Reduced Expression of the Endothelin Receptor Type B Gene in Piebald Mice Caused by Insertion of a Retroposon-like Element in Intron 1*

Received for publication, November 28, 2005, and in revised form, February 24, 2006 Published, JBC Papers in Press, February 24, 2006, DOI 10.1074/jbc.M512618200

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Mice carrying the piebald mutation exhibit white coat spotting due to the regional absence of neural crest-derived melanocytes. We reported previously that the piebald locus encodes the Ednrb gene and that piebald mice express low levels of structurally intact Ednrb mRNA and EDNRB protein (Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A., and Yanagisawa, M. (1994) Cell 79, 1267–1276). Here, we report that both the life span of the Ednrb mRNA and the promotor activity of the Ednrb gene are indistinguishable between wild-type and piebald mice. Introns 2–6 of the Ednrb gene in piebald mice were correctly excised with an efficiency indistinguishable from those in wild-type mice in exon trapping experiments. We found that the piebald allele of the Ednrb gene has a 5.5-kb retroposon-like element in intron 1 possessing canonical sequences of a polyadenylation signal and a splice acceptor site. Abnormal hybrid transcripts carrying exon 1 of the Ednrb gene and a portion of the 5.5-kb element are expressed in piebald mice. The insertion of the 5.5-kb element into a mammalian expression vector markedly reduced the expression of the reporter gene. Premature termination and aberrant splicing of the Ednrb transcript caused by the retroposon-like element in intron 1 lead to a reduced level of the normal Ednrb transcript, which is responsible for the partial loss-of-function phentype of piebald mice.

Melanocytes are specialized melanin-producing cells responsible for coat pigmentation (1). They arise from neural crest cells that leave the apical ridge of the neural tube and migrate dorsally to the somites and through the mesenchymal layer beneath the ectoderm until they eventually enter the epidermis (2, 3). A number of naturally occurring as well as targeted mutations that produce developmental defects in neural crest cell migration, differentiation, or survival have been reported in mice (4–8). These include piebald (s/s) mice, which are one of the spotting mutants that lack neural crest-derived melanocytes in the coat (5). In addition to the coat pigment defect, piebald lethal (s’/s) mice, which carry a severe mutation at the s locus, manifest megacolon (9, 10). This defect is caused by the absence of enteric ganglia, which are also derived from the neural crest, in the distal portion of the colon (11).

A large number of studies have established that the signaling mediated by endothelins plays an essential role in the development of neural crest-derived cell lineages (12–14). Endothelin was originally identified as a potent vasoconstrictive peptide synthesized by vascular endothelial cells (15). Three closely related endothelin peptides (EDN1, EDN2, and EDN3) composed of 21 amino acids have been reported (16–18). The two endothelin receptors EDNRA (endothelin receptor type A) and EDNRB (endothelin receptor type B) belong to the G protein-coupled heptahelical superfamily (19–21). EDNRA preferentially interacts with EDN1 and EDN2, but not with EDN3. EDNRB accepts all three isopeptides equally. EDN3/EDNRB interaction is essential for the development of two neural crest-derived cell lineages, epidermal melanocytes and enteric neurons (22–25).

We demonstrated previously that the Ednrb gene is allelic to the s locus (22). A DNA segment encompassing all of the coding exons of the Ednrb gene is deleted in the s’ genome (22). On the other hand, the s allele expresses reduced levels of structurally normal Ednrb mRNA and EDNRB protein (22). The primary genomic lesion in the s mutation has not been described previously.

Here, we report that the Ednrb’ allele harbors a 5.5-kb retropon-like element in intron 1. This leads to aberrant transcription of the Ednrb gene, resulting in a reduced level of the normal Ednrb transcript, which is responsible for the white coat spotting in s/s mice.

EXPERIMENTAL PROCEDURES

Mice—Wild-type mice (C57BL/6J) and piebald mice (s/s SSL/Le) were obtained from The Jackson Laboratory.

Cell Culture—C6 rat glioma cells were obtained from the American Type Culture Collection (CCL-107). ROS17/2 rat osteosarcoma cells were kindly donated by Dr. Y. Takuwa (University of Tokyo). C6, ROS17/2, and COS-7 cells were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

Isolation and Characterization of the Ednrb Genomic Clones—Genomic DNA prepared from the livers of s/s mice was partially digested with Sau3AI, and the first two nucleotides of the ends were filled in with dATP and dGTP. The DNA was ligated to Xhol-digested AFIXII arms (Stratagene), the ends of which were filled in with dCTP and dTTP. Recombinant DNA was packaged in vitro, and λ phages were plated on Escherichia coli XL1-Blue MRA(P2) (Stratagene). A AFIXII mouse 129/Sv genomic library was purchased from Stratagene. Approximately 2 × 106 phage clones were screened with either the full-length sequence (positions 1–1958) of mouse Ednrb cDNA (GenBank™ accession number U32329) or a 2.5-kb PstI fragment sequence of mouse Ednrb.
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intron 1 (see Fig. 3A). The DNA probe was 32P-labeled using a random primer labeling kit (Roche Applied Science). Plaque hybridization and preparation of recombinant phage DNAs were carried out by standard procedures (26). Positive clones were purified and characterized by restriction endonuclease mapping and Southern blot analysis. DNA sequencing of specific restriction fragments subcloned into the pBluescript II vector (Stratagene) was performed by the dideoxy chain termination method using Sequenase Version 2.0 (U. S. Biochemical Corp.). In addition to M13 universal and reversal primers (U. S. Biochemical Corp.), sequence-specific oligonucleotides were used for sequencing. PCR was performed to determine the sizes of introns. The reaction was cycled 30 times in a cycle profile of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C. Amplified DNA fragments were analyzed by agarose gel electrophoresis.

