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Molecular Identification of a Taste Receptor Gene for Trehalose in *Drosophila*

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The molecular nature of sweet taste receptors has not been fully explored. Employing a differential screening strategy, we identified a taste receptor gene, *Tre1*, that controls the taste sensitivity to trehalose in *Drosophila melanogaster*. The *Tre1* gene encodes a novel protein with similarity to G protein–coupled seven-transmembrane receptors. Disruption of the *Tre1* gene lowered the taste sensitivity to trehalose, whereas sensitivities to other sugars were unaltered. Overexpression of the *Tre1* gene restored the taste sensitivity to trehalose in the *Tre1* deletion mutant. The *Tre1* gene is expressed in taste sensory cells. These results provide direct evidence that *Tre1* encodes a putative taste receptor for trehalose in *Drosophila*.

Olfactory receptors are G protein–coupled seven-transmembrane proteins encoded by a divergent multigene family in vertebrates (*1*) and in *Drosophila* (*2, 3*). Taste receptors are also thought to belong to the superfamily of G protein–coupled receptors (GPCRs) (*4*), and several candidate genes have been reported (*5–8*), but their function as a taste receptor has not been proved. In *Drosophila*, taste sensilla are present on the labellum, tarsi, and wing margins (*9, 10*). In a typical chemosensillum on the labellum, there are four taste sensory cells each of which responds to either water, salt, or sugar. Previously, we showed that there are at least three separate receptor sites for sugars in the sugar receptor cell of *Drosophila* (*11, 12*). The *Tre* gene was identified through studies on natural variants (*12*). Because the *Tre* gene controls taste sensitivity to trehalose without affecting the responses to other sugars, the gene product of *Tre* should function in sugar receptor cells. The *Tre* gene is cytologically mapped to the region between 5A10 and 5B1-3 on the X chromosome (*13*). A P1 clone containing a 90-kb genomic region from 5A9 to 5B1 was used as a starting material to clone the taste receptor gene (Fig. 1A).

To identify the putative *Tre* gene, we performed a differential screening for genes encoded in the P1 clone that might be specifically expressed in chemosensory cells. We took advantage of the *pox-neuro* (*poxn*) gene, which is involved in the developmental decision pathway between mechanosensory and chemosensory cell fates (*14*). In an adult-viable allele of the *poxn* mutant, all external chemosensilla are either transformed into mechanosensilla or are deleted (*15*). In the legs of the wild-type fly, chemosensilla exist on the tarsus, but there are no chemosensilla on the femur. We performed a differential screening with cDNA probes derived from labella, tarsi, and femurs of wild-type and *poxn* mutant flies (*16*).

Southern blot analysis of the subcloned P1 DNA fragments identified one clone that hybridized to the wild-type labella and tarsi probes, but not to the other probes (*17*). A portion of the 8.2-kb clone displayed conserved features of the superfamily of seven-transmembrane domain receptor proteins. The full-length putative *Tre1* cDNA was obtained by reverse transcriptase–dependent polymerase chain reaction (RT-PCR) and 5′ and 3′ rapid amplification of cDNA ends (RACE) (*18*). Sequence analysis revealed that the putative *Tre1* gene contained a 1179–base pair (bp) open reading frame that encodes 392 amino acid residues preceded by an in-frame termination codon. Hydropathy analysis suggests that the *Tre1* cDNA sequence contains seven hydrophobic stretches that represent potential transmembrane domains (Fig. 1C). These domains constitute the regions of maximal sequence similarity to other seven-transmembrane receptors. Although several conserved regions are found between *Tre1* and other GPCRs, the structures of the third and fourth cytoplasmic domains may be unique, because they are longer than the corresponding domains of typical GPCRs. The *Tre1* gene has no similarity to other GPCRs recently identified by database search (*8*). We suggest that the *Tre1* gene may represent a new subclass of GPCRs.

