific binding was defined in the presence of 10^{-6} M unlabeled EDN1 and was ≈5% of total binding in the wild-type membranes. EDNRB binding was determined in the presence of the EDNRA-selective antagonist FR139317 (10^{-6} M) (21).

Cloning of *EDNRB* cDNA. RNA was extracted from frozen tissues (lung, heart, kidney, brain, and intestine) of 5-day-old *sl/sl* and congenic wild-type rats with the RNA STAT-60 reagents (Tel-Test, Friendswood, TX). For Northern blots, total RNA (20 μg per lane) was separated in a formaldehyde/1.1% agarose gel, transferred to a Hybond-N+ membrane (Amersham), and hybridized with random-primed ^{32}P-labeled full-length rat *EDNRB* cDNA (16). For reverse transcription (RT)-PCR, cDNA was synthesized from 2 μg of total RNA with oligo(dT)$_{12-18}$ primers by using SuperScript reverse transcriptase as recommended by the manufacturer (GIBCO/BRL). We used combinations of primers corresponding to nucleotides −106 to −86 and −66 to −46 in the 5′ noncoding region, and 1348–1367 and 1559–1583 in the 3′ noncoding region of the published rat *EDNRB* cDNA sequence (22). cDNA (0.5 μg) was amplified by using buffer F (PCR optimization kit; Invitrogen) for 35 cycles as recommended by the manufacturer. PCR products were cloned into pCR II plasmid (Invitrogen) and sequenced with an Applied Biosystems DNA Sequenator (model 373). Two independent clones derived from separate PCRs with different primer pairs were sequenced from both strands.

Analysis of *EDNRB* Gene. DNA was extracted for Southern blot analysis from frozen livers of 5-day-old *sl/sl* and congenic phenotypically normal rats by proteinase K digestion followed by phenol/chloroform extraction. DNA was digested completely with *Bam*HI, separated in a 1% agarose gel, transferred to a Hybond-N+ membrane, and probed with ^{32}P-labeled full-length rat *EDNRB* cDNA. A portion of the intron 1 sequence of wild-type *EDNRB* was cloned by inverted PCR as described (23). Briefly, DNA was digested with either *Bfa* I or *Nla* III (which cuts near the 3′ end of exon 1), and circularized with T4 DNA ligase at a DNA concentration of 1 ng/μl. Nested PCR primers were used to amplify the circularized fragments (5 ng) for 2 × 30 cycles with buffer B (Invitrogen). First-round primers for *Bfa* I digestion corresponded to nucleotides 346–366 and 377–396, and second-round primers corresponded to nucleotides 341–361 and 390–410 (22). Primers for *Nla* III digestion corresponded to nucleotides 412–431/432–452 (first round) and 402–422/447–467 (second round). PCR products were cloned into the pCR II plasmid and sequenced. To clone the corresponding portion of the *EDNRB^{sl}* allele, we constructed a size-selected genomic DNA library. DNA from a *sl/sl* rat was digested to completion with *Bam*HI and separated in a 0.8% agarose gel. DNA fragments between 2 and 4.3 kb were excised from the gel, removed from agarose by using GeneClean reagents (Bio 101), and further purified by gel filtration chromatography (Sephacryl S-500HR; Pharmacia). DNA fragments were ligated into *Bam*HI-digested, dephosphorylated arms of the ZAP Express λ phage vector (Stratagene) and packaged with Gigapack III Gold packaging extract (Stratagene). About 2 × 10^6 plaques from the unamplified library were screened by hybridization with a 0.4-kb probe fragment from the wild-type *EDNRB* obtained by inverted PCR (see above). DNA inserts from positive phage clones were *in vivo* excised into pBK-CMV plasmid and sequenced with specific internal primers.

