DNA RESEARCH 3, 101–105 (1996) Short Communication

A Mutation in Endothelin-B Receptor Gene Causes Myenteric Aganglionosis and Coat Color Spotting in Rats

Tetsuo KUNIEDA,[1,*] Taeko KUMAGAI,[1] Takehito TSUJI,[1] Tsuyoshi OZAKI,[2]
Hideaki KARAKI,[3] and Hiroshi IKADAI[4]
Faculty of Agriculture, Okayama University, Okayama, Okayama, Japan,[1]
Laboratory of Experimental Animals, National Institute for Physiological Science, Okazaki, Aichi, Japan,[2]
Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Science, The University of Tokyo, Bunkyo-ku, Tokyo, Japan,[3] and
Imamichi Institute for Animal Reproduction, Dejima-mura, Ibaraki, Japan[4]

(Received 24 March 1996)

Abstract

Congenital aganglionosis rat (AR) is a mutant with an autosomal recessive gene (*sl*), which shows megacolon caused by the absence of myenteric ganglion cells and white coat-color with a small pigmented spot on the head. Recently, targeted disruption of the endothelin-B (ET_B) receptor gene (*EDNRB*) in the mouse has been reported to cause aganglionic megacolon and coat color spotting resembling the phenotypes of the *sl/sl* rats. To identify the mutation responsible for the phenotypes of the *sl/sl* rats, we determined the nucleotide sequences of the *EDNRB* genes of the *sl/sl* rats and found that a 301-bp region intervening between direct repeat sequences was deleted in the *EDNRB* gene, and the deletion produces various transcripts due to aberrant splicing.

Key words: aganglionosis; rat; endothelin-B receptor

The congenital aganglionosis rat (AR) was originally described as having an aganglionic bowel caused by an autosomal recessive gene (*sl*).[1] The rat is characterized by megacolon and white coat color with a small pigmented spot on the head. As both myenteric ganglion cells and epidermal melanocytes are derived from neural crest cells,[2] the phenotypes of the mutant rat are thought to be caused by defects in migration and differentiation of the neural crest cells. The mutant *sl/sl* rats have been used as an animal model of human Hirschsprung's disease that is also characterized by the absence of enteric ganglia in the distal colon and incomplete innervation in the gastrointestinal tract.[3]

Recently, targeted disruption of endothelin-B (ET_B) receptor gene (*EDNRB*) in the mouse was reported to cause aganglionic megacolon and coat color spotting,[4] indicating an important role of the ET_B receptor in the migration and differentiation of the cells derived from the neural crest. This suggests the possibility that the abnormal phenotypes of the *sl/sl* rats are also caused by a mutation in the *EDNRB* gene. The ET_B receptor is one of the known mammalian endothelin receptor subtypes belonging to the G-protein-coupled receptor gene family.[5] We, therefore, investigated the nucleotide sequences of the *EDNRB* gene of the *sl/sl* rats in the present study.

Total RNA was prepared from the brains, kidneys, lungs, and eyeballs of the *sl/sl* and normal rats and subjected to RT-PCR using *EDNRB*-specific PCR primers to amplify overlapping regions of the entire coding sequence of the *EDNRB* cDNAs. As shown in Fig. 1, the length and the pattern of the RT-PCR products including the 5′ region of rat *EDNRB* cDNA (fragments 1, 2, and 4) are different between the *sl/sl* and the normal rats, whereas those of the 3′ region (fragment 3) showed no difference. The major band of the RT-PCR products of the *sl/sl* rats amplified by primer pairs Pr1 and Pr4 (fragment 4), Pr1 and Pr3 (fragment 2), and Pr5 and Pr3 (fragment 1) were approximately 300 bp shorter than those of the normal rats. Furthermore, those PCR products of the *sl/sl* rats gave less intensive additional bands in the upper area of the major band. The RT-PCR products of the kidneys, lungs, and eyeballs were similar to those of the brain (data not shown). The mutant rats were, therefore, suggested to have deletions in the 5′ region of the *EDNRB* transcripts. To characterize the deletions, we cloned the RT-PCR products of the *EDNRB* gene and determined the nucleotide sequence of the clones. Consequently, cDNA clones of four different types were identified in the *sl/sl* rats. Comparisons of the nucleotide sequences of the cDNAs of *EDNRB* gene be-

