Hirschsprung disease is a heterogeneous genetic disorder, causative genes of which include the endothelin B receptor (ETB). To investigate the mutations of ETB in Hirschsprung disease, expression of the ETB gene in lymphoblastoid cells from patients and normal healthy adults was examined, and novel mutant transcripts were found. The mutant ETB gene transcripts lacked a 134-bp nucleotide sequence corresponding to exon 5, and some also contained a substitution from A to G at position 950 in exon 4, resulting in an amino acid substitution from glutamine (Q) to arginine (R). This substitution was suspected to be the result of RNA editing because it was not present in the genomic sequence. Transfection experiments using ETB minigenes containing the editing site with or without the gene for double-strand RNA deaminases (ADAR1 and ADAR2) revealed that the deaminases were involved in RNA editing. Furthermore, a c-Myc-tagged mutant ETB protein was not detected by Western blot analysis. The present results show that the mutant ETB transcripts were novel splice variants, which might not be translated, or that the products translated from splice variants might be quickly degraded, presumably because of their instability. The preferential production of this null function ETB by RNA editing/splicing could be involved in the etiology of some cases of Hirschsprung disease.

Endothelins (ETs), one of which was originally discovered as an endothelium-derivered vasoconstricting factor, comprise a family of three isopeptides, termed ET-1, ET-2, and ET-3. They act on two subtypes of G protein-coupled receptors, ETA and ETB (1). ETA exhibits higher affinities, on a subnanomolar order, to ET-1 and ET-2 compared with its affinity for ET-3, whereas ETB accepts all three isopeptides equally. The ET receptors regulate multiple effector pathways such as phospholipase C via Gq, adenylyl cyclase via Gs in smooth muscle cells (ETA), and via Gq in endothelial cells (ETB) (2). Critical determinants in ET receptor domains for subtype-specific Gq/Gs protein coupling were investigated by generating chimeric receptors in two subtypes. It was found that the second and third intracellular loops are important for selective G protein couplings (3).

Physiological and pathological functions of the ET receptor systems have been defined by pharmacological studies as well as by gene targeting. Two lines of evidence arose from mutant mice generated by gene targeting of either the ETB or the ET-3 gene. The mice showed phenotypic similarity to Hirschsprung disease (HSCR) or Shah-Waardenburg syndrome in humans (4, 5). HSCR is a congenital intestinal disorder characterized by the absence of ganglionic cells in the distal portion of the intestinal tract. This absence is primarily the result of premature arrest of cranio-caudal migration of neural crest cells. Critical mutations in the ETB gene locus have also been found among naturally occurring HSCR in humans, mice, and rats (4–14).

In the course of analyzing candidate genes for sporadic cases of Japanese HSCR patients, a novel transcript of the ETB gene was discovered. It was derived from splicing and/or RNA editing of the primary ETB transcript, and it occurred in one of three patients as well as in some normal subjects. The splicing reaction removed the entire fifth exon and resulted in a deletion of both the fourth transmembrane domain and the distal portion of the third extracellular loop from the ETB polypeptide. An observed sequence discrepancy between the genomic and the reverse-transcribed samples suggested that editing of ETB transcripts occurred at the new 5′-splicing site of the shorter transcript. Here, we examine the mechanisms of splicing and RNA editing of the ETB primary transcripts as well as the functional consequences of these post-transcriptional alterations in mammalian cells. Finally, the causal relationship of the production of this mutant ETB to HSCR was assessed.

**MATERIALS AND METHODS**

**Cell Culture**—Lymphoblastoid cells from three patients with HSCR were established by Epstein-Barr virus and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The human neuroblastoma cells (SKNMC), human hepatoma cells (HepG2), human embryonic kidney cells (HEK293), human cervical carcinoma cells (HeLa), and COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

This study was approved by the appropriate institutional review board; appropriate informed consent was obtained from all human subjects.