RESULTS

Both heterozygous *sl/+* and congenic +/+ rats in our breeding colony lack pigment in ≈50% of their coats. Pigmented areas are restricted to the head, tail, and a black dorsal stripe. They are otherwise healthy. Homozygous *sl/sl* rats have dark eyes and a white coat (Fig. 1*A*). Occasionally, they have small pigmented areas on their heads and hips. They become ill and die usually within the first week after birth. Autopsy shows a narrowed distal intestine with proximal dilatation (Fig. 1*B*). Narrowed segments range from partial colonic to total colonic with ileal involvement. Histological examination confirmed the absence of myenteric ganglion cells in the distal intestine (data not shown) (7). The similarity of this phenotype to that observed in mice and humans with mutations of the *EDNRB* gene led us to investigate the rat strain for a mutation in *EDNRB*.

We first examined the presence of functional EDNRB in tissues from *sl/sl* and congenic wild-type rats by radioligand binding assays. EDNRBs were identified by the binding of labeled EDN1 in the presence of the EDNRA-selective antagonist FR139317 (21). A significant number of EDNRB binding sites were detected in lung and kidney membranes from wild-type rats (Fig. 2). EDNRB binding accounted for

FIG. 1. White spotting and megacolon in spotting lethal rats. (*A*) Coat color spotting in a *sl/sl* rat (*Upper*) and congenic homozygous wild-type rat (*Lower*). Note the near complete lack of coat pigment in the mutant animal. (*B*) Dissection of the entire intestine from a *sl/sl* rat (*Upper*) shows distension of the small intestine. Narrowed distal segments of bowel are aganglionic. Arrow indicates the gross anatomical "transitional" zone. (*Lower*) Corresponding specimen from a wild-type littermate. Note the well-formed, compact fecal pellets in the colon, which are absent from the *sl/sl* colon.

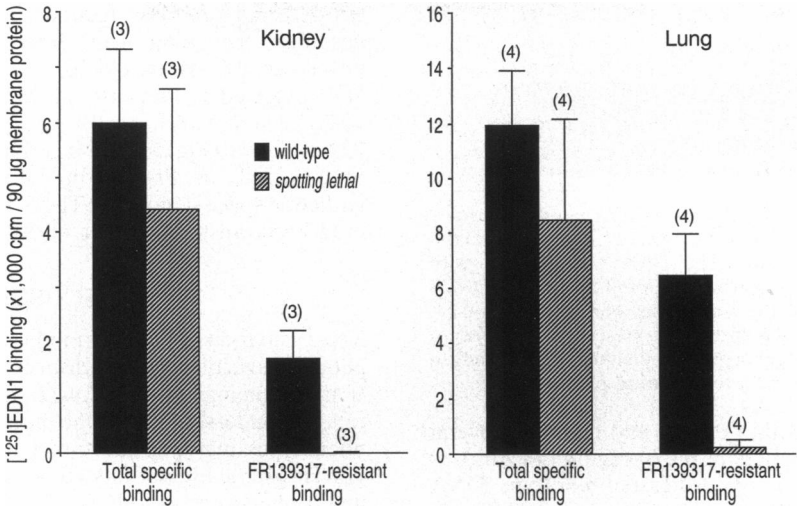

FIG. 2. Functional EDNRBs are undetectable in *sl* tissues. Lung and kidney membranes were prepared from *sl/sl* and congenic wild-type rats, and radioligand binding assays were performed. Specific binding of ^{125}I-EDN1 was determined in triplicate in the presence or absence of the EDNRA-selective antagonist FR139317. ^{125}I-EDN1 binding that was not displaced by FR139317 was used as an index of EDNRB density. Number of animals individually tested is given above each bar. Error bars represent SD.

54% ± 13% (mean ± SD) and 28% ± 9% of total EDN1 binding in wild-type lung and kidney membrane fractions, respectively. In contrast, we detected no appreciable EDNRB binding sites in the lung or kidney from *sl/sl* animals (<3% of total specific EDN1 binding).