By searching the *Drosophila* DNA database with the 5′-flanking genomic sequences of the putative *Tre1* gene, we found that flanking genomic sequences of the P-element in strains in one of the transposon-inserted strains [EP(X) strains (*19*)] completely matched our genomic sequence (Fig. 1B). The EP element is inserted 113 bp upstream to the transcription initiation site in the EP(X)0496 strain. The taste sensitivity of this strain to trehalose was tested with the two-choice preference test (*12*) and was found to be highly sensitive (high-sensitive). We expected that imprecise excision of the P-element should disrupt the promoter region of the *Tre1* gene, and this event might change the trehalose sensitivity. The EP element carries w^+ as a genetic marker, and the element was jumped out by genetically supplying a transposase source (*20*). We tested *w* male flies by two-choice preference tests, using 30 mM trehalose and 2 mM sucrose as the choices. At this concentration of trehalose, nearly 98% of the parental EP(X)0496 flies preferred trehalose (Fig. 2B). Most of the *w* flies preferred trehalose, indicating that the precise excision of the P-element did not impair trehalose sensitivity. Flies that consumed the sucrose side were selected and individually crossed to *C(1)DX* attached-X females. Among about 3000 *w* flies, we isolated 90 lines that were confirmed as showing low sensitivity (low-sensitive) to trehalose. We determined the extent of deletion in all the 90 lines by PCR, using primers flanking the P-element insertion site. There were no amplification products in most of these lines, indicating that a deletion eliminated the primer site(s). Next, several lines were selected, and the extent of deletion was determined by Southern blotting. The results (Fig. 1B) indicated that the deletions removed the putative promoter region and the first exon. In fact,

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RT-PCR analyses indicated that the *Tre1* mRNAs are undetectable in all these lines (Fig. 2A) (*21*). We determined the sequence surrounding the insertion site and confirmed that the strain that showed high sensitivity to trehalose underwent a precise excision event. The *Tre1* mRNA was normal in this line (Fig. 2A, lane 7).

We measured the taste sensitivity to trehalose of two Δ*Tre1* lines by the two-choice preference test with different concentrations of trehalose (Fig. 2B) (*22*). The sensitivity to trehalose can be defined as the PI_{50}, the concentration of trehalose that gives a 50% preference index (PI). For Canton-S, a typical high-sensitive strain, PI_{50} is 10 mM. In the original EP(X)0496 flies, the PI_{50} value is 12 mM, whereas the value is 80 mM in the two Δ*Tre1* lines. Taken together, the disruption of the *Tre1* gene leads to lowering the taste sensitivity to trehalose. Results of the two-choice preference test cannot discern whether trehalose sensitivity alone was altered in the Δ*Tre1* strains. We then examined the proboscis extension reflex by using four different sugar solutions: glucose, fructose, sucrose, and trehalose (*23*). The results demonstrate that the response to trehalose was specifically reduced in the Δ*Tre1* lines (Fig. 3). Since sensitivity to other sugars was unaffected, the sensitivity difference to trehalose should be attributed to a defect in the trehalose receptor. This conclusion is supported by the observation that the nerve responses to trehalose in the labellar chemosensilla were reduced in the Δ*Tre1* mutant, whereas the sucrose sensitivity was unaffected (*24*). This electrophysiological evidence indicated that TRE1 is directly involved in trehalose sensation.

To further confirm that the *Tre1* gene is directly involved in the taste response to trehalose, we established transgenic lines carrying the hs-*Tre1* cDNA gene so that *Tre1* gene expression can be induced by heat shock (*25*). The P[hs-*Tre1*]#1 line showed the highest expression of *Tre1* mRNA after heat shock. Heat shock was tested in the background of the Δ*Tre1* deletion mutant and was found to restore the trehalose sensitivity of the Δ*Tre1* deletion mutant (Fig. 4).

Because the *Tre1* gene was isolated on the basis of its specific expression in taste sensory cells, the gene was likely to be expressed in taste cells. RT-PCR analyses on isolated labella and tarsi preparations showed that the mRNA is expressed in the labella and tarsi of original EP(X) lines but is absent in Δ*Tre1* and *poxn* flies with no taste sensory cells. In situ hybridization experiments showed that the *Tre1* mRNA is present in one of the taste sensory cells beneath a taste bristle (Fig. 5) (*26*). There were no signals in the labellum preparation of *poxn* and in the central brain. Thus, *Tre1* seems to be specifically expressed in taste sensory cells.

