Molecular characterization of four induced alleles at the Ednrb locus

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ABSTRACT The piebald locus on mouse chromosome 14 encodes the endothelin-B receptor (EDNRB), a G protein-coupled, seven-transmembrane domain protein, which is required for neural crest-derived melanocyte and enteric neuron development. A spontaneous null allele of Ednrb results in homozygous mice that are predominantly white and die as juveniles from megacolon. To identify the important domains for EDNRB function, four recessive juvenile lethal alleles created by either radiation or chemical mutagens (Ednrb\(^{17FrS}\), Ednrb\(^{1Chlc}\), and Ednrb\(^{Chlb}\)) were examined at the molecular level. Ednrb\(^{17FrS}\) mice harbor a mutation at a critical proline residue in the fifth transmembrane domain of the EDNRB protein. A gross genomic alteration within the Ednrb gene in Ednrb\(^{Chlc}\) results in the production of aberrantly sized transcripts and no authentic Ednrb mRNA. Supporting previous observations that the degree of spotting in piebald mice is dependent on the amount of EDNRB expressed. Finally, no molecular defect was detected in Ednrb\(^{Chlb}\) mice, which produce normal levels of Ednrb mRNA in adult brain, suggesting that the mutation affects important regulatory elements that mediate the expression of the gene during development.

The piebald Ednrb locus on mouse chromosome 14 encodes the endothelin B receptor (EDNRB), a G protein-coupled, seven-transmembrane (TM) domain receptor that recognizes a family of small peptides known as endothelins, EDN1, EDN2, and EDN3 (1). Mice homozygous for deletion of the Ednrb gene, Ednrb\(^{-}\), are almost completely white with only small pigmented areas on the head and rump due to an early disruption in the development of neural crest-derived melanocytes (2, 3). In addition, these mice usually die 2–3 weeks after birth from megacolon resulting from the absence of enteric neurons in the distal end of the colon (1, 4). A less severe allele of the gene, Ednrb\(^{y}\), generates mice with 10–20% white spotting due to reduced expression of Ednrb mRNA and only rarely exhibiting megacolon. Mutations in Ednrb have been shown to be responsible for a subset of patients with piebaldism, a genetic disorder characterized by aganglionic megacolon (5, 6).

The biologically relevant ligand for EDNRB is probably EDN3, the product of the Edn3 (lethal spotting) gene on mouse chromosome 2. Mice with either a spontaneous or a targeted mutation in Edn3 are almost identical in phenotype to mice lacking EDNRB function (7, 8). Furthermore, recent studies have indicated that EDN3 promotes survival and differentiation of melanoblasts isolated from the neural tube (9, 10).

In addition to the two spontaneous Ednrb alleles, a large number of radiation- and chemical-induced mutations have been isolated in the specific locus test (SLT) at the Oak Ridge National Laboratory (11). In the SLT, mutagenized males were bred to a tester stock homozygous for seven phenotypically visible recessive mutations: non-agouti, brown, chinchilla, pink-eyed dilution, dilute, short ear, and piebald. The mutations that were recovered in the SLT have been invaluable in cloning the target genes, as well as neighboring genes that are deleted in some of the mutations (12–15). They also provide an opportunity to identify important residues, regulatory regions, and mechanisms that are critical for the function of these genes. Furthermore, the molecular analysis of the alleles has yielded insight into the nature of the alterations in the mammalian genome caused by a large variety of mutagens (16, 17).

The induced alleles at piebald fall into two phenotypic classes: a juvenile lethal class that resembles the amelanocytic and aganglionic Ednrb\(^{-}\) phenotype and a second class of mice that die at birth or earlier. The second class is characterized by genomic deletions that extend beyond Ednrb itself and remove outside markers (18, 19). To determine the molecular alterations responsible for four juvenile-lethal piebald mutants (Ednrb\(^{27Pub}\), Ednrb\(^{17FrS}\), Ednrb\(^{1Chlc}\), and Ednrb\(^{Chlb}\)) induced with either radiation or chemicals, both the genomic structure and the expression of Ednrb were analyzed. These studies identified a point mutation in a critical residue that is required for EDNRB function, a probable inversion within the gene itself and two regulatory mutations that affect the expression of the gene. In addition, the nature of molecular changes observed in this study provides additional information about the mechanism of action of mutagens on the germline.