To determine whether this absence of the receptor was the result of abnormal *EDNRB* mRNA expression, we performed Northern blot analysis from several tissues known to express EDNRB in the rat. We found that the expression of *EDNRB* mRNA in lung, brain, and gut was reduced in the *sl/sl* rats compared to normal littermates (Fig. 3). No obvious difference was observed in *EDNRB* mRNA levels in the heart and kidney. In some tissues, Northern blots seemed to suggest a slight increase in the electrophoretic mobility of the *EDNRB* mRNA in *sl/sl* rats compared to the wild-type mRNA (e.g., see lung and brain lanes in Fig. 3).

To further investigate the structure of the *EDNRB* mRNA in *sl/sl* rats, we cloned the cDNA from the lung by RT-PCR. Four different pairs of PCR primers covering the entire EDNRB coding region successfully amplified the *sl* and wild-type *EDNRB* mRNA. RT-PCR from the *sl/sl* animal using several different primer pairs consistently yielded amplified cDNA products that were ≈0.3 kb smaller than the corresponding products from the wild-type rat (data not shown). Restriction mapping of these products revealed the absence of

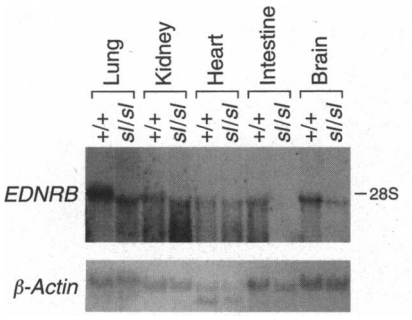

FIG. 3. Reduced levels of *EDNRB* mRNA in *sl* rat. Total RNA was extracted from the designated tissues of wild-type and *sl/sl* rats. RNA was separated in a formaldehyde/1.1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a random-primed full-length rat *EDNRB* cDNA probe and washed in 2× SSC/0.5% SDS at 65°C. The membrane was subsequently rehybridized with a β-actin cDNA.

a *Bgl* I site that was present in the wild-type cDNA at nucleotide 428 on exon 1 (22). We therefore sequenced the RT-PCR products from two different primer pairs, focusing on regions spanning exons 1 and 2. Sequence analysis showed that the *sl* cDNA has a 270-bp deletion starting at nucleotide 213 in exon 1 (see Fig. 5). The entire 3′ end of exon 1 from this point was deleted, but the exon 2 sequence was intact. The deletion encompassed the coding sequence for the first two predicted transmembrane domains of the receptor protein. Wild-type sequence corresponding to the 5′ end of the *sl* deletion, GAG/GTGACC (slash denotes the break point), closely matched the consensus sequence for a splice donor site, (A/C)AGgt(a/g)agt (intron in lowercase letters and obligatory nucleotides underlined). These findings suggested that this cryptic splice donor site may be activated due to a mutation in *sl* rats involving this donor site or the authentic exon 1–intron 1 junction.

To establish that a mutation of *EDNRB* is responsible for the observed phenotype in *sl* rats, we examined *sl/sl* animals and phenotypically normal congenic animals for restriction fragment length polymorphism (RFLP) in the *EDNRB* gene. We initially digested DNA samples from two *sl/sl* animals and two phenotypically normal animals separately with several restriction enzymes and performed Southern blots using a rat *EDNRB* cDNA as a probe. We identified RFLPs for *Pst* I (wild type, 3.2 kb; *sl*, 2.9 kb), *Hin*dIII (wild type, 1.7 kb; *sl*, 1.4 kb), *Xba* I (wild type, 2.5 kb; *sl*, 2.2 kb), *Eco*RI (wild type, 3.3 kb; *sl*, 3.0 kb), and *Bam*HI (wild type, 2.6 kb; *sl*, 2.3 kb). In every case, the normal animals had a hybridizing fragment ≈0.3 kb larger than the *sl/sl* animals, suggesting a possible 0.3-kb deletion in the *sl* chromosome. We further performed *Bam*HI Southern blots with DNA from 11 *sl/sl* animals and 8 phenotypically normal congenic littermates. These blots showed that this *Bam*HI RFLP perfectly cosegregates with the phenotype (Fig. 4). None of the affected animals had the 2.6-kb *Bam*HI fragment. Half of the phenotypically normal animals had both the 2.3- and 2.6-kb bands and are therefore heterozygous. The remainder of the phenotypically normal animals had only the larger band and are therefore homozygous for the wild-type allele.