Construction of Plasmids—To construct a reporter plasmid for the measurement of the promoter activity of the Ednrb gene, a 5-kb BamHI-BssHII genomic fragment starting in the 5′-flanking region and ending in the 5′-noncoding region of exon 1 (171 bp upstream of the initiation codon of the Ednrb gene, at the BssHII site) was linked to the firefly luciferase gene (p5kb-luc). In addition, an 11-kb SphI-BssHII promoter fragment was linked to the luciferase gene (p11kb-luc). The sequence of the luciferase gene was obtained from the reporter plasmid pT7-luc (27). The pT7-luc plasmid was digested with BamHI and BglII to remove the tk gene was obtained from the reporter plasmid pTK-luc (27). The pTK-luc plasmid was digested with BamHI and BglII to remove the herpes simplex virus thymidine kinase promoter and blunt end-ligated to a 1-kb SphI-BssHII genomic fragment from −1.3 kb to 171 bp upstream of the initiation codon (p1kb-luc). To prepare p5kb-luc, the p1kb-luc plasmid was digested with BamHI, and the resulting larger fragment containing a 1-kb BamHI-BssHII fragment from −1.1 kb to 171 bp upstream of the initiation codon was ligated to a 4-kb BamHI genomic fragment from −5.1 to −1.1 kb upstream of the initiation codon. To prepare p11kb-luc, a 10-kb SphI genomic fragment from −11.3 to −1.3 kb upstream of the initiation codon was blunt end-inserted into the unique Sall site of the p1kb-luc plasmid. An Ednrb exon-trapping plasmid harboring sequences of introns 2–4 or introns 5 and 6 of the Ednrb gene was prepared as follows. A 3-kb PvuII Ednrb genomic fragment starting in intron 1 (−0.1 kb upstream of exon 2) and extending to intron 5 (53 bp downstream of exon 5) was blunt end-inserted into the unique NotI site in the intron of the exon-trapping vector pSPL3 (introns 2–4) (Invitrogen). In addition, a 4-kb EcoRI-Sall Ednrb genomic fragment starting in intron 4 (−0.4 kb upstream of exon 5) and extending to 401 bp in exon 7 was blunt end-ligated to the 4-kb Aval fragment of pSPL3, in which 2077 bp of the intron 1 and 13 bp of exon 2 are eliminated (introns 5 and 6). The plasmid containing introns 5 and 6 includes the chimeric exon of the 5′-region of Ednrb exon 7 and the 3′-region of pSPL3 exon 2. To construct a reporter plasmid harboring the 5.5-kb retropon-like element inserted heterologously into a vector intron (see Fig. 6A), the 2.5-kb XbaI-MscI fragment of the pSPL3 plasmid, which contains an intron, splice donor and acceptor sites, and some flanking exon sequences of the human immunodeficiency virus type 1 tat gene, was blunt end-inserted into the unique BglII site between the promoter and reporter regions in the pTK-luc plasmid (pTK-tat-luc). The 6.5-kb EcoRV-Sall fragment carrying part of intron 1 from the Ednrb gene and the 5.5-kb retropon-like element was then blunt end-inserted into the unique NotI site of the tat intron in pTK-tat-luc in the right orientation (pTK-tata-luc) or the reverse orientation (pTK-tatβ-luc).

Transfection—For transfection and the measurement of promoter activity, ROS17/2 or C6 cells were seeded into 6-well culture plates and cotransfected with either a control plasmid (pUC18) or a luciferase reporter plasmid (pTK-luc containing the herpes simplex virus thymidine kinase promoter, p5kb-luc, or p11kb-luc) at 1 µg/well and with a lacZ expression plasmid at 1 µg/well using Lipofectamine and serum-free Opti-MEM I (Invitrogen) following the protocols of the manufacturer. Optimal transfection conditions were 4 µl/well Lipofectamine and a 5-h incubation under serum-free conditions. Forty-eight hours after transfection, the cells were used for the luciferase assay. For transfection in exon trapping experiments, the Ednrb exon-trapping plasmid (50 ng/well) was introduced into COS-7 cells in 6-well culture plates alone or in combination with the 64-bp control exon-containing pSPL3 plasmid (50 ng/well; Invitrogen) using 4 µl/well Lipofectamine and a 12-h incubation under serum-free conditions. After 48 h, the cells were used for RNA isolation. For transfection of the reporter plasmid harboring the 5.5-kb retropon-like element inserted heterologously into a vector intron, COS-7 cells in 6-well culture plates were cotransfected with either pUC18 or a luciferase reporter plasmid at 1 µg/well and with the lacZ expression plasmid at 1 µg/well by the DEAE-dextran method as described (21). After 48 h, the cells were used for the luciferase assay.