MATERIALS AND METHODS

Mouse Strains. The induced piebald alleles were generated at the Oak Ridge National Laboratory as described (11). The mutant mice, generated by treatment of (101/R1 \(\times\) C3H/R1) F\(_1\) hybrid male mice with radiation or chemicals, were bred to the Tester stock with the Ednrb\(^{y}\) allele. The presence of an induced mutation at Ednrb was detected by identifying an offspring with spotted coat. Four heterozygous mutant stocks derived and propagated at Oak Ridge were expanded by mating with SSL/Le Ednrb\(^{y}\)/Ednrb\(^{y}\) mice. SSL/Le Ednrb\(^{y}\)/Ednrb\(^{y}\) and C57BL/6J mice were obtained from the Jackson Laboratory. Ednrb\(^{y}\) and Ednrb\(^{y}\) homozygous mice were obtained by intercrossing SSL/Le Ednrb\(^{y}\)/Ednrb\(^{y}\) mutants.

The PCR-mediated genotyping of Ednrb\(^{27Pub}\) mice with the tightly linked D14Mit8 microsatellite marker has been described (19, 20). The same genotyping assay was used for the Ednrb\(^{1Chlc}\), Ednrb\(^{Chlb}\), and Ednrb\(^{17FrS}\) mutants.

RNA Isolation and Analysis. Total RNA was extracted from brains of 3- to 6-week-old C57BL/6J and homozygous SSL/Le Ednrb\(^{y}\), Ednrb\(^{27Pub}\), Ednrb\(^{1Chlc}\), Ednrb\(^{Chlb}\), and Ednrb\(^{17FrS}\) mutants.

Abbreviations: SLT, specific locus test; UTR, untranslated regions; Ednrb, endothelin-B receptor; Edn3, endothelin 3; TM, transmembrane.

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mice using Trizol reagent (GIBCO/BRL). Poly(A) RNA was purified by use of the Fast Tract system (Invitrogen). Approximately 1 µg of each sample was loaded on a 1% agarose/formaldehyde Mops gel and blotted onto Hybond N+ membrane according to the manufacturer’s instructions (Amersham Life Science). The blot was hybridized in Rapidhyb (Amersham Life Science) to a radiolabeled cDNA probe that encompassed the Ednrb coding region. The blot was stripped and rehybridized with a β-actin cDNA probe as a control for loading. The Northern blot was quantitated by PhosphorImager (DuPont).

DNA Isolation and Analysis. Genomic DNA was isolated from livers of homozygous mice by use of a standard protocol (21). Approximately 15–20 µg of DNA was digested with restriction enzymes under appropriate conditions (New England Bio-Labs). DNA samples were loaded onto a 1% agarose gel in Tris-acetate running buffer. The DNA samples were transferred to nylon filters and hybridized as described above.

Reverse transcriptase-PCR and DNA Sequence Analysis. Poly(A) RNA was transcribed with Superscript II reverse transcriptase with an oligo-dT primer according to the manufacturer’s protocol (GIBCO/BRL). The portion of the Ednrb cDNA covering the ORF was amplified with 5'- and 3'-specific oligonucleotide primers described previously (1). The PCR products were cloned into PCR II plasmid vector (Invitrogen) and sequenced by the dideoxy chain termination method with a radiolabeled oligonucleotide primer (BRL). The portion of the Ednrb coding region was identical to the wild-type Ednrb sequence reported (1). A single point mutation was observed in Ednrb mRNA in mutant mice. One microgram of poly(A) mRNA from the brains of juvenile mice was separated by use of a CHEF Mapper gel electrophoresis system (Bio-Rad). The DNA samples were transferred overnight to Hybond N+ and hybridized as described above.

Pulsed-Field Gel Electrophoresis (PFGE) Analysis. Spleen cells from C57BL/6J and Ednrb mutant mice were isolated, embedded in low-melt agarose blocks, and digested with the appropriate enzymes as described (23). The digested DNA samples were separated on a 1% agarose gel (24) by use of a CHEF Mapper gel electrophoresis system (Bio-Rad). The DNA samples were transferred overnight to Hybond N+ and hybridized as described above.