The aforementioned RT-PCR results taken together with the Southern blot findings suggested a likely 0.3-kb deletion in *EDNRB*, involving the authentic exon 1 splice donor site. We then obtained the wild-type exon 1–intron 1 junction sequence by using inverted PCR. We identified several frequent-cutter

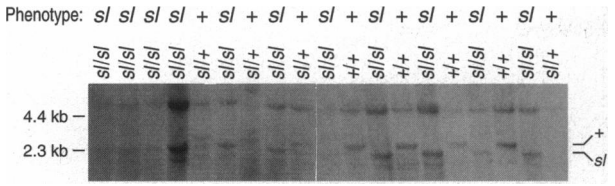

FIG. 4. A RFLP in the *EDNRB* gene cosegregates with the *sl* phenotype. DNA was extracted from the livers of 11 *sl/sl* rats and 8 phenotypically normal littermates. The DNA was digested with *Bam*HI and separated in a 0.8% agarose gel. A full-length rat *EDNRB* cDNA was used as probe, and the membranes were washed in 0.2× SSC/0.1% SDS at 65°C. The 2.6-kb band is present only in phenotypically normal animals. The genotype of individual animals judged by RFLP is indicated together with their clinical phenotype.

endonucleases with sites within exon 1 and designed several pairs of nested inverted primers in the 3′ region of exon 1 as described. Inverted PCR of *Bfa* I-digested wild-type DNA amplified a single fragment of ≈0.4 kb containing the 90-bp 3′ end of exon 1 and subsequent intron sequence. A similar single fragment of 0.45 kb that also contained the exon 1–intron 1 junction was amplified when *Nla* III was used for initial digestion. However, we consistently failed to amplify *sl/sl* DNA in parallel experiments. We then reprobed the *Bam*HI Southern blots with the wild-type *Nla* III inverted PCR product. This probe hybridized with the above-mentioned polymorphic *Bam*HI fragment from both the homozygous *sl/sl* and wild-type rat (data not shown). We used the same probe to further define *Hae* III, *Xba* I, and *Eco*RI RFLPs. The probe identified a single 1-kb *Hae* III fragment in the wild-type rat and a 0.7-kb *Hae* III fragment in the *sl/sl* rat. This probe also hybridized with the previously identified *Xba* I and *Eco*RI polymorphic fragments. These results strongly suggested that the *sl* chromosome has an ≈0.3-kb deletion near or including the exon 1–intron 1 junction.

To define the genomic break points, we cloned the polymorphic *Bam*HI fragment from the *sl* rat. We constructed a size-selected, *Bam*HI-digested *sl* genomic DNA library as described. Screening of this library with the exon 1–intron 1 probe gave 18 positive clones, 3 of which were plaque purified and subjected to further analysis. Nucleotide sequencing revealed that *sl* DNA has a 301-bp deletion starting at nucleotide 229 of exon 1 (Fig. 5). The deletion spans the entire 3′ end of exon 1 and the first 44 bp into intron 1, abrogating the authentic splice donor site. The 5′ end of the genomic deletion is 15 bp downstream of the cDNA deletion.