Luciferase Assay—Transfectants were harvested after 48 h, and extracts were prepared and assayed for enzyme activity as described previously (28, 29). Luciferase and β-galactosidase activities were measured using a Dynatech microtiter plate Model ML3000 luminometer and Model MR5000 spectrophotometer, respectively. Luciferase values were normalized for transfection and harvesting efficiency by measuring β-galactosidase activity, and the results are reported as relative light units.

Preparation and Culture of Mouse Cerebral Astrocytes—Astrocytes were obtained from newborn (first day of life) mouse cerebrum using procedures slightly modified from those of Lim and Bosch (30) and Hertz et al. (31). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a humidified atmosphere of 5% CO2 at 37 °C. They were used for experiments after the third passage.

Northern Blot Analysis and Reverse Transcription (RT)2-PCR—RNA was extracted from frozen tissues of 8-week-old mice or from cultured cells using RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX). For Northern analysis, total RNA (10 µg) was separated on 1.1% agarose gel, transferred to a Hybond-N membrane (Amersham Biosciences), and hybridized with random-primed 32P-labeled probes. The mouse Ednrb cDNA sequence and the 5.5-kb retropon-like element sequence were used as probes. Some probes were obtained by PCR. For RT-PCR, first-strand cDNA was synthesized from 2 µg of total RNA with (dT)12–18 primers using SuperScript reverse transcriptase (Invitrogen) as recommended by the manufacturer. The synthesized cDNA (0.5 µg) was amplified using buffer A included in the PCR optimization kit from Invitrogen and appropriate primers for 35 cycles in a cycle profile of 1 min at 94 °C, 1 min at 55 °C, and 15 min at 72 °C. The amplification products were subcloned into the pCRII cloning vector (Invitrogen) and sequenced using Sequenase Version 2.0. In exon trapping experiments, cDNA synthesis and primary and secondary PCR amplification were performed using an exon trapping system (Invitrogen) as recommended by the manufacturer. The amplification products were subcloned into...
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FIGURE 1. Northern blot analysis using an Ednrb cDNA probe in various tissues of wild-type (+/+) and piebald (s/s) mice. Total cellular RNAs were prepared from the lungs, hearts, livers, kidneys, brains, and intestines of both mice and electrophoresed on 1.1% agarose gel. After being transferred to nylon membranes, the blot was hybridized with a 32P-labeled mouse Ednrb cDNA probe. We used DNA containing exons 1–6 (positions 48–1370) of the mouse Ednrb cDNA (GenBank™ accession number U32330) as a probe. The DNA probe was prepared by PCR. Rehybridization with a β-actin probe is shown as an internal loading standard.

FIGURE 2. Exon trapping in the Ednrb gene from wild-type (+/+) and piebald (s/s) mice. The Ednrb exon-trapping plasmid harboring introns 2–4 (intron 2–4) or introns 5 and 6 (intron 5–6) of the Ednrb gene from +/+ and s/s mice was introduced into COS-7 cells alone or together with a positive (pos.) control plasmid (64-bp control exon-containing pSPL3) as indicated. The puC18 plasmid was used as a negative (neg.) control plasmid. RNA was prepared from the transfected cells, and single-stranded cDNA was synthesized using the prepared RNA. Primary and secondary PCR amplifications of the synthesized single-stranded cDNA were performed using the Invitrogen exon trapping system. The primer sets for both primary and secondary PCRs were designed to anneal to the first and second exons on the vector. The products were analyzed on 2.0% agarose gel and visualized by ethidium bromide staining. The lengths of the DNAs are shown.

RESULTS

We have demonstrated previously that the Ednrb mRNA is structurally intact in terms of the overall length and coding region sequence in s/s mice, but that the expression of Ednrb mRNA in the lung is decreased to ∼25% of the wild-type levels (22). Northern blot analysis of hearts, livers, kidneys, brains, and intestines revealed that the expression of intact Ednrb mRNA is uniformly reduced in all s/s tissues examined except for the liver (Fig. 1). These observations are consistent with our previous finding that s/s mice show ∼27% of the EDNRB density in the kidney compared with wild-type mice as determined by radioligand binding assay (22).

First, we examined whether the decreased Ednrb mRNA level was caused by a shortened life span of the Ednrb mRNA by monitoring the pattern of disappearance of the Ednrb mRNA after the addition of the RNA synthesis inhibitor actinomycin D or 5,6-dichlorobenzimidazole riboside to the culture of mouse cerebral astrocytes. Like s/s tissues, astrocytes from s/s mice exhibited a low level of the Ednrb mRNA with normal length. However, no appreciable difference between wild-type and s/s mice was observed in the pattern of the Ednrb mRNA disappearance from 0 to 16 h after the addition of actinomycin D or 5,6-dichlorobenzimidazole riboside, indicating that the life span of the Ednrb mRNA is normal (data not shown). This excluded the possibility that a less stable mRNA accounts for the reduced steady-state expression of the Ednrb mRNA.