**Isolation of DNA and RNA**—Genomic DNAs were isolated from human lymphocytes, lymphoblastoid cells, and rat liver using the SDS-proteinase K method as described (15). Briefly, the cell pellet was washed three times with 1× phosphate-buffered saline and suspended in buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, pH 8.0, 1% SDS, and 100 μg/ml proteinase K for 3 h to overnight at 37 °C. The DNAs were extracted twice with phenol-chloroform then once with chloroform, and were precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, pH 8.0. Total RNA was isolated from human
The position of each primer is indicated relative to the first A in the initiation ATG. The KpnI sites in the primers (KpnI-WF, KpnI-WR, and KpnI-MR) are underlined.

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex1F</td>
<td>5–20</td>
<td>5'–CACCTGCAGGATCCGCCGCCCTGCAAGCTT–3'</td>
</tr>
<tr>
<td>Ex7R-1328</td>
<td>1305–1328</td>
<td>5'–CACCTGCTAAAGTTGCTATTTTTACT–3'</td>
</tr>
<tr>
<td>Ex4F</td>
<td>801–825</td>
<td>5’–TTTACAAGACAGGAAGATGTT–3’</td>
</tr>
<tr>
<td>Ex6R</td>
<td>1169–1193</td>
<td>5’–CTAAGAGTTTGGTCTACCTTT–3’</td>
</tr>
<tr>
<td>Ex3F</td>
<td>735–764</td>
<td>5’–GAGCTACAAAGGATGTAGTCGCTG–3’</td>
</tr>
<tr>
<td>Ex7R-1246</td>
<td>1217–1246</td>
<td>5’–CTCTCGAGGCTTGGACATCCATCTGCAGGCGGCTACA–3’</td>
</tr>
<tr>
<td>KpnI-WF</td>
<td>21–15</td>
<td>5’–CAGTGAAGATGTTGAGTACAGTGACCTGTATTT–3’</td>
</tr>
<tr>
<td>KpnI-WR</td>
<td>1311–1346</td>
<td>5’–AAATACGAGGCTTGTGACATCCAGGATACCTG–3’</td>
</tr>
<tr>
<td>KpnI-MR</td>
<td>1127–1162</td>
<td>5’–GAGACGAGGGGGGAGGCATACAGAT–3’</td>
</tr>
<tr>
<td>pRC-R</td>
<td>1043–1080</td>
<td>5’–ATATTTTGCCGCCCTGCTGATTTCT–3’</td>
</tr>
<tr>
<td>pME-2R</td>
<td>1267–1288</td>
<td>5’–GATTACAGTGACGCTGTTGTT–3’</td>
</tr>
<tr>
<td>5HT-F</td>
<td>633–656 (exon 3 of 5-HTpR)</td>
<td>5'--GAAGGCACGGGGGAGGGGCAAACAACAGAT--3'</td>
</tr>
<tr>
<td>5HT-INTR</td>
<td>Intron 3 of 5-HTpR</td>
<td>5’--GGACTACAAAGGAAGTTATCTGCGAATCTG--3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGACTACAAAGGAAGTTATCTGCGAATCTG</td>
</tr>
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</table>

lymphocytes, lymphoblastoid cells, and cultured cells using isogen reagent (Nippon Gene Co. Tokyo, Japan) according to the manufacturer's instructions.

Oligonucleotides—The oligonucleotides used for PCR amplification are listed in Table I. Numbering of the nucleotide sequence starts at the adenine nucleotide of the initiating ATG in the human ETB (16). The primers Ex1F and Ex7R-1328 were used for amplification of full-length cDNA corresponding to the ETB gene. The primers Ex4F and Ex6R were used for amplification of a region from exon 4 to exon 6 of the ETB transcripts or gene. The primers Ex3F and Ex7R-1246 were used for amplification of a region extending from exon 3 to exon 7 of the ETB gene. The primers 5HT-F and 5HT-INTR were for PCR amplification of the rat 2C subtype of the serotonin receptor (5-HTpR) (17). Specific primers (pRC-R and pME-2R) for the expression vectors pRC/CMV2 (Invitrogen, Groningen, The Netherlands) and pME18S (18) were used for amplification of transcripts from expression plasmids.