Communicated by Yoshihide Hayashizaki

* To whom correspondence should be addressed. Tel. +81-86-251-8314, Fax. +81-86-254-0714, E-mail: tkunieda@cc.okayama-u.ac.jp

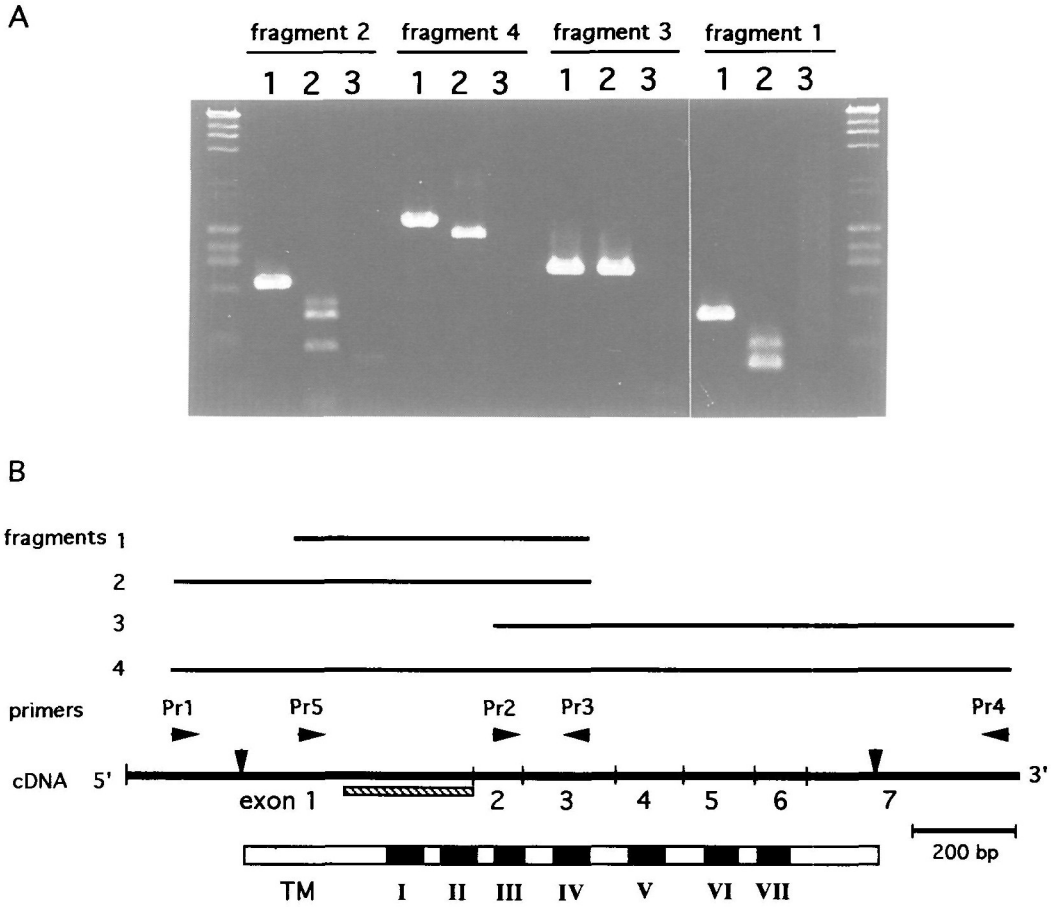

Figure 1. RT-PCR analysis of the rat *EDNBR* cDNAs. (A) RT-PCR products of the *EDNRB* gene of *sl/sl* and normal rats. Total RNA was prepared from brains of *sl/sl* and normal rats using the guanidine thiocyanate-sarkosyl method.[23] First-strand cDNA was synthesized from 10 μg of total RNA using oligo d(T) primers and SuperScript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD). PCR amplification was carried out for 40 cycles consisting of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 120 s. The 50-μl reaction mixture contained 0.4 μg of each primer, 2.5 U of *Taq* polymerase (Gibco BRL), 200 mM of dNTPs, and *Taq* buffer containing 1.5 mM Mg^{2+}. Five microliters of the PCR products were electrophoresed through a 1.2% agarose gel. Four fragments (fragments 1, 2, 3, and 4) were amplified using the sets of primers as indicated in B. Lanes: 1, normal rat; 2, *sl/sl* rat; 3, without template cDNA. Left and right lanes contain DNA size markers of *Hin*dIII digested λ DNA and *Hae* III digested ϕX 174 DNA. (B) Schematic representation of the rat *EDNRB* RT-PCR fragments and position of the PCR primers. Rat *EDNRB* RT-PCR fragments (fragments 1 to 4), amplified from brain RNAs of the *sl/sl* and normal rats, are shown in comparison with the *EDNRB* cDNA. The location and orientation of the primers are indicated by horizontal