Second, we examined the possibility of decreased promoter activity of the Ednrb gene by constructing reporter plasmids in which the expression of the firefly luciferase gene was driven by Ednrb promoter fragments from s/s and wild-type (129/Sv) mice. We isolated phage clones containing DNA segments that included the 5′-flanking region and exon 1 of the Ednrb gene from both wild-type and s/s mice as demonstrated by restriction mapping, oligonucleotide hybridization, and partial sequencing. SphI, BamHI, and BssHII restriction sites were conserved between the Ednrb promoter regions of wild-type and s/s mice (in the 5′-flanking region, −11.3 and −1.3 kb upstream of the initiation codon for Spほ; in the 5′-flanking region, −5.1 and −1.1 kb upstream of the initiation codon for BamHI; and in the 5′-noncoding region of exon 1, 171 bp upstream of the initiation codon for BssHII in the Ednrb gene of both mice). A 5-kb BamH1-BssHII fragment from −5.1 kb to 171 bp upstream of the initiation codon or an 11-kb SphI-BssHII fragment from −11.3 kb to 171 bp upstream of the initiation codon in both mice was used as the promoter in luciferase reporter plasmids (designated as p5kb-luc and p11kb-luc, respectively), and the reporter plasmids were introduced into C6 glioma and ROS17/2 osteosarcoma cells, which express endogenous EDNRB, for the luciferase assay. The luciferase expression directed by the 5-kb Ednrb fragment in both ROS17/2 and C6 cells was indistinguishable between wild-type and s/s mice (data not shown). The 11-kb Ednrb fragment-directed luciferase expression in both cells was also indistinguishable between the two genotypes (data not shown). Mock-transfected cells expressed luciferase levels that were not significantly different from the background of the luminometer. These results indicate that promoter activity in the 11-kb 5′-region of the Ednrb gene is normal. Moreover, the nucleotide sequences of a 453-bp region upstream of the initiation codon of the Ednrb gene (containing the 221-bp 5′-noncoding region of the mouse Ednrb mRNA reference sequence (GenBank™ accession number NM_007904)) are identical in wild-type and s/s mice, suggesting that exon 1 and the proximal promoter of Ednrb are structurally intact (data not shown). This excluded the possibility that an abnormal cis-element in the 11-kb 5′-region of the Ednrb allele contributes to the reduced mRNA expression.

Third, we examined the intron structure of the Ednrb gene. Eleven and 12 positive clones that covered all introns of the Ednrb gene as demonstrated by restriction mapping, Southern hybridization, and partial sequencing were isolated from genomic DNA libraries of wild-type (129/Sv) and s/s mice, respectively. Of the 11 and 12 clones each, three overlapping clones were used for a detailed characterization of the two mouse genomic sequences. The nucleotide sequences of the exon-intron junctions of the Ednrb gene from both mice were determined (data not shown). The sizes of exons 2–6 in the Ednrb gene of both mice were 113, 205, 150, 134, and 109 bp, respectively, and are in complete concordance with those of the reported mouse Ednrb genomic structure (GenBank™ accession number NT_039609). The sizes of introns 1–6 (except for Ednrb intron 1) in both mice were ∼20.0, 0.2, 1.5, 0.6, 0.6,
Figure 3. Insertion of a 5.5-kb retroposon-like DNA element into Ednrb intron 1 in piebald mice. A, restriction maps of intron 1 in the Ednrb gene from wild-type (+/+) and piebald (s/s) mice. The introns and flanking sequences are drawn as solid lines, and exons 1 and 2 are represented by shaded boxes. The inverted triangle indicates the insertion point. The open boxes and double lines show the direct repeats and the core sequence of the 5.5-kb retroposon-like element, respectively. Recognition sites for restriction enzymes are shown.
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and 2.3 kb, respectively, and are consistent with those of the reported mouse Ednrb genomic structure (GenBankTM accession number NT_039609). Surprisingly, the size of intron 1 in s/s mice was 5.5 kb longer than that in wild-type mice (see below). All of the 5’- and 3’-splice sites in Ednrb introns 1–6 of both mice conformed to the GT-AG rule (32); and in addition, adenosine residues in presumed branch sites within introns 1–6 were conserved between the two genotypes (data not shown) (33).

Subsequently, to investigate the precision and efficiency of excision of Ednrb introns 2–6 during RNA splicing, we performed exon trapping experiments in which the RT-PCR-amplified product from COS-7 cells transfected with a genomic insert-containing exon-trapping vector represented a chimeric transcript produced by the pairing of vector (pSPL3) and genomic (Ednrb) exons (34). The Ednrb exon-trapping plasmid harboring the sequences of introns 2–4 or introns 5 and 6 of the Ednrb gene from wild-type and s/s mice was introduced into COS-7 cells alone or together with the 64-bp control exon-containing pSPL3 plasmid as a positive control. We detected RT-PCR-amplified products (pSPL3) and genomic (Ednrb) introns 2–6 during RNA splicing, we performed exon trapping experiments in which the RT-PCR-amplified product from COS-7 cells transfected with a genomic insert-containing exon-trapping vector represented a chimeric transcript produced by the pairing of vector (pSPL3) and genomic (Ednrb) exons (34). The Ednrb exon-trapping plasmid harboring the sequences of introns 2–4 or introns 5 and 6 of the Ednrb gene from wild-type and s/s mice was introduced into COS-7 cells alone or together with the 64-bp control exon-containing pSPL3 plasmid as a positive control. We detected RT-PCR-amplified products of the expected length (779 and 808 bp, respectively, when introns 2–4 and introns 5 and 6 are excised) in cells transfected with the wild-type and s/s Ednrb exon-trapping plasmids (Fig. 2). The ratio of the transcripts produced by the Ednrb exon-trapping plasmid to those produced by the cotransfected positive control plasmid (244 bp) was not significantly different between the two genotypes (Fig. 2). We detected no PCR product in mock-transfected cells. These findings indicate that introns 2–6 of the Ednrb gene in s/s mice are correctly excised with an efficiency indistinguishable from those in wild-type mice during splicing. Together with the results of the structural analysis of Ednrb introns 2–6, the data suggest that the sequences of Ednrb introns 2–6 do not have any detrimental effects on gene expression.