cDNA Synthesis, PCR Amplification, Cloning and Sequencing of ETB Fragments—10 µg of total RNA was used for cDNA synthesis with 100 pmol of random hexamers (Takara Shuzo Co., Tokyo). 50 µl of cDNAs was synthesized with Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) or avian myeloblastosis virus reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. The conditions for PCR were as follows: an initial 5-min incubation at 94 °C followed by 30 amplification cycles of denaturation at 94 °C for 30 s, annealing at 57 °C or at 60 °C for 30 s, and extension at 72 °C for 30 s for the small fragments, or 5 min for the fragments larger than 1.0 kb. Each 50-µl reaction contained 100 ng of genomic DNA or 10 ng of plasmid DNA, or else it contained a 1-µl aliquot of each cDNA solution, with 10 pmol of each primer, 2.5 units of Taq polymerase (Stratagene) or Pfu DNA polymerase (Stratagene) or Vent DNA polymerase (New England Biolabs). When the reaction conditions were specified by the manufacturers were followed. At least two different PCR products generated from reverse transcribed cDNAs were subcloned into pGEM-T Easy Vector (Promega Corp.).

A 1.3-kb cDNA fragment covering the entire coding region of ETB was generated using primers Ex1F and Ex7R-1328. The 387-bp and 253-bp cDNA fragments covering the region from exon 4 to exon 6 were amplified using Ex4F and Ex6R primers, and a 1.5-kb genomic fragment extending from exon 4 to exon 6 was amplified. A 5.0-kb genomic fragment including exons 3–7 was amplified with primers Ex3F and Ex7R-1246. The primer set of Ex4F and pRC-R was used for amplification of a region extending from exon 3 to exon 7 of the ETB gene.

The PCR products were separated on agarose gels, recovered, and subcloned into plasmids. Sequencing was performed on both strands by a 377A DNA autosequencer (Perkin-Elmer Cetus, Foster City, CA). No point mutations were detected during this study except the A/G substitution at nucleotide position 950 in the ETB gene.

Allele-specific Oligonucleotide Hybridization—10 µl of PCR-amplified DNA was fractionated on a 1.5% agarose gel then denatured with NaOH, neutralized, and blotted onto nitrocellulose filters (Amersham Biosciences) in 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate) for 6 h to overnight. The filters were cross-linked by UV irradiation, baked at 80 °C for 1 h, and prehybridized in 6 × SSC, 0.5% SDS, 1 × Denhardt's solution, and 10 µg/ml sonicated serum sperm DNA for 2 h at 37 °C.

Allele-specific oligonucleotides designated SVA (5’–AGCAAGCTTTCTTCTGTTG3’) and SVG (5’–AGCGGGTCTTTGTGTTG3’) contained A or G at nucleotide position 950 (underlined), respectively, and were labeled using T4 polynucleotide kinase (Takara Shuzo Co.) and [32P]ATP (6,000 Ci/mmol, Amersham Biosciences). Unincorporated [32P]ATP was removed by several precipitations. Purified probes were added to the prehybridization buffer at a concentration of 1 × 106 cpm/ml and incubated for 6 h to overnight at 37 °C for hybridization. Blots were then washed three times at room temperature for 10 min in washing buffer containing 5 × SSC and 0.1% SDS and then washed at 46 or 52 °C for 30 min. The blots were analyzed using a PhosphorImager (Amersham Biosciences). For a second hybridization, the old probe was stripped by pouring a buffer (0.1% SSC and 0.1% SDS) at 80 °C onto the blots and then rinsing with 2 × SSC. The washed blot was examined using the PhosphorImager to verify complete removal of the radioactive plasmids.

