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Genetics

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*^{27Pub}, *Ednrb*^{17FrS}, *Ednrb*^{1Chlc}, and *Ednrb*^{3Chlo}) were examined at the molecular level. *Ednrb*^{27Pub} 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*^{3Chlo} results in the production of aberrantly sized transcripts and no authentic *Ednrb* mRNA. *Ednrb*^{17FrS} mice exhibited a decreased level of *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*^{1Chlc} 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* or *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*$^{s-l}$, 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*^{s}, 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 pediatric Hirschsprung disease, 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*$^{s-l}$ 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*^{3Chlo}) 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 × C3H/R1) F_1 hybrid male mice with radiation or chemicals, were bred to the Tester stock with the *Ednrb*^{s} 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*^{s}/*Ednrb*^{s} mice. SSL/Le *Ednrb*^{s}/*Ednrb*$^{s-l}$ and C57BL/6J mice were obtained from the Jackson Laboratory. *Ednrb*^{s} and *Ednrb*$^{s-l}$ homozygous mice were obtained by intercrossing SSL/Le *Ednrb*^{s}/*Ednrb*$^{s-l}$.

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*^{3Chlo}, 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*$^{s-l}$, *Ednrb*^{27Pub}, *Ednrb*^{1Chlc}, *Ednrb*^{3Chlo}, and *Ednrb*^{17FrS}

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

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

Alleles	Mutagen	Treated germ cell stage	Homozygous phenotype	
			Viability	White spotting, %
$Ednrb^{s}$	Spontaneous		Viable	10–20
$Ednrb^{s\text{-}l}$	Spontaneous		Juvenile lethal	>90
$Ednrb^{17FrS}$	Radiation (γ rays)	Stem cell spermatogonia	Juvenile lethal	>90
$Ednrb^{3Chlo}$	Chlorambucil	Early spermatids	Juvenile lethal	>90
$Ednrb^{1Chlc}$	Chlorambucil	Early spermatids	Juvenile lethal	>90
$Ednrb^{27Pub}$	^{239}Pu-citrate (α-rays)	Stem cell spermatogonia	Juvenile lethal	>90

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 *Endrb* 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 an Applied Biosystems 373 DNA Sequenator. Both strands were completely sequenced by use of specific internal primers.

Probes and DNA. *Ednrb* cDNA was isolated by digestion of the *PCR*II–*Ednrb* plasmid with *Eco*RI, and the insert was labeled with [^{32}P]dCTP (DuPont/NEN) by random primer synthesis (22). A P1 clone containing the *Ednrb* genomic region was obtained from a Genome Systems mouse 129/Sv genomic library (St. Louis, MO) by PCR screening with oligonucleotide primers covering exon 1 (5′- GGAGCAAGCTGTAACATGCAATCGCC-3′ forward and 5′-GATCTCCCAGAGCCAGACTGGCGATC-3′ reverse) and exon 7 (5′-TCGTGTTTGTGCTGCTGGTGCCA-3′ forward and 5′-TTTGGCAAAGGTTTCATTTTGTTT-3′ reverse). Genomic fragments from the *Ednrb* P1 were subcloned into pBluescript SK (Stratagene), and genomic probes were labeled 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 $Ednrb^{s\text{-}l}$ 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 mutagens 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, $Ednrb^{s\text{-}l}$ mice did not express *Ednrb* mRNA. $Ednrb^{17FrS}$ mice exhibited a greatly decreased level of *Ednrb* mRNA (5% of the wild type) whereas $Ednrb^{3Chlo}$ 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. 4*A*). Similar results were obtained with other *Ednrb*-expressing tissues (data not shown). Finally, $Ednrb^{27Pub}$ and $Ednrb^{1Chlc}$ 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 $Ednrb^{27Pub}$, $Ednrb^{17FrS}$ and $Ednrb^{1Chlc}$ mice, primers from the 5′- and 3′-untranslated regions (UTRs) were used to amplify *Ednrb* cDNA transcribed from brain mRNA. Sequence analysis of $Ednrb^{1Chlc}$ and $Ednrb^{17FrS}$ 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 $Ednrb^{27Pub}$ 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)