DISCUSSION

Several hereditary rodent models exist for congenital aganglionic megacolon or Hirschsprung disease. They are s^l mice, *ls* mice, dominant megacolon (*Dom*) mice, and spotting lethal rats. An understanding of the molecular basis of these mutant phenotypes may provide direct insight into the etiology of Hirschsprung disease. Indeed, we recently reported that the s^l and *ls* phenotype as well as a familial form of human Hirschsprung disease are caused by mutations involving the EDNRB/EDN3 receptor–ligand pair (18–20). In this paper, we have extended these findings and shown that the *EDNRB* gene is also responsible for the *sl* mutant phenotype in the rat. We showed that RFLPs within *EDNRB* cosegregate with the recessive *sl* phenotype, supporting a causal relationship. A 301-bp chromosomal deletion in the $EDNRB^{sl}$ allele was revealed, including the last 265 bp of exon 1. The absence of the remaining intron 1 sequence (and of an additional 5′ 15 bp of exon 1) from the $EDNRB^{sl}$ cDNA, together with the similarity of the sequence at the 5′ end of the cDNA deletion to a splice donor consensus sequence, indicate the use of a cryptic splice donor site in mRNA processing in *sl*. A decrease in the *EDNRB* mRNA expression compared to wild-type animals is also demonstrated. Reduced mRNA expression is demonstrated in those tissues that normally express high levels of EDNRB (16) (see Fig. 3). The apparent tissue-specific variation in the levels of the mutant *EDNRB* mRNA suggest