Preparation of Expression Plasmids—For construction of the ETB minigenes, primer sets of Ex4F/Ex6R and Ex3F/Ex7R-1246 were used to obtain a 1.5-kb and 5.0-kb PCR product, respectively. The 1.5-kb fragment containing exons 4–6 and the 5.0-kb fragment containing exons 3–7 of the ETB gene were amplified from human lymphocytes and were subcloned into pRC/CMV2. The direction and the nucleotide sequence were confirmed by sequencing.

Expression plasmids containing a rat double-strand RNA (dsRNA)-specific adenosine deaminase (DRADA)R (19) or a rat dsRNA-specific editase 1 (REDA2) were kind gifts from Dr. Higuchi at the Max Plank Institute in Heidelberg. A human ADAR2 cDNA (20) was obtained from Dr. Gerbe at the University of Basel, and the 3.0-kb insert of the human ADAR2 cDNA was transferred into pRC/CMV2.

Epitope Tagging and Mammalian Expression of Wild-type and Spliced ETB—The wild-type and mutant ETB sequences in plasmid vectors were amplified with the 5’-primer KpnI-WF and 3’-primer KpnI-MR to remove the stop codon and to add KpnI restriction enzyme sites. All PCR fragments were subcloned in-frame into the upstream region of the c-myc gene in the pBS vector (Stratagene), creating a fusion gene for a carboxyl-terminally tagged ETB. The fusion genes were next subcloned into the expression vector pME18S (18). The nucleotide sequences and the insert directions within these plasmids, designated ETB-W (wild-type ETB), ETB-SVA (mutant that skips exon 5), and ETB-SVG (mutant that skips exon 5 and substitutes nucleotide A to G at position 950), were confirmed by DNA sequencing.

Transfection Analysis—Cells grown in 150 × 150-mm tissue culture plates were transfected with 10 µg of plasmids using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 2 days after transfection as described previously (21). Cells were harvested after washing with phosphate buffer, and total RNA was extracted.

Western Blot Analysis—COS-7 cells that had been transfected with 10 µg of ETB-W or mutant genes ETB-SVA and ETB-SVG for c-Myc-tagged ETB were harvested by scraping the dish with buffer (0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.1 mM EGTA, 1 µM pepstatin, 1 µM leupeptin). They were then suspended in the sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Samples were subjected to SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Daichiikagaku Co., Tokyo) and blocked with 5% skim milk for 12 h. After washing, the membrane was incubated with anti-c-Myc mouse IgG antibody for 2 h at room temperature, washed with phos-
phosphate-buffered saline with 0.05% Tween 20, containing 1% bovine serum albumin and then incubated with horseradish peroxidase-linked whole anti-mouse IgG antibody (Amersham Biosciences). The signals were detected with ECL Western blotting reagent (Amersham Biosciences).

RESULTS

Analysis of cDNAs Encoding the Human ETB Splice Variant—The entire coding sequences of the ETB transcripts were obtained from lymphoblastoid cells prepared from patients with HSCR. RT-PCR and nucleotide sequence analysis revealed that several subcloned inserts derived from a patient (HD8) contained the wild-type sequence (Fig. 1, Wild), but some inserts lacked the 134-bp nucleotide stretch corresponding to the entire exon 5 (Fig. 1, SVA). The shortened ETB transcript had the same amino acid sequence as that of the wild-type up to the proximal region of the third intracellular protein loop (C3) and then a completely different peptide sequence of 19 amino acids which was followed by an early stop codon (Fig. 1). The deduced polypeptide sequence consisted of 336 amino acid residues with a calculated molecular mass of \( \text{H}11011 \) 37 kDa. This is different from the size of the wild-type ETB, which has 442 amino acid residues with a calculated molecular mass of 49 kDa. Furthermore, among the spliced clones a nucleotide substitution from A to G at position 950 in exon 4 was detected (Fig. 1, SVG). This A to G alteration resulted in an amino acid change from glutamine (Glu\(^{317}\)) to arginine (Arg\(^{317}\)) at the distal portion of the C3 domain. This substitution was detected only in a subpopulation of the spliced transcripts and not in the normal sized (Table II). The A to G substitution at this nucleotide position in the cDNA sequence was present not only in several clones from the same PCR, but also in clones representing PCRs from different first strand cDNAs.