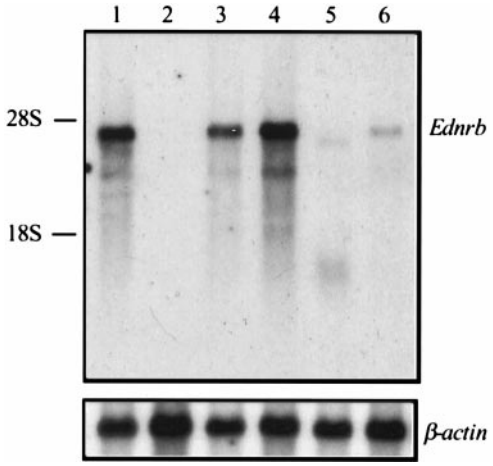

FIG. 1. Expression of *Ednrb* mRNA in mutant mice. One microgram of poly(A) mRNA from the brains of juvenile mice was separated on an agarose gel, transferred to a membrane filter, and hybridized with a radiolabeled *Ednrb* cDNA probe. Lanes 1–6 are, in order: C57BL/6J, $Ednrb^{s\text{-}l}$, $Ednrb^{27Pub}$, $Ednrb^{1Chlc}$, $Ednrb^{3Chlo}$, and $Ednrb^{17FrS}$ homozygous mice. The blot was stripped and rehybridized with β-actin cDNA as a control.

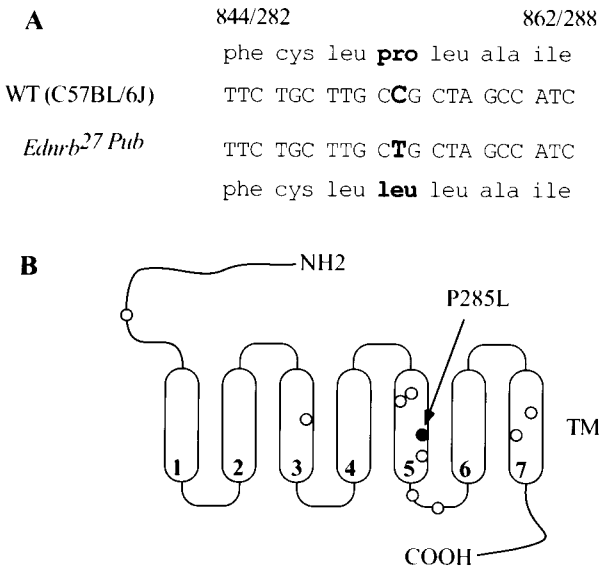

FIG. 2. A point mutation in *Ednrb*^{27Pub}. (*A*) DNA sequences of wild-type *Ednrb* (C57BL/6J) and *Ednrb*^{27Pub} from nucleotides 841–865 of the protein coding region with the corresponding amino acids are shown. The arrow indicates the C-to-T transition at nucleotide 854 and the change in amino acid sequence. (*B*) Schematic diagram of the structure of EDNRB with open circles indicating positions of previously identified mutations in human patients with Hirschsprung disease (5). The closed circle indicates the position of the substitution in *Ednrb*^{27Pub}. The open ovals represent the TM domains.

in the amino acid sequence (Fig. 2). This mutation was confirmed in genomic DNA because it creates a restriction enzyme polymorphism, introducing a *Bbv*I 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 *Ednrb*^{27Pub} mice because this difference was not found in the other Oak Ridge-derived mice.

Analysis of the *Ednrb* Genomic Locus. To determine whether there were changes in the genomic structure at *Ednrb* that could account for the mutant phenotypes in *Ednrb*^{17FrS}, *Ednrb*^{3Chlo}, and *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 *Ednrb* cDNA. No alterations were observed for *Ednrb*^{27Pub}, *Ednrb*^{17FrS}, or *Ednrb*^{1Chlc}. A difference, however, was observed in the size of the largest *Bam*HI fragment in *Ednrb*^{3Chlo} DNA (Fig. 3*A*). This *Bam*HI 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. 4*A*; ref. 25). To further localize the mutation, a 2.4-kb *Nco*I/*Hin*dIII fragment (probe B in Fig. 4*A*) located ≈7.0 kb downstream of the first exon was hybridized to genomic DNA. It detected restriction fragment length polymorphisms between C57BL/6J and *Ednrb*^{3Chlo} with every restriction enzyme tested (Fig. 3*B*). In most cases, probe B hybridized to a single fragment in C57BL/6J DNA and to two different bands in *Ednrb*^{3Chlo} DNA. This result, combined with the observation that *Ednrb*^{3Chlo} produces two different truncated transcripts, suggests that an inversion, with one breakpoint located within the 2.4-kb *Nco*I/*Hin*dIII 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 *Ednrb* gene. To detect any changes in larger genomic regions in *Ednrb*^{17FrS} and *Ednrb*^{1Chlc}