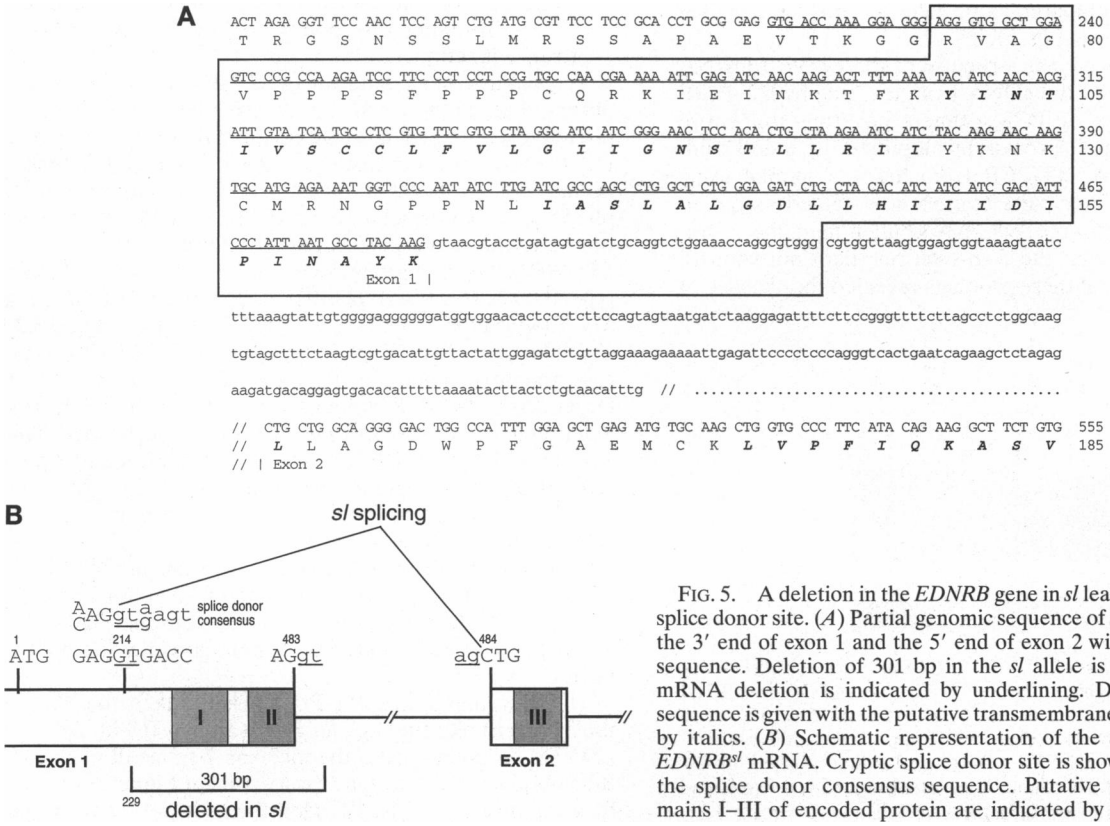

FIG. 5. A deletion in the *EDNRB* gene in *sl* leads to use of a cryptic splice donor site. (*A*) Partial genomic sequence of rat *EDNRB* showing the 3′ end of exon 1 and the 5′ end of exon 2 with a partial intron 1 sequence. Deletion of 301 bp in the *sl* allele is indicated by a box. mRNA deletion is indicated by underlining. Deduced amino acid sequence is given with the putative transmembrane segments indicated by italics. (*B*) Schematic representation of the aberrant splicing of $EDNRB^{sl}$ mRNA. Cryptic splice donor site is shown in comparison to the splice donor consensus sequence. Putative transmembrane domains I–III of encoded protein are indicated by shaded boxes.

that the effect of the mutation on processing efficiency or stability of the transcripts may vary in different tissues. EDN1 binding studies with kidney and lung membranes demonstrate that the *sl* mutation completely abrogates production of functional EDNRB. This is consistent with the finding that the mRNA deletion removes the coding sequence for the first two transmembrane domains of the heptahelical receptor. Deletion of any of the seven transmembrane domains of the β-adrenergic receptor has been shown to abrogate functional expression (24). Thus, we establish the molecular basis of the *sl* mutation and the critical importance of EDNRB for normal development of epidermal melanocytes and enteric neurons in rats. The description in the original report (7) that the recessive *sl* phenotype was observed in multiple F_2 offspring from a normal laboratory female rat and a male rat of unknown wild origin is consistent with the idea that the *sl* allele was inherited from this wild rat.

EDNRB accepts all three endothelin isopeptides with equal affinity. We previously demonstrated that mice with targeted and natural (*ls*) mutations of the *EDN3* gene also exhibit coat color spotting and aganglionic megacolon (19). This indicates that EDN3 is the physiologically relevant ligand of EDNRB for development of epidermal melanocytes and enteric neurons in mice. Presumably, the interaction of EDN3 with EDNRB is also essential for the development of these cell lineages in rats. In contrast, *EDN1* "knockout" mice exhibit a distinct set of developmental abnormalities including craniofacial defects due to failure of normal development of the cephalic neural crest-derived mesenchyme of pharyngeal arch tissues (25). The expression of coat color spotting and aganglionic megacolon in EDN3-deficient mice indicates that EDN1 cannot compensate for the function of EDN3 in the development of epidermal melanocytes and enteric ganglia, even though EDN1 levels in the plasma are higher than EDN3 (26, 27). This suggests that, although endothelin peptides are small soluble extracellular molecules, they do not function as circulating hormones in these developmental situations.

When and where the critical EDNRB–EDN3 interaction occurs requires further investigation. The skin phenotype in mice with mutations of the *EDNRB* gene is caused by a disruption in the migration, proliferation, and/or differentiation of precursors of melanocytes (28, 29). Pavan and Tilghman (30) recently showed that the s^l mutation acts shortly after neural crest cells leave the neural plate and prevents the appearance of melanoblasts identifiable by the differentiation marker TRP-2. Previous studies showed that EDNRB is expressed by cultured melanocytes and melanoma cell lines and stimulation of these receptors leads to proliferation and chemokinesis (31, 32), supporting the idea that mutations of *EDNRB* act in neural crest-derived precursor cells.

In the intestine, the lack of EDN3/EDNRB signaling may reduce the initial number, proliferation, survival, and/or migration potential of the enteric neuroblasts. In EDN3- and EDNRB-deficient animals, the defect is confined to the colon, with the small intestine almost always innervated normally. This suggests that EDN3/EDNRB signaling normally occurs relatively late in intestinal development. The possibility cannot be excluded, however, that EDNRB activation takes place earlier when cells migrate from the vagal neural crest through the proximal intestine. Such activation might be needed to preprogram the crest-derived cells for successful colonization of the colon.