To determine whether the skipping of exon 5 was unique to this patient and whether the A/G difference at position 950 was a genetic polymorphism, we examined DNA and RNA from lymphoblastoid cells of the patient, lymphocytes of normal adults, and cultured cells. Using the primers Ex4F and Ex6R, the region corresponding from exon 4 to exon 6 in the genomic DNA or RNA was amplified. From the genomic DNA, only a single band of a 1.5-kb fragment was generated by PCR with each of the primer sets. DNA sequence analysis revealed that all of the 1.5-kb fragments were derived from the patient’s DNA as well as from controls containing an A at position 950, which was consistent with the published sequence (16) (Table II). When ETB transcripts were analyzed in five individuals and in cultured cells, both the smaller sized 253-bp PCR fragment and the normal sized 387-bp fragment were found in all types of cells (Figs. 2 and 3).

A Southern blot analysis was performed on RT-PCR products from lymphoblastoid cells, lymphocytes, and cultured cells (HeLa, HepG2, SKNMC, and HEK293 cells) using 15-mer synthetic oligonucleotide probes specific to either A or G at position 950, as described under “Materials and Methods.” The probes were designed to cover the splicing junction between exons 4 and 6, so as to detect only the spliced form of the ETB transcript. Specific conditions for detecting this single nucleotide difference were established for both probes. After washing the blot at 46 °C, the signal from the A-specific oligonucleotide

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**FIG. 1.** Nucleotide sequences for ETB cDNA from a patient with HSCR. A, partial nucleotide sequences from the patient (HD8) corresponding to the junction between exons 4 and 5 (Wild) or to the junction between exons 4 and 6 (SVA, SVG). The entire coding region of ETB (1.3 kb) was amplified using primers Ex1F and Ex7R-1328. The amplified cDNAs were subcloned into the pGEM-T vector and sequenced. Nucleotide and deduced amino acid sequences of each cDNA are indicated. The wild-type sequence is indicated in B (Wild), and the mutant cDNA (SVA) sequence skips exon 5, which corresponds to a 134-bp deletion within the coding region. The sequence of the mutant cDNA (SVG) shows a nucleotide substitution from A to G at position 950, resulting in the conversion from glutamine to arginine.
(SVA) was still detected from the control SVA sample (Figs. 2C and 3B, lanes A), whereas the signal was not found from the SVG sample (Figs. 2B and 3B, lanes G). On the other hand, washing the blot at 52 °C was enough to remove the G-specific probe (SVG) from the SVA sample (Figs. 2B and 3C, lanes A), whereas the signal was still detected from the SVG sample (Figs. 2C and 3C, lanes G). No signal from the wild-type 387-bp fragment was detected using either probe (Fig. 2, B and C, lanes W, and Fig. 3, B and C, lanes W). Under these conditions, the signal from the SVA sample was detected in all of the 253-bp DNA bands from each cell type (Fig. 2B, lanes 1–6 and Fig. 3B, lanes 1–4), but the signal intensity differed among the SVG samples (Fig. 2C, lane 1–6 and Fig. 3C, lanes 1–4). Recent results revealed that the frequency of SVG occurrence was different depending on individuals or on cell types. Sequence analysis of the 387-bp DNA contained the nucleotide sequences of exons 4, 5, and 6, whereas the 253-bp DNA skipped the 134-bp region corresponding to exon 5. Among the 253-bp fragments that skipped exon 5, some were accompanied by a nucleotide substitution of A (SVA) to G (SVG) at position 950 in the subcloned fragments, and this observation held for all cell types. Because there is no published report of a pseudogene for ETB, our data indicated that the 134-bp deletion in exon 5 in the ETB transcript is the result of alternative splicing.