RESULTS

Expression of Ednrb mRNA. The Oak Ridge juvenile lethal mice homozygous for induced mutations in Ednrb are almost completely white and die as juveniles from megacolon. They closely resemble Ednrb1-4 mice in which the Ednrb gene is deleted completely (1). The alleles generated by the SLT were produced either by radiation or the chemical mutagen chlorambucil (Table 1). Because these mutations have been reported to cause large lesions in the genome (16), it was likely that at least some of these mutants were deleted for the Ednrb gene and, therefore, would not express Ednrb mRNA. To determine if this were the case, poly(A) RNA from brains of 3- to 6-week-old C57BL/6J and homozygous mutant mice were examined by Northern analysis with the coding region of the Ednrb cDNA as a probe (Fig. 1). As expected, Ednrb1-4 mice did not express Ednrb mRNA. Ednrb1T7F5S mice exhibited a greatly decreased level of Ednrb mRNA (5% of the wild type) whereas Ednrb2Chlo mutants failed to express any full length transcript but rather displayed two transcripts, 650 bp and 3.9 kb in length. By use of exon-specific probes, it was determined that the 650-bp transcript contained only the first exon whereas the larger transcript contained the rest of the coding region (data not shown; see Fig. 4A). Similar results were obtained with other Ednrb-expressing tissues (data not shown). Finally, Ednrb27Pub and Ednrb1Chlo mutants expressed normal levels of Ednrb mRNA. Thus, none of the four juvenile lethal alleles is a deletion of the gene itself.

Sequence Analysis of Ednrb Transcripts. To examine whether mutations in the Ednrb coding region were responsible for the coat color and megacolon phenotype in Ednrb27Pub, Ednrb1T7F5S and Ednrb1Chlo mice, primers from the 5'- and 3'-untranslated regions (UTRs) were used to amplify Ednrb cDNA transcribed from brain mRNA. Sequence analysis of Ednrb2Chlo and Ednrb7T7F5S cDNA revealed that the Ednrb protein coding region was identical to the wild-type C57BL/6J sequence reported (1). A single point mutation was observed in Ednrb27Pub in the fifth TM domain of the protein. This C-to-T transition at nucleotide 854 of the coding region resulted in a proline-to-leucine change at residue 285 (P285L).

Table 1. Origin and phenotype of the juvenile lethal piebald (Ednrb) alleles

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Mutagen</th>
<th>Treated germ cell stage</th>
<th>Homozygous phenotype</th>
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<tbody>
<tr>
<td>Ednrb1</td>
<td>Spontaneous</td>
<td>Viable</td>
<td>White spotting, %</td>
</tr>
<tr>
<td>Ednrb1-4</td>
<td>Spontaneous</td>
<td>Viable</td>
<td>10–20</td>
</tr>
<tr>
<td>Ednrb1T7F5S</td>
<td>Radiation (γ-rays)</td>
<td>Stem cell spermatagonia</td>
<td>Juvenile lethal &gt;90</td>
</tr>
<tr>
<td>Ednrb2Chlo</td>
<td>Chlorambucil</td>
<td>Early spermatids</td>
<td>Juvenile lethal &gt;90</td>
</tr>
<tr>
<td>Ednrb2Chlo</td>
<td>Chlorambucil</td>
<td>Early spermatids</td>
<td>Juvenile lethal &gt;90</td>
</tr>
<tr>
<td>Ednrb27Pub</td>
<td>239Pu-citrate (α-rays)</td>
<td>Stem cell spermatagonia</td>
<td>Juvenile lethal &gt;90</td>
</tr>
</tbody>
</table>

FIG. 1. Expression of Ednrb mRNA in mutant mice. One microgram of poly(A) mRNA from the brains of juvenile mice was separated on an agarose gel, transferred to a membrane filter, and hybridized with a radiolabeled Ednrb cDNA probe. Lanes 1–6 are, in order: C57BL/6J, Ednrb1-4, Ednrb1T7F5S, Ednrb1Chlo, Ednrb2Chlo, and Ednrb27Pub homozygous mice. The blot was stripped and rehybridized with β-actin cDNA as a control.
in the amino acid sequence (Fig. 2). This mutation was confirmed in genomic DNA because it creates a restriction enzyme polymorphism, introducing a \textit{BbvI} site (data not shown). It is highly unlikely that this amino acid substitution is simply a strain-specific polymorphic difference between the wild-type C57BL/6J and \textit{Ednrb}27\textsubscript{Pub} mice because this difference was not found in the other Oak Ridge-derived mice.