Studies with aggregation chimeras between wild-type and *ls/ls* embryos labeled with transgenic or endogenous markers demonstrate that the *ls/ls* neuroblasts can migrate normally (together with wild-type neuroblasts) to the rectum (33, 34). These studies also show that neuroblast migration is dependent on the proportion of *ls/ls* neuroblasts present in the chimeric gut. In chimeric animals where the *ls/ls* neuroblasts predominate, there is a failure of migration of both the wild-type and mutant neuroblasts. When the wild-type neuroblasts predominate, both the wild-type and mutant neuroblasts migrate normally. More recently, Kapur obtained similar results in analogous studies with chimeras between s^l/s^l and wild-type embryos (41). Thus, the s^l/s^l neuroblasts migrate normally to the rectum together with wild-type neuroblasts in chimeras that do not exhibit aganglionosis. Both the wild-type and s^l/s^l neuroblasts fail to colonize the distal intestine in those chimeric mice that manifest aganglionic megacolon. These findings indicate a nonneuroblast autonomous action of the *ls* and s^l mutations. On the other hand, human myenteric ganglia have been shown to express EDNRB and endothelin immunoreactivity (35). Our preliminary *in situ* hybridization experiments also suggest that EDNRB is expressed by enteric neuroblasts in mice.

Collectively, these findings suggest that intercellular signals downstream of the activation of EDNRB by EDN3 are important for colonization of the hindgut by neural crest-derived cells. This critical intercellular signaling may occur between neuroblasts or between neuroblasts and non-crest-derived mesenchyme. Studies by Jacobs–Cohen and colleagues (36) show that primary cultures of hindgut from *ls/ls* embryos will not support normal colonization by wild-type or mutant neuroblasts, in contrast to similar cultures of wild-type hindgut. Gershon and colleagues (37) have proposed that an excess of laminin expression in the developing *ls* hindgut may promote premature differentiation of the neuroblast prior to completion of migration. Endothelins can influence the production of extracellular matrix components (38). These observations suggest the possibility that regulation of extracellular matrix components may represent the hypothetical downstream effect of EDNRB stimulation. Mutations in the gene encoding RET, an orphan receptor tyrosine kinase, and an unidentified gene (*Dom*) also impair colonization of the gut by enteric neuroblasts (6, 39). RET is expressed in neural crest-derived enteric neuroblasts from their early stages of colonization (40). The *Dom* gene product and the hypothetical ligand for RET are obvious candidates for the secondary intercellular signal produced by cells expressing EDNRB.

The *EDNRB* gene is clearly important for the development of epidermal melanocytes and enteric neurons in the mouse, human, and rat. However, it has remained controversial what homeostatic roles EDNRB plays in the adult animal. EDNRB is expressed in a variety of tissues including endothelial cells, vascular, airway and intestinal smooth muscles, myocardium, neurons in peripheral and central nervous systems, certain endocrine tissues, and airway and renal epithelial cells. EDNRB stimulation exerts a whole variety of biological effects in these cells, including vasodilatation, vasoconstriction, bronchoconstriction, stimulation and inhibition of hormone release, cellular proliferation, regulation of contractility of myocardium and gastrointestinal smooth muscle, and modulation of ion transport in kidney and gut (10). Animals lacking EDNRB may therefore prove invaluable in studies of adult physiology in health and disease. Many physiological parameters can be more accurately determined in rats than in mice. Indeed, the rat has been the species in which the largest body of information has been accumulated regarding the pharmacological actions of EDNRB agonists and antagonists (14). If the *sl* rat can be rescued from the lethal megacolon phenotype by delivery of a transgene, surgery, or other means, it may prove valuable in our understanding of (patho)physiology in which endothelin receptor type B plays a significant role.

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