The observed A to G substitution in the ETB transcript might be attributable to the misincorporation of G in the place of A by MMLV reverse transcriptase or by Taq polymerase. To examine this possibility, RT-PCR analysis was performed. The MMLV reverse transcriptase was replaced by avian myeloblastosis virus reverse transcriptase, and Taq polymerase was replaced by M13 DNA polymerases from the hyperthermophilic archaeabacteria Pyrococcus furiosus (Pfu DNA polymerase, Stratagene) and Thermococcus litoralis (Vent DNA polymerase, New England Biolabs). Neither of the two reverse transcriptases in combination with one of the three DNA polymerases affected the outcome of the experiment, and identical nucleotide sequencing results were obtained irrespective of the enzyme used (data not shown). Hence, generation of the transcript with a G at position 950 could not be attributed to the use of any particular enzyme.

These results suggest that the spliced ETB transcripts specifically undergo RNA editing in which the A is converted to G, resulting in the replacement of glutamine by arginine (at the Q/R site). The dsRNA-specific adenosine deaminases (DRADA/ADAR1 and RED1/ADAR2) have been shown to be involved in alternative splicing.

The observed A to G substitution in the ETB transcript might be attributable to the misincorporation of G in the place of A by MMLV reverse transcriptase or by Taq polymerase. To examine this possibility, RT-PCR analysis with several additional enzymes was performed. The MMLV reverse transcriptase was replaced by avian myeloblastosis virus reverse transcriptase, and Taq polymerase was replaced by M13 DNA polymerases from the hyperthermophilic archaeabacteria Pyrococcus furiosus (Pfu DNA polymerase, Stratagene) and Thermococcus litoralis (Vent DNA polymerase, New England Biolabs). Neither of the two reverse transcriptases in combination with one of the three DNA polymerases affected the outcome of the experiment, and identical nucleotide sequencing results were obtained irrespective of the enzyme used (data not shown). Hence, generation of the transcript with a G at position 950 could not be attributed to the use of any particular enzyme.
ADAR2 was introduced into HEK293 cells, a signal from the edited transcript increased specifically in the spliced variant (Fig. 3C, lanes 5 and 6). An increased signal from the edited transcript using HEK293 cells was also obtained in transfection experiments using the human ADAR2 (data not shown).

Next, minigenes of length 1.5 and 5.0 kb were constructed using human ETB genomic fragments extending from exon 4 to exon 6 and from exon 3 to exon 7, respectively. These minigenes were expressed transiently in HEK293 cells to examine whether or not the single nucleotide conversion had occurred. The transcripts derived from the transfected minigenes were specifically detected by RT-PCR by using the plasmid sequence as the annealing site for a lower primer. Therefore, the endogenous ETB transcript was not amplified. When the 1.5-kb minigene was transfected, a PCR fragment having the expected size of 358 bp, which is 134 bp smaller than the wild-type transcript using HEK293 cells was also obtained in transfec- tion experiments using the human ADAR2 (data not shown).

Next, minigenes of length 1.5 and 5.0 kb were constructed using human ETB genomic fragments extending from exon 4 to exon 6 and from exon 3 to exon 7, respectively. These minigenes were expressed transiently in HEK293 cells to examine whether or not the single nucleotide conversion had occurred. The transcripts derived from the transfected minigenes were specifically detected by RT-PCR by using the plasmid sequence as the annealing site for a lower primer. Therefore, the endogenous ETB transcript was not amplified. When the 1.5-kb minigene was transfected, a PCR fragment having the expected size of 358 bp, which is 134 bp smaller than the wild-type transcript (492 bp), was present in the cells (Fig. 4A, lanes 1 and 2). Southern blot analysis using the oligonucleotide probes specific to the SVA or SVG sequence revealed that the SVA signal was present in the 358-bp products from the 1.5-kb minigene (Fig. 4A, lanes 1 and 2), and the SVG signal was also detected in SKNMC (Fig. 4C, lane 2) but was barely detectable in HEK293 cells (Fig. 4C, lane 1). This result may suggest that the transcripts from the exogenous ETB minigene could be edited efficiently in SKNMC but not as efficiently in HEK293 cells, as observed for the endogenous ETB transcript (Fig. 3C, lane 6). Next, the 1.5-kb ETB minigene was transfected into cells along with the rat ADAR1 or ADAR2 cDNA to test whether these enzymes mediate the site-specific modification of ETB transcripts from the minigenes. Increased SVG signals were detected in HEK293 cells cotransfected with the 1.5-kb minigene and rat ADAR1 (lane 3) and rat ADAR2 (lane 4). Similar results were obtained with the 5.0-kb minigene containing exons 3–7 with rat ADAR1 and ADAR2 cDNA (data not shown). Also, the SVG signals that were detected by Southern blot analysis were confirmed by sequence analysis (Table II). The frequency of the edited clones increased...
The 33-kDa protein found in the cells transfected with ETB-W appear quickly.