arrowheads. Vertical arrows stand for translation initiation and termination codons. Hatched line denotes the region commonly deleted in the four types of RT-PCR fragments of the *sl/sl* rat. The structure of the ET_B receptor is presented at the bottom. Transmembrane domains (TM I to VII) are displayed as filled boxes. The nucleotide sequence of the primers are: Pr1 (5′-GTGCTGCAGTTCAGAGGCGTGGCTGGGTAGC-3′), Pr2 (5′-ATCACAGTGTTGAGTCTATGTGCTCT-3′), Pr3 (5′-CATCAAAACCTATGGCTTCAGGGACAG-3′), Pr4 (5′-GTCCTGCAGATGGCTTTCTTAGGTTGTAAAC-3′), and Pr5 (5′-GCGTCGACGCCACCCACTAAGACCTCC-3′).

tween normal and mutant rats revealed that the shortest type of transcript (transcript 1) had a 270-bp deletion and the second one (transcript 2) had a 255-bp region substituted with a 17-bp sequence. In the remaining two types (transcripts 3 and 4), an additional 44-bp sequence was inserted into transcripts 1 and 2, respectively (Fig. 3). The commonly deleted region in the four types of transcripts is located between nucleotide 429 to 683 of the rat *EDNRB* cDNA sequence reported by Sakurai et al.[5] The 3′ end of the deleted region corresponds to the boundary between exons 1 and 2 of human and bovine *EDNRB* cDNAs[6,7] (Fig. 1B), suggesting that these transcripts arise from aberrant splicing.

To characterize the mutation of the *EDNRB* gene that causes the aberrant transcripts, we investigated the genomic structure of the *EDNRB* gene of the *sl/sl* rats. The cloned RT-PCR products consisting of 0.7-kb and 1.0-kb fragments corresponding to exons 1 and 2 (probe