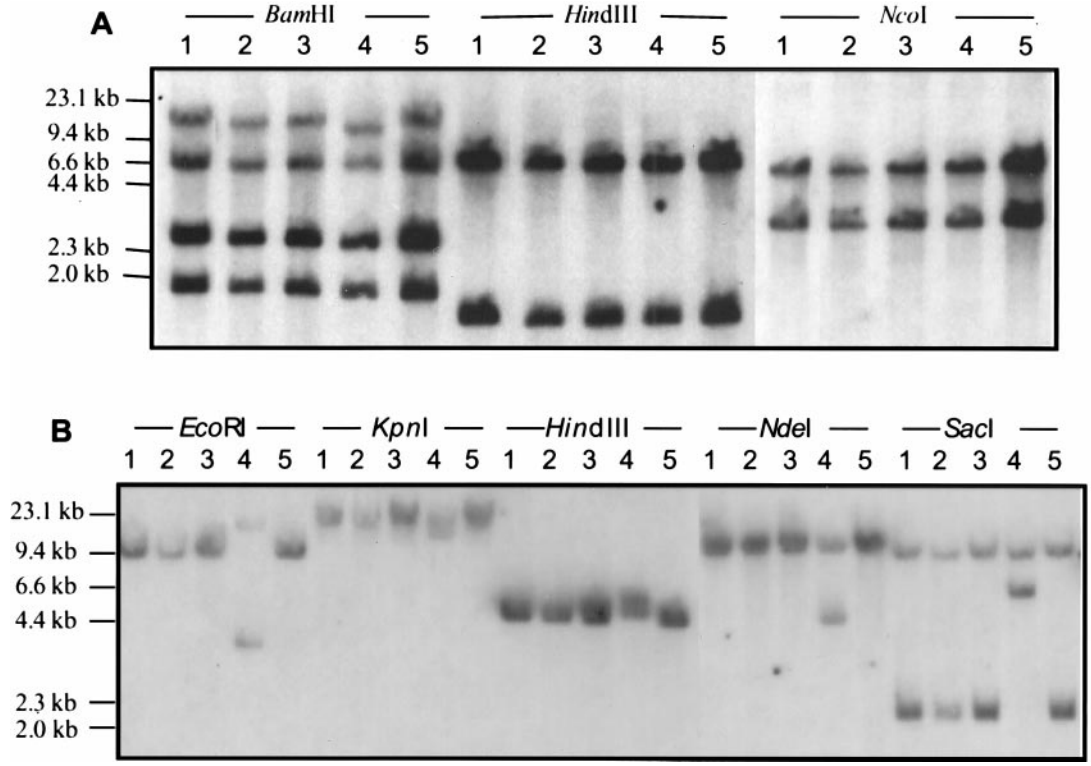

FIG. 3. The genomic structure of *Ednrb*. (*A*) Genomic DNAs were digested with the restriction enzymes indicated and separated by agarose gel electrophoresis. The DNAs were transferred to a nylon membrane and hybridized to a radiolabeled *Ednrb* cDNA that contained the protein coding region. Lanes 1–5, in order, correspond to: C57BL/6J, *Ednrb*^{27Pub}, *Ednrb*^{1Chlc}, *Ednrb*^{3Chlo}, and *Ednrb*^{17FrS} homozygous mice. (*B*) Genomic DNA was treated as in *A* and hybridized with the *Nco*I/*Hin*dIII intron 1 genomic probe (probe B) in Fig. 4*A*. The lanes are in the same order as in **A**. The DNA standard was bacteriophage λ DNA digested with *Hin*dIII (New England Bio-Labs).