**Analysis of the \textit{Ednrb} Genomic Locus.** To determine whether there were changes in the genomic structure at \textit{Ednrb} that could account for the mutant phenotypes in \textit{Ednrb}17FrS, \textit{Ednrb}3Chlo, and \textit{Ednrb}1Chlc, Southern analysis was performed. Genomic DNA from the mutants and C57BL/6J mice were digested with several restriction enzymes and hybridized with the coding region of \textit{Ednrb} cDNA. No alterations were observed for \textit{Ednrb}27\textsubscript{Pub}, \textit{Ednrb}17FrS, or \textit{Ednrb}1Chlc. A difference, however, was observed in the size of the largest \textit{BamHI} fragment in \textit{Ednrb}3Chlo DNA (Fig. 3A). This \textit{BamHI} band contains exon 1 and the first intron; further restriction mapping suggested that the difference occurred within the large, conserved, 20- to 25-kb first intron of the gene (Fig. 4A; ref. 25). To further localize the mutation, a 2.4-kb \textit{NcoI}/\textit{HindIII} fragment (probe B in Fig. 4A) located \textasciitilde7.0 kb downstream of the first exon was hybridized to genomic DNA. It detected restriction fragment length polymorphisms between C57BL/6J and \textit{Ednrb}3Chlo with every restriction enzyme tested (Fig. 3B). In most cases, probe B hybridized to a single fragment in C57BL/6J DNA and to two different bands in \textit{Ednrb}3Chlo DNA. This result, combined with the observation that \textit{Ednrb}3Chlo produces two different truncated transcripts, suggests that an inversion, with one breakpoint located within the 2.4-kb \textit{NcoI}/\textit{HindIII} probe, has occurred, thereby separating exon 1 from the rest of the gene. All other mutants displayed restriction patterns identical to wild-type DNA with all the probes tested.

The Southern analysis was limited to the genomic structure of the immediate vicinity of the \textit{Ednrb} gene. To detect any changes in larger genomic regions in \textit{Ednrb}17FrS and \textit{Ednrb}1Chlc.
When probe B was used, no changes were observed for generated at Oak Ridge National Laboratory were analyzed probe B detected changes in Not digested with either detected in Ednrb Ednrb1–4 correspond to DNA from C57BL enzymes indicated and separated on a pulsed-field gel. After transfer (exon is indicated. Below the line are the positions of probes A and B. represent the exons of the gene. The EdnrbEdnrbBss EdnrbBssHII homozygous mice, respectively. The size standards repre- sent the Saccharomyces cerevisiae chromosomes (Bio-Rad).

and to further confirm that an inversion has occurred in EdnrbEdnrb3Chlo, pulsed-field gel electrophoresis was performed. A 680-kb BssHII fragment was detected in C57BL/6J DNA and all mutant DNAs with probe A, which hybridizes ~3.5 kb upstream of the BssHII site in the 5′ UTR (data not shown). When probe B was used, no changes were observed for EdnrbEdnrb17FrS and EdnrbEdnrb3Chlo mutants (Fig. 4B). As expected, probe B detected changes in EdnrbEdnrb3Chlo DNA. A 570-kb BssHII fragment was detected in C57BL/6J DNA whereas 650- and 150-kb BssHII bands were obtained in EdnrbEdnrb3Chlo DNA. Similarly, a 850-kb fragment was observed in C57BL/6J DNA digested with either NotI or EagI, but two different bands were detected in EdnrbEdnrb3Chlo. The same patterns were observed when the blot was hybridized with a cDNA probe covering exons 2–7 (data not shown). Thus, this long range restriction analysis lends additional support to the proposal that the EdnrbEdnrb3Chlo mutation is caused by an inversion, with one of the breakpoints occurring within the gene itself. Furthermore, the analysis rules out gross genomic alterations in EdnrbEdnrb17FrS and EdnrbEdnrb3Chlo mutants, at least within ~1.25 megabases (sum of the BssHII fragments detected by probe A and B) surrounding the Ednrb locus.