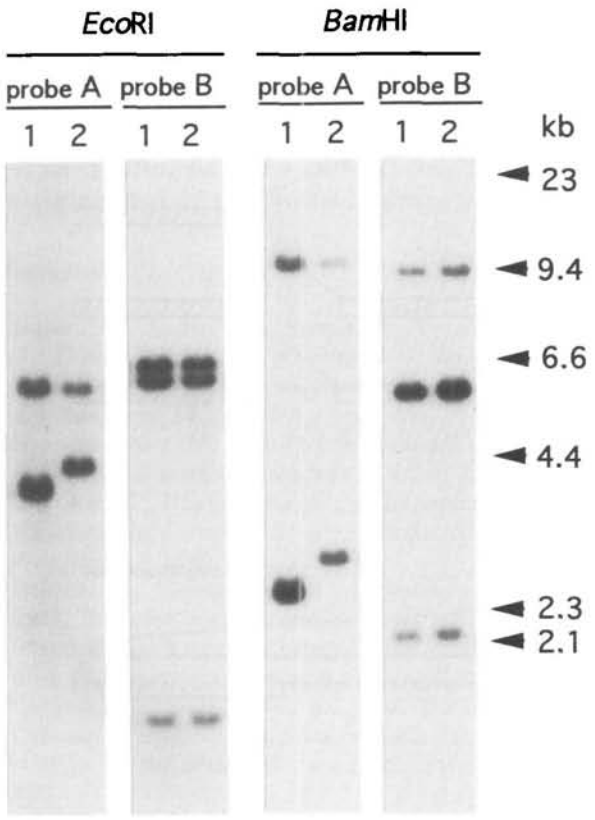

Figure 2. Southern blot analysis of *EDNRB* gene of *sl/sl* and normal rats. The cloned PCR products consist of 0.7-kb fragments corresponding to exons 1 and 2 (A), and 1-kb fragment corresponding to exons 3 to 7 (B) of the *EDNRB* cDNA were labeled by random priming with [α-^{32}P]dCTP, and used as probes. High-molecular-weight genomic DNA was isolated from the livers of *sl/sl* (Lane 1) and normal (Lane 2) rats by phenol extraction, digested with *Eco*RI or *Bam*HI endonucleases, separated by 0.7% agarose gel electrophoresis, and transferred to nylon membrane filters. The filters were hybridized with hybridization solutions containing the ^{32}P-labeled probes, 50% formamide, 5× Denhardt's solution, 0.5% SDS, and 5× SSC at 50°C for 24 hr, washed twice in 1× SSC, 0.1% SDS at room temperature and once in 0.1× SSC, 0.1% SDS at 50°C, and exposed to X-ray films for 48 h with an intensifying screen at −70°C. The *sl/sl* rat gave 3.8-kb *Eco*RI and 2.5-kb *Bam*HI fragments hybridizing to probe A, whereas the normal rat gave 4.1-kb *Eco*RI and 2.8-kb *Bam*HI fragments.

A) and to exons 3 to 7 (probe B) of the *EDNRB* gene, respectively, were used as a probe for the Southern blot hybridization of genomic DNAs of the mutant and normal rats. As shown in Fig. 2, probe A showed restriction fragment length polymorphisms (RFLPs) in *Eco*RI and *Bam*HI digests. The *sl/sl* rats gave 3.8-kb *Eco*RI and 2.5-kb *Bam*HI fragments, whereas the normal rats gave 34.1-kb *Eco*RI and 2.8-kb *Bam*HI fragments. Probe B showed no RFLP between the *sl/sl* and normal rats. There is an approximately 300 bp difference between the 5′ region of the *sl/sl* and normal rats in each enzyme digest, indicating that the *sl/sl* rats have a deletion in the 5′ region of the *EDNRB* gene. To identify the exact region of the deletion, we amplified the region spanning exon 1 and intron 1 from the genomic DNAs by using primers flanking this region, and sequenced the amplified fragments. Comparison of the nucleotide sequence between the *sl/sl* and normal rats revealed that the *sl/sl* rats carry a deletion of 301-bp region consisting of the 255-bp sequence of the exon 1 and 46-bp sequence of the intron 1. Notably, we found direct repeat sequences consisting of 16 nucleotides in the 3′ end and the 5′ adjacent sequence flanking the deleted region (Fig. 3). Eleven of the 16 nucleotides were identical in these two direct repeats. We also identified inverted repeat sequences consisting of eight nucleotides just upstream of the direct repeats in exon 1.

Recently Gariepy et al.[8] have also reported the 301-bp deletion in the *EDNRB* gene of the *sl/sl* rats, but we further revealed that the deletion produces various transcripts and that the deleted region intervenes between the direct repeats. As shown in Fig. 3, transcripts 1 and 3 presumably arise from the cryptic splice donor site located 15 bp upstream of the deleted region while transcripts 2 and 4 arise from the site located 17 bp downstream. The nucleotide sequence of the upstream cryptic splice site coincides with that of the common splice donor site.[9] The additional 44-bp sequence inserted into transcripts 3 and 4 may be derived from the intronic sequence by aberrant splicing, because we could not detect any transcripts having the 44-bp sequence in the normal rats (data not shown). These findings indicate that the loss of the authentic splicing donor site of the *EDNRB* gene results in aberrant splicing which generates various transcripts.