In summary, we have identified a putative taste receptor gene, *Tre1*, in *Drosophila* and conclude that the product of the *Tre1* gene likely functions as a taste receptor for trehalose. First, disruption of the *Tre1* gene lowered the trehalose sensitivity of sugar receptor cells while leaving sensitivity to other sugars intact. Second, overexpression of the

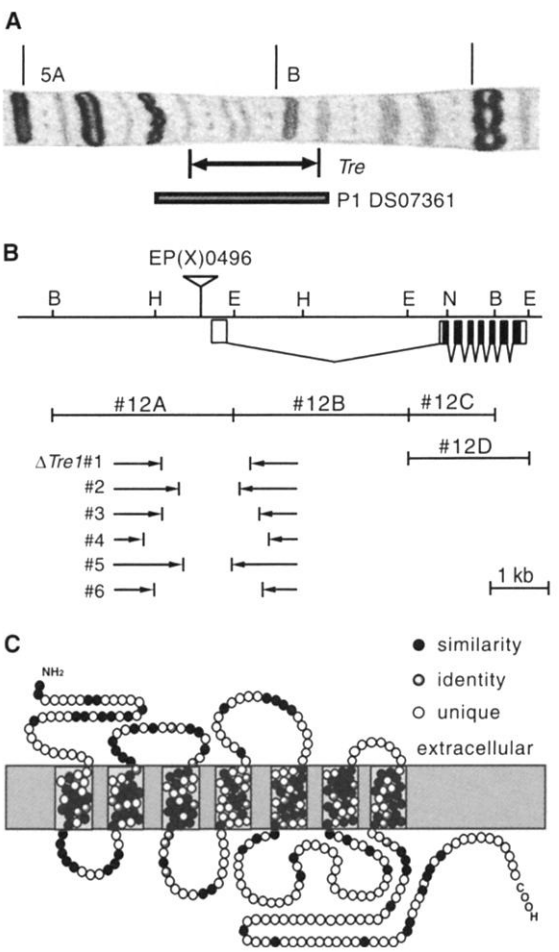

Fig. 1. (**A**) *Tre* is mapped between 5A10 and 5B1-3 on the X chromosome (*13*), and the P1 clone DS07361 covers this interval. (**B**) A restriction map of genomic region where the putative trehalose receptor gene was identified. Identified exons of the putative trehalose receptor gene are boxed. Filled and open boxes show coding and noncoding regions, respectively. The *Tre1* gene is separated into eight exons. The 5′ termini of cDNA contained noncoding sequence of 247 bp. The first exon is far from the second exon for about 4 kb. Four genomic clones (#12A, #12B, #12C, and #12D) are shown. B, Bam HI; E, Eco RI; H, Hind III; N, Not I. One EP(X) line has an insertion upstream to the *Tre1* coding sequence. The extent of deletion in Δ*Tre1* lines is shown by arrowheaded lines. (**C**) The topology is based on the structure of the other members of the G protein–coupled receptor family, with the NH_2-terminal region extracellular. The membrane is shown by shaded box. Similarity was examined by comparing TRE1 with other GPCRs, chicken melatonin receptor, mouse neuropeptide Y receptor, human dopamine receptor, and scallop Go-coupled rhodopsin.

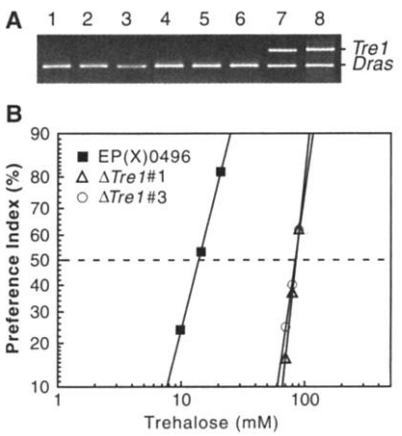

Fig. 2. (**A**) Comparison of *Tre1* mRNA level in EP(X)0496 and Δ*Tre1* lines as revealed by RT-PCR. Lane 1 to 6: imprecise excision lines (trehalose low-sensitive), Δ*Tre1* #1 to #6. Lane 7: a precise excision line (trehalose high-sensitive). Lane 8: the original EP(X)0496. *Dras* was used as an internal control in RT-PCR reactions. Using *Tre1*-specific primers, a single 889 bp fragment was amplified. (**B**) Taste sensitivity curves for trehalose in EP(X)0496 and Δ*Tre1* lines #1 and #3. Each point represents data using 200 to 250 flies from at least five independent experiments. SEM of each point of data is within 10%. The sensitivity difference is statistically significant by *t* test ($P < 0.01$).