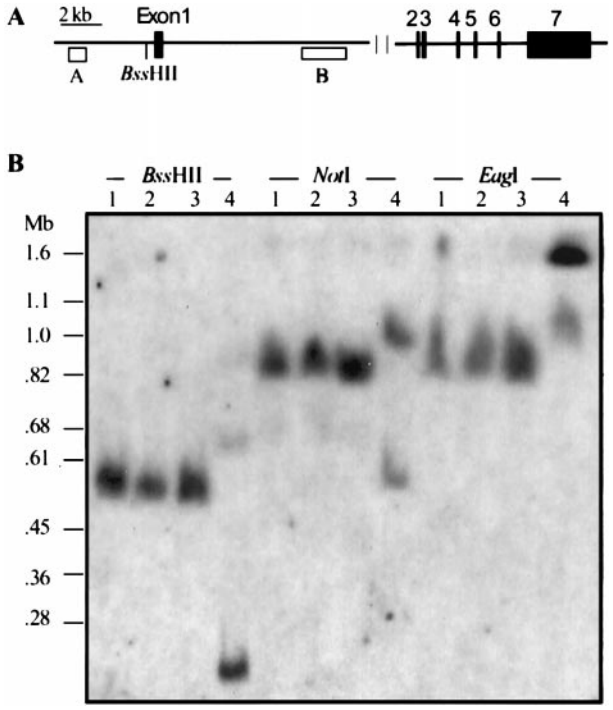

FIG. 4. Pulsed-field gel electrophoresis analysis of *Ednrb*. (*A*) Schematic diagram of the murine *Ednrb* locus. The black rectangles represent the exons of the gene. The *Bss*HII site upstream of the first exon is indicated. Below the line are the positions of probes A and B. (*B*) Genomic DNAs embedded in agarose plugs were digested with the enzymes indicated and separated on a pulsed-field gel. After transfer to a nylon membrane, the DNAs were hybridized with probe B. Lanes 1–4 correspond to DNA from C57BL/6J, *Ednrb*^{1Chlc}, *Ednrb*^{3Chlo}, and *Ednrb*^{17FrS} homozygous mice, respectively. The size standards represent the *Saccharomyces cerevisiae* chromosomes (Bio-Rad).

and to further confirm that an inversion has occurred in *Ednrb*^{3Chlo}, pulsed-field gel electrophoresis was performed. A 680-kb *Bss*HII fragment was detected in C57BL/6J DNA and all mutant DNAs with probe A, which hybridizes ≈3.5 kb upstream of the *Bss*HII site in the 5′ UTR (data not shown). When probe B was used, no changes were observed for *Ednrb*^{17FrS} and *Ednrb*^{1Chlc} mutants (Fig. 4*B*). As expected, probe B detected changes in *Ednrb*^{3Chlo} DNA. A 570-kb *Bss*HII fragment was detected in C57BL/6J DNA whereas 650- and 150-kb *Bss*HII bands were obtained in *Ednrb*^{3Chlo} DNA. Similarly, a 850-kb fragment was observed in C57BL/6J DNA digested with either *Not*I or *Eag*I, but two different bands were detected in *Ednrb*^{3Chlo}. 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 *Ednrb*^{3Chlo} 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 *Ednrb*^{17FrS} and *Ednrb*^{1Chlc} mutants, at least within ≈1.25 megabases (sum of the *Bss*HII 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 *Ednrb*$^{s-l}$ mice, which harbor a complete deletion of the *Ednrb* gene (Table 1). The *Ednrb*^{27Pub} 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. 2*B*, 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 *Ednrb*^{3Chlo} mice (Fig. 4*A*). We favor the likelihood that the rearrangement is an inversion because there is no clear reduction in the litter sizes born to *Ednrb*^{3Chlo} 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 *Ednrb*^{3Chlo} 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 initiating 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

Table 2. Summary of juvenile lethal *piebald* alleles

Alleles	Genomic alteration	RNA	Sequence difference in coding region	Nature of mutation
Ednrb^{27Pub}	No	Normal	Yes	CCG to CTG/P285L
Ednrb^{17Frs}	No	Decreased	No	?
Ednrb^{3Chlo}	Yes	Truncated transcripts	—	Chromosomal inversion
Ednrb^{1Chlc}	No	Normal	No	?

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*^{s} allele, which expresses ≈25% of wild-type *Ednrb* mRNA levels, exhibit 10–20% spotting. A heteroalleleic combination of *Ednrb*^{s}/*Ednrb*$^{s\text{-}l}$, which would express ≈12.5% of normal mRNA levels, displays an intermediate phenotype of 40–60% spotting, and *Ednrb*$^{s\text{-}l}$ homozygotes are almost completely white. Megacolon, on the other hand, almost exclusively occurs in the *Ednrb*$^{s\text{-}l}$/*Ednrb*$^{s\text{-}l}$ 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*^{17FrS} 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*^{17FrS} homozygotes are indistinguishable from null *Ednrb*$^{s\text{-}l}$ 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*^{ch}) mice in response to different levels of melanocortin 1 receptor (MC1R), a seven-TM receptor coupled to G_s signaling (36).

The most difficult allele to explain is *Ednrb*^{1Chlc}, 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 translation initiation sites were wild type as well (37). It is possible that a mutation affects the large 3′ UTR, which accounts for ≈3 kb of the 4.5-kb *Ednrb* transcript (Fig. 4*A*). 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*^{1Chlc} 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 mutagens 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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