The existence of the direct repeats at the 5′ and 3′ ends of the deleted region suggests possible mechanisms for the generation of the 301-bp deletion. Deletion or duplication of particular sequences intervening between direct repeats have been reported in several human mutations.[10,11] These deletion mutations are believed to be generated by replication slippage arising from illegitimate pairing between two complementary sequences at the replication or by unequal crossing over resulting in either deletion or duplication of a particular sequence by crossing over between the direct repeats at meiosis. The 16-bp direct repeats might, therefore, be responsible for the deletion of the *EDNRB* gene. The inverted repeats located at the site adjacent to the direct repeats are also likely to play an important role in the generation of the deletion through the formation of a particular second structure of single-stranded DNA.

The substitutions in the transcripts 2, 3, and 4 cause frame-shift mutations that introduce premature termination codons at codons 78, 114, and 78, respectively. Transcript 1, however, has an in-frame deletion that causes an internal deletion of 90 amino acids in the products of the

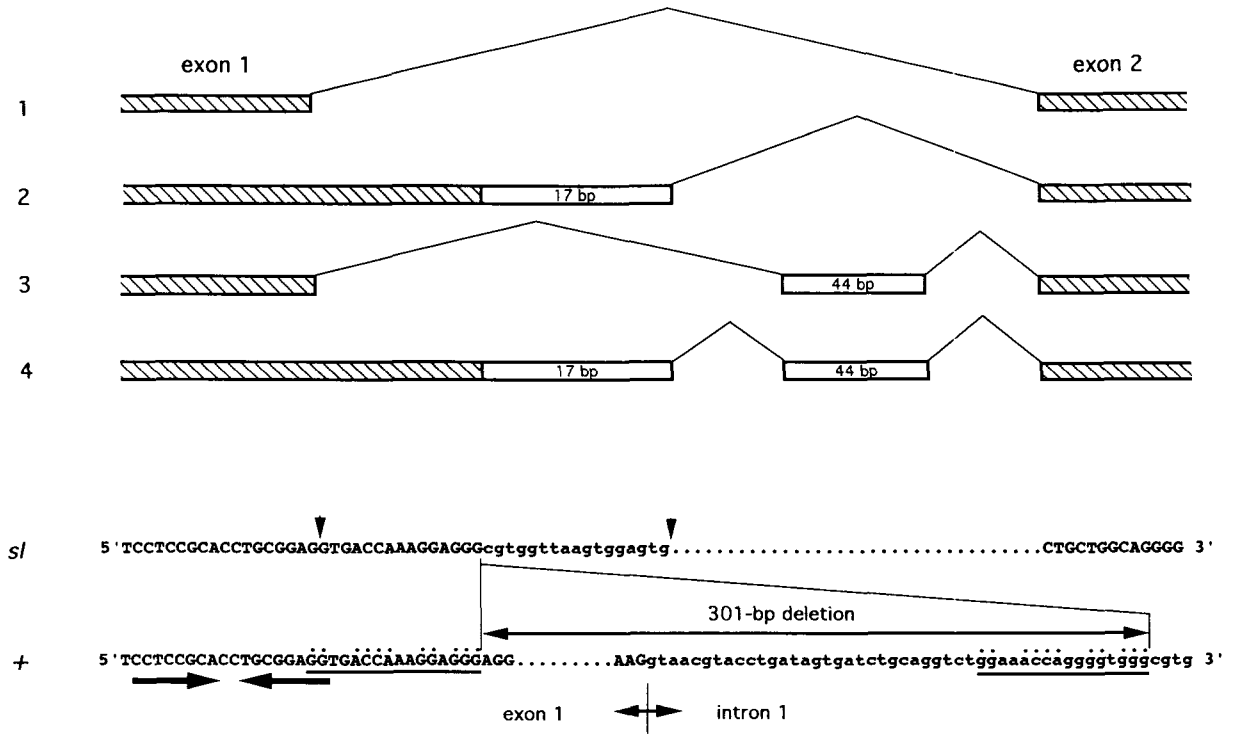

Figure 3. A partial nucleotide sequence of the *EDNBR* gene of *sl/sl* and normal rats and a schematic illustration showing the four types of the transcripts of the mutant *EDNBR* gene (1, 2, 3, and 4). The cDNA and genomic DNA of the *EDNBR* gene were amplified by primers Pr1 and Pr2, and Pr5 and Pr7 (5'-TCTCTTGACATAAAGCCAGGACC-3'), respectively. The amplified fragments were subcloned into pCRII vector (Invitrogen, San Diego, CA) and sequenced in both orientations by the dideoxy chain terminating method using an automated DNA sequencer (SQ 5500, Hitachi, Tokyo, Japan). The splicing forms of the transcripts, having a 270-bp deletion and the substitution of a 255-bp sequence with a 17-bp sequence, are indicated in 1 and 2, respectively. The splicing forms of the transcripts having an additional 44-bp sequence are indicated in 3 and 4. *sl* and + indicate mutant and wild-type alleles, respectively. Hatched boxes and capital letters indicate the regions corresponding to the exons, and open boxes and lower-case letters indicate the regions corresponding to the intron. Underlines and horizontal arrow in the sequence of the wild-type allele denote direct repeats and inverted repeats, respectively. Dots indicate nucleotides identical in the direct repeats. Vertical arrows stand for cryptic splicing donor sites on the mutant allele. Broken line in *sl* allele corresponds to the nucleotide sequence of intron 1 which presumably includes the 44-bp sequence inserted into transcripts 3 and 4, and that in the + allele corresponds to the nucleotide sequence of exon 1.