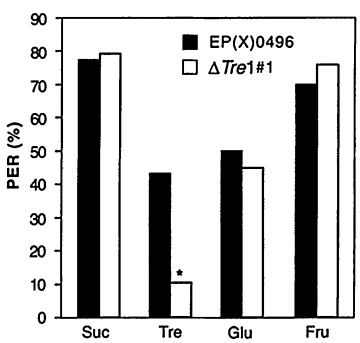

Fig. 3. The taste sensitivity to trehalose is affected in Δ*Tre1* lines. Proboscis extension reflex was examined using 30 males of EP(X)0496 and Δ*Tre1*#1. Sucrose (5 mM), trehalose (15 mM), glucose (40 mM), and fructose (40 mM) were used as stimulants. Asterisk indicates significantly different by the chi-square test ($P < 10^{-7}$). Different batches of flies and other Δ*Tre1* lines were tested, and they gave similar results.

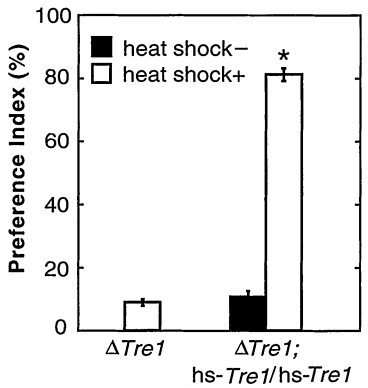

Fig. 4. Induced overexpression of TRE1 restored the trehalose sensitivity in Δ*Tre1* flies. Two-choice preference tests were performed between 2 mM sucrose and 80 mM trehalose. Δ*Tre1* #1 flies carry two copies of P[hs-*Tre1*]#1 on the autosome. Percentages show the proportion of flies preferred the trehalose side. Flies aged 0 to 2 days after emergence were heat-shocked at 36°C for 1 hour with an interval of 24°C for 7 hours. This regime was continued for 24 hours. Flies were tested 3 hours after the last heat-shock. Error bars: SEM. Asterisk indicates significantly different by *t* test ($P < 0.0001$).

Tre1 transgene restored the response to trehalose. Third, the *Tre1* gene was specifically expressed in putative sugar receptor cells. Because the *Tre1* gene identified in this study was isolated from the genomic clone where *Tre* is mapped, we think that the mutation(s) of the *Tre1* gene are involved in the natural variation (*27*). If we assume that TRE1 is the sole receptor for trehalose, the null mutant of *Tre1* (Δ*Tre1*) should show no response to trehalose. The Δ*Tre1* flies still respond to higher concentrations of trehalose, and this response would be mediated by another unidentified receptor for trehalose, although we cannot exclude the possibility that deletion mutants are not null. In fact, we have identified two other genes in the *Drosophila* genome with similarity to *Tre1*, and we think that TRE1 belongs to a novel family of G protein–linked transmembrane receptors that may operate as taste receptors. The function of the clone gene should be investigated using expression systems, as has been successfully applied in the studies of olfactory receptors (*28–31*).

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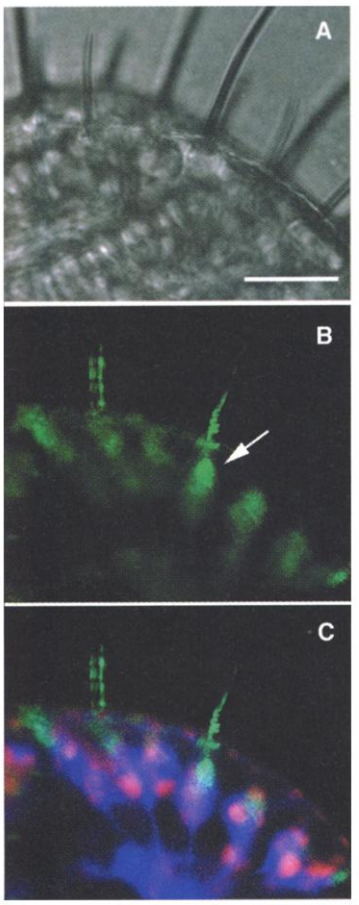