Finally, we focused on an abnormality in intron 1 of the Ednrb gene. Comparison of intron 1 in wild-type and s/s mice indicated that a 5.5-kb DNA element is inserted ~15 kb downstream of exon 1 and ~5 kb upstream of exon 2 in the Ednrb gene (Fig. 3A). Nucleotide sequences of the insert and its flanking regions were determined for the wild-type and s/s DNAs (Fig. 3B). The entire length of the insert DNA is 5056 bp, and the nucleotide sequence data have been submitted to the GenBankTM/EBI Data Bank with accession number AB244236. The first 507 bp at the 5’- and 3’-ends of the insert DNA have the same sequences and are highly homologous to the 526-bp direct terminal repeats in insertion sequences of the nonagouti (a) and the black-and-tan (a′) alleles of the agouti locus (35). The 507-bp direct repeat carries sequences of a canonical AATAAA polyadenylation signal (36) and of a T cluster (TTTCTTTT) 49 nucleotides downstream of the signal (36). The T cluster is conserved in the areas for transcription termination and 3’-processing and usually lies between 5 and 30 nucleotides downstream of the polyadenylation signal (36). Moreover, canonical sequences of a splice acceptor site (37) and of a branch site 54 nucleotides upstream of the acceptor site (33) are present. A search for the core sequence of the 5.5-kb element in the data base revealed that a 460-bp 3’-region (positions 3957–4446), a 273-bp internal region (positions 3098–3370), and a 164-bp internal region (positions 2177–2340) in the core sequence of the 5.5-kb element (GenBankTM accession number AB242436) are highly homologous to positions 3187–3647 (GenBankTM accession number AF030884) of the mouse early transposon (ETn) element near the Pafaha-ps2 gene (38), positions 345–616 (GenBankTM accession number X57268) of the mouse ETn-related t haplo-typical-specific element within the H-2 complex (39), and a short stretch of a retroviral pol gene (positions 3695–3860 of GenBankTM accession number AF246633) in the mouse ETn-related MusD element (40), respectively, showing only partial sequence similarity. At the 5’- and 3’-ends of the insert DNA, there is a 6-bp repeat sequence (AGAAGA) that is found once in the wild-type DNA. These findings suggest that the 5.5-kb DNA insert in intron 1 of the Ednrb gene is an as yet uncharacterized retroposon-like element.

We detected decreased levels of intact Ednrb mRNA in s/s mice (~25% of the levels in wild-type mice) by Northern blot hybridization using exons 1–6 of the Ednrb cDNA as a probe. An additional RNA larger than the intact RNA seemed to be detected in s/s mice by this Northern blot hybridization (Fig. 1). In addition, the data suggested that the promoter and exon 1 of the Ednrb gene are intact, but that intron 1 has the 5.5-kb retroposon-like element carrying canonical sequences of a polyadenylation signal and a splice acceptor site. Thus, the expression of the Ednrb gene was re-examined using DNA probes containing exon 1 or exons 2–6 of the Ednrb cDNA. As shown in Fig. 4A, Northern blot hybridization using an exon 1 probe detected a 6.5-kb RNA (~75% of the amount of the wild-type Ednrb mRNA) as well as an RNA of normal size (4.4 kb; ~75% of the amount of the wild-type Ednrb mRNA) in s/s mice. When a DNA probe containing exons 2–6 was used, the 4.4-kb RNA (~25% of the amount of the wild-type Ednrb mRNA), but not the 6.5-kb species, was detected in s/s mice (Fig. 4B). In wild-type mice, both probes detected only the 4.4-kb RNA. These findings suggest that the Ednrb mRNA is initiated normally in s/s mice, but that ~75% of the initiated mRNA is terminated before exon 2. Moreover, a DNA segment indicated in Fig. 3B in the retroposon-like element hybridized with the 6.5-kb RNA in s/s mice, but not in wild-type mice (Fig. 4C).