transcript. As the deleted region of the transcript corresponds to the first and second transmembrane domains of the receptor (Fig. 1B), the products have neither first nor second transmembrane domains of the G-protein-coupled heptahelical receptor, suggesting complete loss of the function of the receptor. Deletion of any of the transmembrane domains of the β-adrenergic receptor, which also belongs to the G-protein-coupled heptahelical receptor gene family, has resulted in loss of the receptor function.[12] The loss of the ET_B receptor function that produces phenotypes similar to the *sl/sl* rat has also been reported in mice with knock out of the *EDNRB* gene and s^l/s^l mutant mice.[4] Furthermore, the *EDNRB* gene has been reported to be expressed in human myenteric ganglion neurons,[13] and the ligands for the ET_B receptor to stimulate the proliferation and chemokinesis of human melanocytes and melanoma cells.[14,15] We, therefore, concluded that the 301-bp deletion in the *EDNRB* gene is responsible for the myenteric aganglionosis and the coat color spotting phenotypes of the *sl/sl* rats.

The human Hirschsprung's disease is a heterogeneous genetic disorder with autosomal dominant, autosomal recessive, and polygenic forms.[16] One of the autosomal dominant diseases has been found to be caused by a mutation in the *RET* proto-oncogene,[17] which is a member of a receptor tyrosine kinase gene family originally isolated by transforming activity in NIH3T3 cells.[18,19] Missense mutations in the *EDNRB* gene have recently been found in the cases of autosomal recessive and autosomal dominant Hirschsprung's disease.[20,21] The *sl/sl* rat, therefore, is an authentic model for such types of human Hirschsprung's disease. The present findings for the *sl/sl* rat also indicate that the rat is an excellent model for investigating the *in vivo* function of the ET_B receptor.

The *EDNRB* gene is expressed in various tissues and organs including brain, kidney, lung, heart and endothelial cells,[22] but the precise functions of the ET_B receptor, except for those in the vascular endothelium, are still yet unknown. Further investigations of the *sl/sl* rats should reveal novel functions of the *EDNRB* gene in various organs, tissues, and developmental stages of the mammals.