Fig. 5. Fluorescent in situ hybridizations were done on whole-mount preparations of labella. **(A)** A Nomarski image shows the location of taste bristles. Positive signal of *Tre1* mRNA is shown as green in (B) and (C). Counter-staining of nuclei was done with propidium iodide [red (C)]. Taste sensory cells are identified by staining with monoclonal antibody 22C10 [blue (C)]. Bar, 30 μm. **(B and C)** *Tre1* mRNAs were detected in one of the sensory cells in a labellum [arrow in (B)], using an antisense probe. Colocalization of the *Tre1* mRNA signal in one of the taste sensory cells was observed for other cell clusters at different confocal planes. Signals along the taste bristles are nonspecific.

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16. Flies were raised on a standard medium at 25°C. Canton-S or w^{1118} was used as a wild-type strain. $poxn^{70\text{-}23}$/*CyO* was crossed to a deficiency strain, *Df(2R)42WMG/CyO* and $poxn^{70\text{-}23}$/*Df(2R)42WMG* flies were used. Labella, tarsi, and femurs were cut with a razor blade from 100 wild-type and *poxn* mutant flies. mRNA was extracted, and first-strand cDNAs were synthesized by SuperScript II RT (Stratagene) and were poly(A)-tailed. DIG-labeled PCR-amplified cDNA was used as a probe for the differential screening.
17. The P1 DNA was digested with a restriction endonuclease, Bam HI, and genomic DNA fragments were subcloned. They were classified on the basis of the restricted digestion pattern of each insert, and 19 independent fragments were obtained. We also screened a P1 genomic mini-library but could not find another clone that contains a taste-specific transcript. Each subcloned plasmid DNA was subjected to Southern blot analysis. Hybridization was carried out at 68°C for 20 hours after adding the cDNA probe of either CS or *poxn*. Differential screening identified one genomic clone. The clone was digested with a restriction enzyme, Eco RI, and three clones, #12A, #12B, and #12C, were obtained. We determined 8.2 kb of the DNA sequence. One of the clones, #12C (1.6 kb), was found to contain a gene encoding a protein that displayed conserved features of the superfamily of seven transmembrane domain receptor proteins. The predicted cDNA sequence indicated that the clone #12C contained sequences from the NH_2-terminus to the fifth transmembrane domain. We isolated a clone, #12D, from P1 genomic clones digested with Eco RI. The #12D contained the remaining sequence of the gene.
18. cDNAs derived from mRNA prepared from 100 labella of wild-type flies were subjected to RT-PCR amplification with the primers in TM2 and TM7, and a PCR product was purified and cloned. The sequence between the second transmembrane domain and the seventh transmembrane domain of the *Tre1* gene was determined. At the same time, the predicted exon/intron boundary structure was confirmed by performing PCR using different primers (Fig. 1B). Subsequently, the full-length *Tre1* cDNA was obtained by the 5′ RACE and 3′ RACE method. The sequence of the 5′ primer (TM2) was 5′-CTTCGTCATTTCGCTAAGCA-3′, corresponding to the sequence in the second transmembrane domain. The sequence of the 3′ primer (TM7) was 5′-ATCGGGTTAATGACACTGGA-3′, corresponding to the sequence in the seventh transmembrane domain.
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20. Males of EP(X)0496 were mated to females of w^{1118}; *Dr*^{1}/*TMS*, *Sb*^{1} P[*ry*$^{+}$, Δ2-3]99B [H. M. Robertson *et al.*, *Genetics* **118**, 461 (1988)]. F_1 males carrying the *TMS* balancer chromosome were crossed to attached-X females, *y w f*, and descending *w*; *Sb*$^{+}$ males were tested by the two-choice preference test between 30 mM trehalose and 2 mM sucrose. Flies that preferred sucrose were selected and individually mated to attached-X females. The low-sensitive phenotype of established strains were reexamined at the next generation. Some w^{+} flies from the F_2 cross were randomly picked and individually mated to attached-X females. These strains were used to confirm that precise excision of the P-element resulted in the trehalose high-sensitive phenotype.
21. Total RNA was isolated from 30 heads or from labella and tarsi from 80 flies. RT-PCR were performed using a primer set of *Dras* (5′-ACGAAGCAGTGCAACATCGAC-3′ and 5′-ATCCTGCTCGATGAAGGGACG-3′) as an internal control and *Tre1*-specific primers in TM2 and TM7 sequences.
22. All behavioral tests were done at 24° to 25°C. Two-choice preference test was performed as described (*12*). We used 2 mM of sucrose, colored red, as a control and varying concentrations of trehalose, which was colored blue.
23. Proboscis extension reflex was examined as previously described [K.-I. Kimura, T. Shimozawa, T. Tanimura, *J. Exp. Zool.* **239**, 393 (1986)]. Flies aged 0 to 1 day after eclosion were maintained on fresh medium for 1 day. Flies were starved for 16 hours but were supplied with water. Before the test with sugar solutions, the prothoractic tarsi of a fly was touched with a drop of water and if they extended the proboscis, water was fully fed. This procedure was repeated between stimulations with sugar solutions.
24. Nerve responses were recorded from the labellar chemosensilla by the tip-recording method (*10*). Numbers of nerve impulses (±SD) originated from the sugar receptor cell 0.2 to 0.4 s after the onset of stimulations were counted. The number of impulses to 10 mM sucrose was 6.1 ± 2.5 (n = 34) and 6.7 ± 2.8, (n = 34) in EP(X)0496 and Δ*Tre1*#1, respectively. The number to 60 mM trehalose was 7.3 ± 2.6