**DISCUSSION**

In this study, four recessive juvenile-lethal piebald alleles generated at Oak Ridge National Laboratory were analyzed for mutations at the Ednrb locus (summarized in Table 2). Phenotypically, these mice are indistinguishable from EdnrbEdnrb1 mutant mice, which harbor a complete deletion of the Ednrb gene (Table 1). The EdnrbEdnrb2Pub allele is a C to T transition at nucleotide 854 of the Ednrb-coding region, which leads to a proline-to-leucine substitution at amino acid residue 285 (Fig. 2). This proline, situated in the TM domain V, is conserved among both endothelin-B receptors and endothelin-A receptors as well as among more distantly related members of the G protein-coupled seven-TM family (26, 27).

The highest level of sequence conservation in endothelin receptor family members is contained within the TM spanning domains (27). Biochemical studies have demonstrated that different TM domains contribute to ligand binding and intracellular signal transduction properties of the receptors (28, 29). To date, nine mutations in the coding regions of the Ednrb gene have been identified in human patients with Hirschsprung disease (5). Three of the nine mutations lead to premature termination of the protein whereas the other six are missense mutations. As shown in Fig. 2B, five of these nine mutations occur near TM V (three within the TM V and two within the intracellular domain connecting TM V and TM VI). Although the sample size is small, mutations identified to date suggest that this region is critical for EDNRB function. One of these mutations in TM V, W276C, has been demonstrated to affect the intracellular signal response of EDNRB (29). One of the six missense mutation occurs in a highly conserved proline (P383) in the helix of TM VII (30). There are conserved prolines in TM helices IV, V, VI, and VII of the seven-TM receptor family members, and these prolines have been suggested to play a role in the packing of the helices (26). Therefore, mutations at these prolines would be expected to disrupt the arrangement of the helices and cause drastic changes in the structure of the receptors.

The chemotherapeutic agent chlorambucil has been shown to induce gross chromosomal rearrangements such as deletions and translocations in post-stem cell stages of spermatogenesis (31, 32). Thus, it was not unexpected that a chromosomal rearrangement within the first intron of Ednrb was found in EdnrbEdnrb3Chlo mice (Fig. 4A). We favor the likelihood that the rearrangement is an inversion because there is no clear reduction in the litter sizes born to EdnrbEdnrb3Chlo heterozygotes, which would have been expected in most animals with translocation (32). The Southern analysis in Fig. 3, in which the copy number and organization of the rest of the Ednrb gene appear to be unaffected, also lends support to the notion that the rearrangement is an inversion. This alteration in the genome has resulted in the separation of exon 1 from rest of the coding region. The first exon encodes the N-terminal extracellular domain, as well as TM I and TM II, whereas exons 2–7 encode the rest of the protein. It is unlikely that the two aberrant EdnrbEdnrb3Chlo transcripts produce a functional protein because studies with β-adrenergic receptor family members suggest that disruption of any of the seven TM domains will have a deleterious effect on receptor function (33). The 5′ transcript, which presumably initiates at the authentic Ednrb promoter, is 650 bp in length and contains only a small portion of the functional receptor. The larger 3′ transcript is probably initi- ating from a cryptic promoter near the inversion breakpoint because the level of this transcript is very low in all cells examined (Fig. 1); however, we cannot rule out the possibility

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<tr>
<th>Alleles</th>
<th>Genomic alteration</th>
<th>RNA</th>
<th>Sequence difference in coding region</th>
<th>Nature of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdnrbEdnrb2Pub</td>
<td>No</td>
<td>Normal</td>
<td>Yes</td>
<td>CCG to CTG/P285L</td>
</tr>
<tr>
<td>EdnrbEdnrb17FrS</td>
<td>No</td>
<td>Decreased</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>EdnrbEdnrb3Chlo</td>
<td>Yes</td>
<td>Truncated transcripts</td>
<td>No</td>
<td>Chromosomal inversion</td>
</tr>
<tr>
<td>EdnrbEdnrb27Pub</td>
<td>No</td>
<td>Normal</td>
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that the gene is transcribed from a neighboring promoter with the resulting transcript being unstable.