References

1. Ikadai, H., Fujita, H., Agematsu, Y., and Imamichi, T. 1979, Observation of congenital aganglionosis rat (Hirschsprung's disease rat) and its genetical analysis, *Cong. Anom.*, **19**, 31–36.
2. Bronner-Fraser, M. 1994, Neural crest cell formation and migration in developing embryo, *FASEB J.*, **8**, 699–706.
3. Swenson, O., Rheinlander, F., and Diammond, I. 1949, Hirschsprung's disease, a new concept of the etiology, *New Engl. J. Med.*, **241**, 551–556.
4. Hosoda, K., Hammer, R. H., Richardson, J. A. et al. 1994, Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice, *Cell*, **79**, 1267–1276.
5. Sakurai, T., Yanagisawa, M., and Takuwa, Y. 1990, Cloning of a cDNA encoding a nonisopeptide-selective subtype of the endothelin receptor, *Nature*, **348**, 732–735.
6. Arai, H., Nakao, K., Takaya, K. et al. 1993, The human endothelin-B receptor gene, *J. Biol. Chem.*, **268**, 3463–3470.
7. Mizuno, T., Saito, Y., Itakura, M. et al. 1992, Structure of the bovine ET_B endothelin receptor gene, *Biochem. J.*, **287**, 305–309.
8. Gariepy, C. E., Cass, D. T., and Yanagisawa, M. 1996, Null mutation of endothelin receptor type B gene in spotting lethal rats causes aganglionic megacolon and white coat color, *Proc. Natl. Acad. Sci. USA.*, **93**, 867–872.
9. Padgett, R. A., Grabowski, P. J., Konarska, M. M. et al. 1986, Splicing of messenger RNA precursors, *Ann. Rev. Biochem.*, **55**, 1119–1150.
10. Emmeric, J., Chadeuf, G., Alhen-Gelas, M. et al. 1994, Molecular basis of antithrombin type I deficiency: The first large in-frame deletion and two novel mutations in exon 6, *Thromb. Haemost.*, **72**, 534–539.
11. Tiller, G. E., Rimoin, D. L., Murray, L. W., and Cohn D. H. 1990, Tandem duplication within a type II collagen gene (COL2A1) exon in an individual with spondyloepiphyseal dysplasia, *Proc. Natl. Acad. Sci. USA.*, **87**, 3889–3893.
12. Dixon, R. A. F., Sigal, I. S., Candelore, M. R. et al. 1987, Structural features required for ligand binding to the β-adrenergic receptor, *EMBO J.*, **6**, 3296–3275.
13. Inagaki, H., Bishop, A. E., Escrig, C., Wharton, J., Allen-Mersh, T. G., and Polak, J. M. 1991, Localization of endothelinlike immunoreactivity and endothelin binding sites in human colon, *Gastroenterology*, **101**, 47–54.
14. Yada, Y., Higuchi, K., and Imokawa, G. 1991, Effects of endothelins on signal transduction and proliferation in human melanocytes, *J. Biol. Chem.*, **266**, 18352–18357.
15. Yohn, J. J., Smith, C., Stevens, T. et al. 1994, Human melanoma cells express functional endothelin-1 receptor, *Biochem. Biophys. Res. Commun.*, **201**, 449–457.
16. Bardner, J. A., Sieber W. K., Garver, K. L., and Chakravarti, A. 1990, A genetic study of Hirschsprung disease, *Am. J. Hum. Genet.*, **46**, 568–560.
17. Romeo, G., Ronchetto, P., Luo, Y. et al. 1994, Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease, *Nature*, **367**, 377–378.
18. Takahashi, M., Ritz, J., and Cooper, G. M. 1985, Activation of a novel human transforming gene, ret, by DNA rearrangement, *Cell*, **42**, 581–588.
19. Kunieda, T., Matsui, M., Nomura, M., and Ishizaki, R. 1991, Cloning of an activated human ret gene with a novel 5′ sequence fused by DNA rearrangement, *Gene*, **107**, 323–328.
20. Puffenberger, E. G., Hosoda, K., Washington, S. S. et al. 1995, A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease, *Cell*, **79**, 1257–1266.
21. Amiei, J., Attié, T., Jan, D. et al. 1996, Heterozygous endothelin receptor B (*EDNRB*) mutations in isolated Hirschsprung disease, *Hum. Mol. Genet.*, **5**, 355–357.
22. Sakamoto, A., Yanagisawa, M., Sawamura, T. et al. 1993, Distinct subdomains of human endothelin receptors determine their selectivity to ET_A-selective antagonist and ET_B-selective agonist, *J. Biol. Chem.*, **268**, 8547–8553.
23. Chomczynski, P. and Sacchi, N. 1987, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, *Anal. Biochem.*, **162**, 156–159.