(n = 24) and 1.8 ± 2.3 (n = 26). n, number of recordings from different chemosensilla. Trehalose response difference was statistically significant by t-test ($P < 0.001$).
25. Transgenic flies carrying *Tre1* cDNA under the heat-shock promoter were generated by P element–mediated germ line transformation. A 1.4-kb Eco RI–Xho I fragment containing the entire *Tre1* cDNA was cloned into the Eco RI site in the P-element transformation vector, pKB255 [K. Basler, unpublished data; K. Basler, K. Yen, A. Tomlinson, E. Hafen, *Genes Dev.* **4**, 728 (1990)], placing it under the control of the *Drosophila* hsp70 promoter. This construct was then used to generate germ line transformants by injecting into w^{1118}; Dr^1/TMS, Sb^1 P[ry^+, Δ2-3]99B embryos. Transformants were selected, and homozygous lines were established.
26. In situ hybridization was done as described [T. R. Bhatt, P. A. Taylor III, F. M. Horodyski, *Biotechniques* **23**, 1000 (1997)] with minor modifications. The DIG-labeled antisense and sense riboprobes were produced by in vitro transcription by T7 and Sp6 RNA polymerase with a 244-bp *Tre1* cDNA fragment as a template, respectively. Antidigoxygenine antibody conjugated to peroxidase (Pierce) was applied at a dilution of 1:500 overnight at 4°C, and signals were enhanced with biotin-tyramide (NEN). After tyramide signal amplification reaction, streptavidin-TC (Caltag) was applied at a dilution of 1:100. Sensory neurons were stained with a neuron-specific monoclonal antibody 22C10 using anti-mouse IgG antibody conjugated to Cy5 (Chemicon International Inc.) diluted to 1/100 as the secondary antibody. Nuclei were counter stained with propidium iodide. The triple-stained images were visualized with a Zeiss LSM410 confocal laser scan microscope equipped with a Kr/Ar and a He/Ne laser.
27. We have sequenced *Tre1* cDNA clones obtained from trehalose high-sensitive and low-sensitive strains and found a few possible mutation sites, but further study is needed to verify that the mutation is directly involved in the trehalose sensitivity difference.
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32. We are grateful to K.-I. Kimura for *poxn* and K. Basler for plasmid. EP lines and other fly strains were provided by the Bloomington stock center and the Berkeley *Drosophila* Genome Project. The monoclonal antibody 22C10 was obtained from the Developmental Studies Hybridoma Bank at The University of Iowa. We thank T. Hirozane for her initial extensive attempt on this project and T. Hirozane and T. Tamura for their help in sequencing. We also thank Y. Mitsuiki, M. Haruta, M. Yamasaki for their technical assistance and C. Montell for critically reading the early version of our manuscript. Supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and by a Human Frontier Science Program grant to T.T. The *Tre1* cDNA sequences have been deposited in DDBJ (accession number AB034204). This paper is dedicated to the memory of the late Dr. Toshihide Kikuchi.