Therefore, we examined the possibility that the 6.5-kb RNA expressed in s/s mice is a hybrid transcript carrying Ednrb exon 1 and the retroposon-like element sequences. Sequence analysis of segments trapped as a exon in the exon-trapping vector pSPL3, into which the retroposon-like element-containing fragment was inserted, revealed that a splice acceptor site in the 5’-direct repeat was used for the pairing with the splice donor site of the vector (data not shown). RNAs from wild-type and s/s mice were then analyzed by RT-PCR. RNA from the s/s mice (but not from the wild-type mice) produced DNA fragments (220 or 1720 bp) that were derived from chimeric RNA resulting from the pairing of the splice donor site in Ednrb exon 1 and the splice acceptor site in the 5’-direct repeat of the retroposon-like element (Fig. 5A). Furthermore, 3’-RACE resulted in amplification of a fragment (553 bp) that was derived from RNA terminated at a polyadenylation site 28 nucleotides downstream of the polyadenylation signal in the 3’-direct repeat of the retroposon-like element in s/s mice, but not in wild-type mice (Fig. 5B). The larger fragment detected in both genotypes resulted from a nonspecific amplification as demonstrated by sequencing. These results indicate that there is a hybrid transcript carrying (~1-kb Ednrb exon 1 and the ~5.5-kb retroposon-like element portion in s/s mice, which is paired at a splice acceptor site in the 5’-direct repeat and ends at a polyadenylation site in the 3’-direct repeat. It is likely that ~75% of the transcripts normally inti-

indicated. The 2.5-kb PstI fragment used for library screening is indicated by a bar. 8, nucleotide sequences of exon 1 and the 5.5-kb retroposon-like element in intron 1 of the Ednrb gene in s/s mice. The junction between exon 1 and intron 1 in the Ednrb gene is indicated by a closed triangle. The ends of the 5’- and 3’-direct repeats are indicated. The sequences of the canonical polyadenylation signal and T cluster downstream of the signal are underlined, and the polyadenylation site detected in 3’-RACE (see Fig. 5B) is indicated by a closed inverted triangle. The canonical sequences of the splice acceptor site and the branch site upstream of the acceptor site are doubly underlined, and the splice junction detected by RT-PCR (see Fig. 5A) is indicated by an open triangle. The sequences of exon 1 and intron 1 of the wild-type Ednrb gene are identical to the corresponding sequence in s/s mice except for the 5.5-kb retroposon-like element. The 6-bp repeats of the wild-type DNA are boxed. The DNA segment used as a probe for the Northern blot in Fig. 4C is indicated by a solid line above the sequence. The oligonucleotide primers used for PCR in Fig. 5 are shown by arrows.
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FIGURE 4. Northern blot analysis of aberrant Ednrb transcripts in piebald mice. Total cellular RNAs were prepared from the lungs, brains, intestines, and hearts of both wild-type (+/+ ) and piebald (s/s) mice and electrophoresed on 1.1% agarose gel. After transfer to nylon membranes, the blot was hybridized with a 32P-labeled probe. We used DNA containing exon 1 (A) or exons 2–6 (B) or the DNA segment indicated in Fig. 3A as a probe. The DNA probe were prepared by PCR. The DNA probe for exon 1 or exons 2–6 carries sequence 48–660 or 711–1370 of the mouse Ednrb cDNA (GenBankTM accession number U323299), respectively. Rehybridization with a β-actin probe is shown as an internal loading standard.

FIGURE 5. RT-PCR and 3′-RACE analyses of aberrant Ednrb transcripts in piebald mice. In RT-PCR analysis (A), single-stranded cDNA was synthesized using total cellular RNAs from the lungs and brains of both wild-type (+/+) and piebald (s/s) mice and (dT)12–18 primers, and the cDNA containing the sequence of the aberrant Ednrb transcript was amplified using combinations of primers MEB1 and MEB9 or MEB12 (indicated in Fig. 3B). In 3′-RACE analysis (B), single-stranded cDNA was synthesized using total cellular RNAs from the lungs and brains of both mice and adapter primers, and the 3′-end of the aberrant Ednrb transcript was amplified using primer MEB18 (indicated in Fig. 3B). The products were analyzed on 2.0% agarose gel and visualized by ethidium bromide staining. The lengths of the DNAs are shown.

transfected cells expressed luciferase levels that were not significantly different from the background of the luminometer. These findings indicate that the 5.5-kb retroposon-like element has the ability to reduce gene expression if it is inserted into the intron in the right orientation.

DISCUSSION

In this study, we have shown that the Ednrb gene in s/s mice carries an insertion of a 5.5-kb retroposon-like element that includes canonical sequences of a polyadenylation signal and a splice acceptor site. This insertion causes premature termination and aberrant splicing in ~75% of the normally initiated Ednrb mRNA in s/s mice, resulting in reduced levels of the structurally intact Ednrb transcript (~25% of the wild-type levels) (Fig. 7). The levels of the intact Ednrb mRNA seen in s/s mice are compatible with both the reduced EDNRB density to ~27% of the wild-type levels and the mild anomaly of white coat spotting in ~20% of the body surface area in s/s mice (22). Furthermore, this is consistent with our finding that insertion of the 5.5-kb retroposon-like element into a heterologous intron in a reporter plasmid reduces luciferase expression to ~25% of the levels seen in the reporter plasmid without the insertion. The premature termination occurs at the polyadenylation site in the 3′-direct repeat of the retroposon-like element, and aberrant splicing occurs at the splice acceptor site in the 5′-direct repeat of the retroposon-like element (Fig. 7). Thus, as shown in Fig. 6, if the element is inserted into the intron in the reverse orientation, which destroys both sites, the retroposon-like element is unable to reduce expression of a heterologous gene.