and/or protein produced from these DNA sequences might dis-

cause the wild-type receptor of this 50-kDa protein is processed

products that were translated from the ETB transcripts. Be-

than did the ADAR1 enzyme (Table II).

The mutant ETB transcripts skipped the 134-bp nucleotide

sequence corresponding to exon 5, and the same splicing vari-

tations mentioned above, but mutant as well as full-length

polypeptide translated from the splice variant described here

might exhibit a severe functional impairment, if expressed

in alterations in the cytoplasmic domain that reduce the capa-

bility of the cell to conduct signal transduction (29). Thus,

alternative RNA splicing may play a functional role by gener-

physiologically diverse receptor activities (29). The skipping

of a 134-bp stretch of DNA corresponding to exon 5 would

result in the synthesis of smaller ETB polypeptides, with an

entirely different polypeptide sequence starting at the C3 do-

main. Because the intracellular carboxyl-terminal domain of

the seven-helix transmembrane receptors is critical for G pro-

tein coupling and functional responses (28, 30). On the other hand, the variant

with a different amino acid sequence at the C4 domain results

in the synthesis of smaller ETB polypeptides, with an

nucleotide region corresponding to a polypep-

tide section from the distal portion of C3 to the proximal side of

the third extracellular protein loop. From this splicing site a

frameshift resulted in an addition of 19 amino acids that are

completely different from the wild-type sequence. The open

reading frame of the spliced transcript was terminated by an

eye termination codon that was generated.

So far, several splice variants have been reported for the

ETB gene in humans (28, 29) as well as in other organisms (30).

The splice variant of ETB having an insertion of 10 amino acid

residues at the C2 domain and the variant that is 14 amino

acids shorter than the wild-type ETB receptor exhibit similar

functional responses (28, 30). On the other hand, the variant

with a different amino acid sequence at the C4 domain results

in alterations in the cytoplasmic domain that reduce the capa-

bility of the cell to conduct signal transduction (29). Thus,

alternative RNA splicing may play a functional role by gener-

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of a 134-bp stretch of DNA corresponding to exon 5 would

result in the synthesis of smaller ETB polypeptides, with an

entirely different polypeptide sequence starting at the C3 do-

main. Because the intracellular carboxyl-terminal domain of

the seven-helix transmembrane receptors is critical for G pro-

tein coupling and functional responses (1, 2), the smaller

polypeptide translated from the splice variant described here

might exhibit a severe functional impairment, if expressed

stably in vivo.

A substantial number of ETB clones were also found which

contained an A to G conversion at nucleotide position 950 in

exon 4, resulting in an amino acid change from glutamine to

arginine (Q/R conversion). Several possible explanations for a

site-specific difference between the genomic and cDNA

sequences can be made. First, the presence of different copies

of the gene, some of them bearing the mutation and some having

the normal sequence, could explain the discrepancy found here.

This could happen if ETB is a member of a family of highly

homologous genes or if allelic variants of the gene are present,

This could happen if ETB is a member of a family of highly

homologous genes or if allelic variants of the gene are present,

The positions of molecular mass standards (in kDa) are indicated.