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Activating Mineralocorticoid Receptor Mutation in Hypertension Exacerbated by Pregnancy

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Hypertension and pregnancy-related hypertension are major public health problems of largely unknown causes. We describe a mutation in the mineralocorticoid receptor (MR), S810L, that causes early-onset hypertension that is markedly exacerbated in pregnancy. This mutation results in constitutive MR activity and alters receptor specificity, with progesterone and other steroids lacking 21-hydroxyl groups, normally MR antagonists, becoming potent agonists. Structural and biochemical studies indicate that the mutation results in the gain of a van der Waals interaction between helix 5 and helix 3 that substitutes for interaction of the steroid 21-hydroxyl group with helix 3 in the wild-type receptor. This helix 5–helix 3 interaction is highly conserved among diverse nuclear hormone receptors, suggesting its general role in receptor activation.

Although blood pressure is normally reduced throughout gestation, about 6% of pregnancies are complicated by the development of hypertension, raising the risk of pre-eclampsia, a hypertensive disorder of pregnancy that increases maternal and perinatal mortality (*1*). The factors responsible for these developments are unknown; however, the prompt resolution of many cases with delivery suggests pregnancy-specific factors.

Mutations that change renal salt reabsorption alter blood pressure (*2*). For example, heterozygous loss-of-function mutations in the mineralocorticoid receptor (MR; locus symbol *NR3C2*), a member of the nuclear receptor family, cause pseudohypoaldosteronism type 1 (PHA1), a disease featuring salt wasting and hypotension (*3*). Normally, activation of MR by the steroid hormone aldosterone raises renal salt reabsorption by increasing activity of the epithelial sodium channel of the distal nephron.

To determine if gain-of-function mutations in *MR* could cause increased renal salt reabsorption and hypertension, we screened *MR* in 75 patients with early onset of severe hypertension (*4*). A 15-year-old boy with severe hypertension, suppressed plasma renin activity, low serum aldosterone, and no other underlying cause of hypertension was heterozygous for a missense mutation, resulting in substitution of leucine for serine at codon 810 (Fig. 1A). The S810L mutation lies in the MR hormone-binding domain (HBD), altering an amino acid that is conserved in all MRs from *Xenopus* to human (*5*) but not found in other nuclear receptors (Fig. 1B). S810L was not detected in 160 control chromosomes.

The impact of MR bearing S810L (MR_{L810}) was assessed by clinical and biochemical studies. Twenty-three relatives of the proband were evaluated. Remarkably, 11 had been diagnosed with severe hypertension before age 20, a rare trait in the general population (*6*), whereas the remainder had unremarkable blood pressures (Fig. 1C). These findings suggest Mendelian segregation of hypertension. MR_{L810} precisely cosegregated with early-onset hypertension in this family (Fig. 1C), providing strong evidence of linkage. The maximum lod score (logarithm of the odds ratio for linkage) was 5.24 at a recombination fraction of zero (odds of 174,000:1 in favor of linkage) (*7*).

Comparison of the clinical features of MR_{L810} carriers and noncarriers revealed a marked increase of blood pressure among carriers even though they were taking antihypertensive medication, as well as suppression of aldosterone secretion (Table 1). There was a nonsignificant trend toward lower serum potassium among carriers, and there were no significant effects of gender or age on phenotypic expression of MR_{L810}. Of note, three deceased pedigree members with early-onset hypertension all died of heart failure before age 50.

To investigate the functional effects of the

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