The 5.5-kb retroposon-like element has a 507-bp direct repeat at both the 5′- and 3′-ends, which is highly homologous to the 526-bp direct terminal repeats in insertion sequences of the nonagouti (a) and black-and-tan (a′) alleles of the agouti locus (35). It is regrettable that the 526-bp direct repeats have not been investigated in detail, except for the proposal that they are involved in homologous recombination for a-to-a′ reverse mutation (35). The retroposon-like element also shows sequence similarity to ETn (38) or its relatives, the t haplotype-specific element (39) and the MusD element (40) both of which share homology with ETn in a 460-bp 3′-region and in 273- and 164-bp internal regions of the core sequence, respectively. The ETns are a family of murine retrotransposon-like elements possessing long terminal repeats, and ~1000 copies are present in the mouse genome (41). These elements are abundantly transcribed in early mouse embryogenesis, although they...
contain mainly non-retroviral noncoding sequences of unknown origin (41), except for a short stretch of a retroviral \textit{pol} gene (40). The significance of these partial sequence similarities among the 5.5-kb retroposon-like element, ETn, and its relatives is currently unclear, whereas the MusD elements, classified as long terminal repeat-containing and retrotransposon-like and as possessing \textit{gag}, \textit{pro}, and \textit{pol} genes homologous to those in type D viruses or betaretroviruses (40), are suggested to provide the proteins necessary for ETn retrotransposition in \textit{trans} (42). However, it is

**FIGURE 6.** Effect of the retroposon-like element on expression of a heterologous gene. \textit{A}, construction of the reporter plasmid carrying the 5.5-kb retroposon-like element. The luciferase (\textit{LUC}) reporter construct pTK-tat\(\alpha\)-luc and its derivatives carrying the 5.5-kb DNA element in the right orientation (pTK-tat\(\alpha\)-luc) and in the reverse orientation (pTK-tat\(\beta\)-luc) are shown schematically. Plasmids pTK-tat\(\alpha\)-luc, pTK-tat\(\alpha\)-luc, and pTK-tat\(\beta\)-luc contain the herpes simplex virus thymidine kinase (\textit{TK}) promoter, \textit{HIV}, human immunodeficiency virus. \textit{B}, luciferase activity in the transfected COS-7 cells with the luciferase reporter plasmid. COS-7 cells were cotransfected with either the control plasmid (pUC18) or luciferase reporter plasmid (pTK-tat\(\alpha\)-luc, pTK-tat\(\alpha\)-luc, or pTK-tat\(\beta\)-luc) as indicated and with a \textit{lacZ} expression plasmid. Cell lysates were prepared and then assayed for luciferase activity, which is expressed as relative light units (RLU) and represents the mean ± S.E. of triplicate assays normalized to \(\beta\)-galactosidase activity as an internal control.

**FIGURE 7.** Schematic representation of the \textit{Ednrb} transcripts in piebald mice. The introns and flanking sequences of the \textit{Ednrb} gene are represented by a solid line, and the exons are shown as closed rectangles. The open boxes and double lines show the direct repeat and the core sequence of the 5.5-kb retroposon-like element inserted into intron 1 of the \textit{Ednrb} gene, respectively. The open and closed inverted triangles indicate the splice acceptor site in the 5'-direct repeat and the polyadenylation site in the 3'-direct repeat, respectively. The normal and abnormal \textit{Ednrb} transcripts in piebald mice are presented at the top and bottom, respectively. The sizes of the transcripts are indicated in parentheses. Exons and the retroposon-like element portion in the genomic DNA and their corresponding regions in the transcripts are connected by solid lines. The percentages of each transcript in all of the normally initiated \textit{Ednrb} mRNA are indicated.
Characterization of the Ednrb Mutation in the Piebald Allele

interesting to note that ETn insertions occur in at least 19 mouse loci (43), such as the T locus (44), the Fas locus (45, 46), and the Adcy1 locus (41), producing loss-of-function mutations in most of these cases.

Here, a search of the 507-bp direct repeat for canonical consensus sequences revealed that, in addition to sequences of a canonical polyadenylation signal, a T cluster of 49 nucleotides downstream of the signal, a canonical splice acceptor site, and a canonical branch site 54 nucleotides upstream of the acceptor site, there exist a TATA box-like sequence (TATAAT) (47) 68 nucleotides upstream of the polyadenylation signal and a consensus sequence for AP-2 binding (CCCCCGAACGGG) (48) 181 nucleotides upstream of the polyadenylation signal. There is no conventional CCAAT box (49) in the direct repeat. The location of the TATA box-like promoter element, AP-2-binding site, and polyadenylation signal in the direct repeat suggests that the retroponson-like element is transcribed through the direct repeat and that the resulting transcript has a terminal redundancy as in retrotransposon or retrovirus possessing retroviral long terminal repeats (50). Furthermore, there is a duplication of six nucleotides of host DNA at the insertion site, which has been recognized in the integration process of retrotransposon or retrovirus (50). In addition, the retroponson-like element reveals no long open reading frames and no significant homology to known retroviral genes, except for a short stretch of a retroviral pol gene, but contains a 17-bp sequence (TGGCGCCACAGTGGAGG) with 16 of 17 matches with the consensus sequence for AP-2 binding (CCCCCGAACGAGG) (48) 181 nucleotides upstream of the polyadenylation signal. There is no conventional CCAAT box (49) in the direct repeat. The location of the TATA box-like promoter element, AP-2-binding site, and polyadenylation signal in the direct repeat suggests that the retroponson-like element is transcribed through the direct repeat and that the resulting transcript has a terminal redundancy as in retrotransposon or retrovirus possessing retroviral long terminal repeats (50).