Lanes 1 and 4, RNA from COS-7 cells transfected with the ETB-W (wild-type) fused with c-Myc. Lanes 2 and 5, RNA from COS-7 cells transfected with the ETB-SVA fused with c-Myc. Lanes 3 and 6, RNA from COS-7 cells transfected with the mutant ETB-SVG fused with c-Myc.

DISCUSSION

Proper functioning of the ET system is now recognized as an

important requirement for a wide variety of cellular physiologi-

cal processes as well as for the development of certain organs.

Mutations in the ETB gene may lead to HSCR when ETB

proteins are functionally altered. Among the reported ETB

mutations, the mutation C109R resulted in a mutant ETB with

a lowered affinity to ET-1, and the mutations W276C and

S390R impaired intracellular signaling events (11). On the other hand, the ETB mutation N104I was found in a patient,

and the mutations, the mutation C109R resulted in a mutant ETB with

a lowered affinity to ET-1, and the mutations W276C and

S390R impaired intracellular signaling events (11). On the other hand, the ETB mutation N104I was found in a patient,

S390R impaired intracellular signaling events (11). On the other hand, the ETB mutation N104I was found in a patient,

S390R impaired intracellular signaling events (11). On the other hand, the ETB mutation N104I was found in a patient,
any of these situations, clearly showed A at position 950 in all of the PCRs from genomic DNA. Furthermore, previous studies demonstrated that a single gene encodes the ETB enzyme (16). We were consistently unable to find the conversion in the genome, and a single band was generated by genomic DNA amplification. The altered transcripts were therefore considered to be derived from same gene, and it was also concluded that there is no ETB pseudogene.

Another possible explanation for the observed base change is that an artifact was introduced either by the reverse transcriptase or by the DNA polymerase. We were particularly concerned that misincorporation by one of the polymerases used in RT-PCR amplification of ETB RNA might give rise to this type of result. It was reported previously that the polymerase activity exhibited no proofreading function and was notoriously error-prone, with misincorporation of inappropriate nucleotides at frequencies ranging from 1 in 10⁻⁴ to 1 in 10⁻³ (31). However, sequencing of ETB transcripts generated with avian myeloblastosis virus or MMLV reverse transcriptase showed that an A or G nucleotide at position 950 of ETB occurred with nearly equal frequencies from at least some of the RNA sources. When reconstitution experiments were performed using the minigenes, no evidence for misincorporation of nucleotides by reverse transcriptase was obtained. The mostable Taq polymerase is also known to create point mutations at a frequency of ~1 in 600-bp in PCR-amplified DNA (32, 33). This plausible source of artifacts in our observations was ruled out by replacing Taq polymerase with either Pfu or Vent DNA polymerase. Identical nucleotide sequencing results were obtained after RT-PCRs were carried out by several different reverse transcriptases or DNA polymerases. Using either of the two reverse transcriptases in combination with any one of the three DNA polymerases did not affect the outcome of the experiment. The results showed that neither the reverse transcriptase, the Taq polymerase, nor experimental artifacts were responsible for generating the transcript with a G at position 950. Hence, this series of experiments may rule out the following possible mechanisms for generating the nucleotide conversion: (i) the existence of pseudogenes; (ii) RT artifacts; or (iii) PCR artifacts. We then examined the possible RNA editing at position 950 in ETB RNA which results in a glutamine (CAG) to arginine (CGG) substitution at amino acid residue 317 (Q/R site).

Several gene-editing phenomena have been described in the past few years, in which editing can lead to predetermined modification of the coding potential of genes (34–36). There are at least four kinds of adenosine deaminases that act on mammalian RNA (37). Among these deaminases, the RED1/ADAR2 enzyme, the most stable polymerase is also known to create point mutations at a frequency of 1 in 10⁻³/H₁₁₀₀₂ (31). However, sequencing of ETB transcripts generated by RNA editing/splicing is causally related to the mutagenic HSCR or not.

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