Ednrb function, as revealed by the degree of spotting, has been shown to be highly sensitive to gene dosage. Mice homozygous for the milder spontaneous Ednrb allele, which expresses \( \approx 25\% \) of wild-type Ednrb mRNA levels, exhibit 10–20\% spotting. A heteroallelic combination of Ednrb\(^{f1}\)/Ednrb\(^{b1}\), which would express \( \approx 12.5\% \) of normal mRNA levels, displays an intermediate phenotype of 40–60\% spotting, and Ednrb\(^{b4}\) homozygotes are almost completely white. Megacolon, on the other hand, almost exclusively occurs in the Ednrb\(^{f1}\)/Ednrb\(^{b1}\) mice (4, 7). On the basis of these observations, it has been suggested that developing melanoblasts and enteric neuroblasts may respond to different concentrations of EDNRB (1). In Ednrb\(^{f1}\)/Ednrb\(^{b1}\) homozygotes, the level of the Ednrb transcript is greatly reduced, to \( < 5\% \) of wild type levels. This reduction is likely due to either a mutation in a transcriptional regulatory element of the Ednrb gene or a change within the gene that affects the stability of the transcript. Of interest, a similar effect was observed in a radiation-induced recessive viable agouti allele in which agouti mRNA was decreased while the genomic structure and the sequence of the cDNA appeared to be unaltered (34). Because the Ednrb\(^{f1}\)/Ednrb\(^{b1}\) homozygotes are indistinguishable from null Ednrb\(^{b1}\) mice (Table 1), it appears that, between 5 and 12.5\%, there must be a lower limit requirement for EDNRB for the survival of both melanoblasts and enteric neurons.

The sensitivity of these cell lineages to the concentration of EDNRB, in addition to unlinked genetic variation (35), may explain the variable penetrance observed in human patients with Hirschsprung disease (5). For example, for the W276C mutation in human patients, the signal transduction properties of the receptor are diminished but not completely absent, which suggests that total abrogation of EDNRB function is not required for the disease state (29). Concentration dependence on coat color on protein level has been observed for other melanogenic proteins, for example the phenotypic consequence of lowered tyrosinase activity in chinchilla (c\(^{b}\)) mice in response to different levels of melanocortin 1 receptor (MC1R), a seven-TM receptor coupled to G, signaling (36).

The most difficult allele to explain is Ednrb\(^{b1}\)/MC1R, for which no alterations were identified in the level of Ednrb mRNA, the sequence of the Ednrb ORF, or the structure of the genomic region. Sequence analysis revealed that the transcription initiation sites were wild type as well (37). It is possible that a mutation affects the large 3′ UTR, which accounts for \( \approx 3\) kb of the 4.5-kb Ednrb transcript (Fig. 4A). If the mutation is located in the 3′ UTR, however, it does not affect the stability of the transcript because the mRNA levels in Ednrb\(^{b1}\)/MC1R mice are comparable to wild type (Fig. 1). More likely, a mutation within the transcript itself may affect the translatability of the mRNA, a possibility that is consistent with the identification of translational control elements in 3′ UTRs of developmentally regulated Drosophila genes such as nanos and caudal (38–40). Alternatively, it is possible that a small change (such as a deletion or rearrangement) that cannot be detected by the assays performed in this study may have disrupted an important temporal transcriptional regulatory element. It has been established that Ednrb gene function is required before e10.5 for the development of melanoblasts (3). Thus, the loss of a regulatory element that is required early in development would be expected to display a piebald phenotype. Of interest, a deletion of a DNA segment upstream of the Ednrb gene has been implicated in one instance of Hirschsprung disease (5).

The range of mutations characterized in this study add to the body of work documenting the effects of various mutagens on the mammalian genome. Early studies had led to the conclusion that the predominant class of mutations generated by radiation and chlorambucil treatment during specific stages of spermatogenesis were large genomic lesions (41). To some extent, this conclusion may have been biased by the fact that investigators were focused on mutations that resulted in neonatal and prenatal phenotypes (reviews: refs. 16 and 17). When viable radiation-induced mutations at albino (c) and agouti (a) loci were analyzed, over 50\% (14 of 27 at c and 2 of 3 at a) of the mutations showed no alteration in coding sequences by Southern analysis (34, 42). Further analysis of the agouti mutants demonstrated that these mice carried mutations in the coding sequence and possibly at the promoter/regulatory region. A recent survey of chemical- and radiation-induced viable mutations at the short ear locus also uncovered alleles with no apparent genomic lesion (43). Thus, these mutations are capable of generating a wide spectrum of changes in the genome.

In conclusion, four mutations at the Ednrb locus that disrupt the development of neural crest-derived melanocytes and enteric neurons have been analyzed. Further studies on mutations in mice and human patients will be useful in identifying the critical residues, molecular mechanism, and regulatory elements for EDNRB function.

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