We have demonstrated that a hybrid transcript carrying ~1-kb Ednrb exon 1 and the ~5.5-kb retroponson-like element portion is expressed in s/s mice. It ends at the polyadenylation site in the 5′-direct repeat and is spliced at the splice acceptor site in the 5′-direct repeat. The first in-frame stop codon is 28 bp downstream of exon 1 in the hybrid transcript; and as a result, the hybrid transcript encodes a putative truncated protein with the 281 residues encoded by exons 2–7 replaced with an arbitrary peptide of nine residues: VCGPPRTH. This truncation results in a deletion of the first extracellular loop through the cytoplasmic C-terminal tail of EDNRB and then abrogates functional expression of EDNRB. It has already been shown that deletion of the first two transmembrane domains of EDNRB abrogates its functional expression in spotting lethal rats (52). It may be presumed that a fraction of the transcription of the Ednrb mRNA in s/s mice is terminated at the putative polyadenylation site in the 5′-direct repeat because the 5′-direct repeat also harbors both a potential polyadenylation signal and a T cluster, as does the 3′-direct repeat in the 5.5-kb retroponson-like element. However, 3′-RACE using a primer in intron 1 of the Ednrb gene flanking the 5.5-kb element in the 5′-region and an adapter primer that targets the poly(A) tail failed to amplify any sequence in s/s mice (data not shown). Furthermore, Northern analysis could not detect the band (~16 kb) expected when the Ednrb mRNA transcription ends in the 5′-direct repeat. We therefore suggest that the Ednrb RNA transcription is not terminated at the putative polyadenylation site in the 5′-direct repeat. About 25% of the initiated Ednrb mRNA in s/s mice escapes termination in the 3′-direct repeat and is terminated at the same polyadenylation site as wild-type mRNA. Provided that the normally terminated mRNA is aberrantly spliced at the splice donor and acceptor sites of Ednrb exon 1 and the 5′- or 3′-direct repeat of the retroponson-like element, respectively, the resulting transcript should encode the same truncated protein as described above. We could not obtain any evidence of the existence of the normally terminated but aberrantly spliced Ednrb mRNA in s/s mice. Almost all of the normally terminated Ednrb mRNA in s/s mice is of normal size and at steady-state levels consistent with the EDNRB density levels in s/s mice. In other words, almost all of the normally terminated Ednrb mRNA is normally spliced and then encodes structurally intact protein. Therefore, although the splice acceptor site in the retroponson-like element is activated in the presence of the authentic acceptor site.

We reported previously that s/s, s/s, s/s, and s/s+ mice, in which the relative ratio of Ednrb expression is about 0:12.5:25:50%, show a “graded” coat color phenotype in the extent of white spotting, having a white coat in >95, 40–50, ~20, and ~0% of the body surface area, respectively (22). This indicates that the extent of white spotting is precisely dependent on the dosage of Ednrb expression. In contrast, the megacolon phenotype occurs in the s/s, s/s, or s/s+ mice, but almost never in the s/s, s/s, or s/s+ mice (6, 9). This is compatible with the idea that the two neural crest-derived cell lineages require different minimal threshold levels of Ednrb expression (22). However, with the s allele, Ednrb expression might be more severely impaired in the melanocyte lineage in a cell type-selective manner. Our present data demonstrate that the expression of structurally intact Ednrb mRNA is uniformly reduced in most of the s/s tissues, including the intestine. Furthermore, not only Ednrb gene expression, but also heterologous gene expression is reduced because of the insertion of the 5.5-kb retroponson-like element into the intron of the gene, reflecting the generalized ability of the 5.5-kb inserted element to reduce gene expression. These findings argue against the possibility that the Ednrb mRNA is expressed in a cell type-selective manner. Thus, it is likely that ~12.5% of the wild-type levels of EDNRB density is sufficient for the normal development of myenteric ganglion neurons, whereas 25–50% of the wild-type levels is required for complete development of epidermal melanocytes. We could not detect reduced expression of structurally intact Ednrb mRNA in s/s liver. The premature termination and aberrant splicing of the Ednrb transcript are likely to occur in s/s liver because the 6.5-kb RNA seems to be detected. The insertion of the 5.5-kb retroponson-like element may enhance liver-specific Ednrb transcription.

Acknowledgments—We thank David J. Mangelsdorf and Trish Willy for luciferase assays, Cheryl E. Gariepy for editing the manuscript, and Damiane deWit for technical assistance.
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