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Trehalose sensitivity in *Drosophila* correlates with mutations in and expression of the gustatory receptor gene *Gr5a*

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Drosophila taste gene _Tre_ is located on the distal X chromosome and controls gustatory sensitivity to a subset of sugars [1, 2]. Two adjacent, seven-transmembrane domain genes near the _Tre_ locus are candidate genes for _Tre_. One (_CG3171_) encodes a rhodopsin family G protein receptor [3, 4], and the other (_Gr5a_) is a member of a chemosensory gene family encoding a putative gustatory receptor [5–7]. We carried out molecular analyses of mutations in _Tre_ to elucidate their involvement in the gustatory phenotype. Here, we show that _Tre_ mutations induced by P element-mediated genomic deletions disrupt _Gr5a_ gene organization and the expression of _Gr5a_ mRNA, while disruption of the _CG3171_ gene or its expression was not always associated with mutations in _Tre_. In flies with the spontaneous mutation _Tre_^{01}, both _CG3171_ and _Gr5a_ mRNAs are transcribed. Coding sequences of these two candidate genes were compared among various strains. A total of three polymorphic sites leading to amino acid changes in _CG3171_ were not correlated with the gustatory phenotype. Among four nonsynonymous sites in _Gr5a_, a single nucleotide polymorphism leading to an Ala218Thr substitution in the predicted second intracellular loop cosegregated with _Tre_^{01}. Taken together, the mutation analyses support that _Gr5a_ is allelic to _Tre_.

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Results and discussion

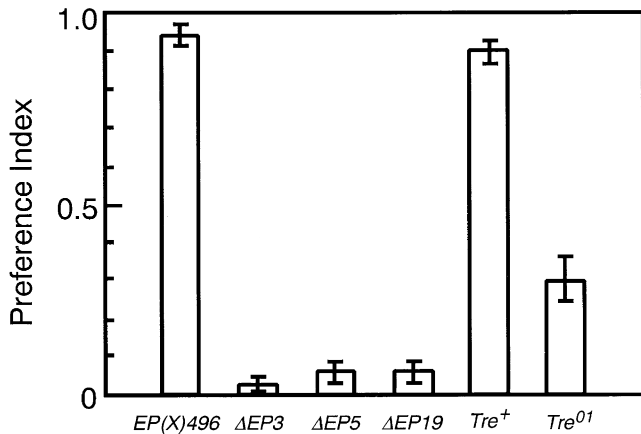
P element-induced *Tre* mutations

Drosophila locus *Tre* is mapped at 5A10 to 5B1-2 on the X chromosome and controls gustatory sensitivity to a disaccharide trehalose (α-D-Glucopyranosyl-α-D-glucopyranoside) [1, 2]. Flies carrying a spontaneous mutation *Tre*^{01} show a decreased gustatory sensitivity to trehalose. Two seven-transmembrane receptor genes *Gr5a* [6, 7] and *CG3171* [3, 4] have been identified previously in the region where the *Tre*^{01} is mapped. A single P element insert *EP(X)496* (GenBank accession number AQ025347) that maps directly between the two genes was used to generate *Tre* mutations by activating imprecise P element excisions [8]. A total of 22 independent *Tre* mutations were recovered. The gustatory phenotype, assessed by a feeding preference test (see Materials and methods and the Supplementary material available with this article online), is shown in Figure 1 for some of the P excised mutants. Males of a wild-type *Tre*$^{+}$ strain, *w cx Tre*$^{+}$, produced the mean proportion of flies choosing the trehalose solution, or preference index (PI), of 0.90, while *w cv Tre*^{01} males carrying a spontaneous mutation *Tre*^{01} gave a low PI value of 0.30 ($p < 0.01$). *EP(X)496* was wild-type for *Tre*, since the strain gave a PI value of 0.95. Males carrying the P excised *Tre* mutations *Tre*$^{\Delta EP3}$, *Tre*$^{\Delta EP5}$, or *Tre*$^{\Delta EP19}$ gave significantly low PIs of 0.06 or less ($p < 0.01$). Complementation tests between the P mutations and *Tre*^{01} confirmed that the P mutations are allelic to *Tre* (data not shown), but these P mutations of *Tre* apparently show a more severe phenotype than the spontaneous mutation *Tre*^{01} ($p < 0.01$).

Induced *Tre* mutations are genomic deletions uncovering *CG3171* and/or *Gr5a*

The two *Tre* candidates *CG3171* [3, 4] and *Gr5a* [5–7] are adjacent genes on the genome facing the 5′ end (GenBank accession number AE003435). The insertion of *EP(X)496* is located between the two genes and is less than 0.1 kbp upstream of the transcription start site (GenBank accession number AB042625) of *CG3171* and 0.7 kbp upstream of the putative start codon of *Gr5a* (Figure 2). *Tre*$^{\Delta EP3}$ (GenBank accession number AB066610) has a 2.1 kbp genomic deletion uncovering 0.7 kbp toward *Gr5a* and 1.4 kbp toward *CG3171*. In *Tre*$^{\Delta EP5}$ (GenBank accession number AB066611), we identified a 2.5 kbp deletion that spans 0.9 and 1.6 kbp in the directions of *Gr5a* and *CG3171*, respectively. The gene structure of *CG3171* in *Tre*$^{\Delta EP3}$ and *Tre*$^{\Delta EP5}$ is disrupted by the absence of the promoter, exon 1, and part of intron 1. *Gr5a* gene is also

Figure 1



Comparisons of the gustatory preference for 20 mM trehalose against 2 mM sucrose among wild-type and *Tre* mutant males. Bars indicate 95% limit of reliance of the mean preference index. The number of flies (n) of *EP(X)496* was 209; of *w* $Tre^{\Delta EP3}$, 261; of *w* $Tre^{\Delta EP5}$, 227; of *w* $Tre^{\Delta EP19}$, 220; of *w cx* Tre^{+}, 374; and of *w cv* Tre^{01}, 260.

disrupted in $Tre^{\Delta EP5}$ since it uncovers the promoter, the 5′ leader, and 133 bp downstream of the start codon. Deletion in $Tre^{\Delta EP3}$ leaves 44 bp intact in the 5′ leader, but at least 58 bp are truncated in addition to the deletion of the promoter. Therefore, $Tre^{\Delta EP3}$ and $Tre^{\Delta EP5}$ are expected to be double mutations of both *CG3171* and *Gr5a*.

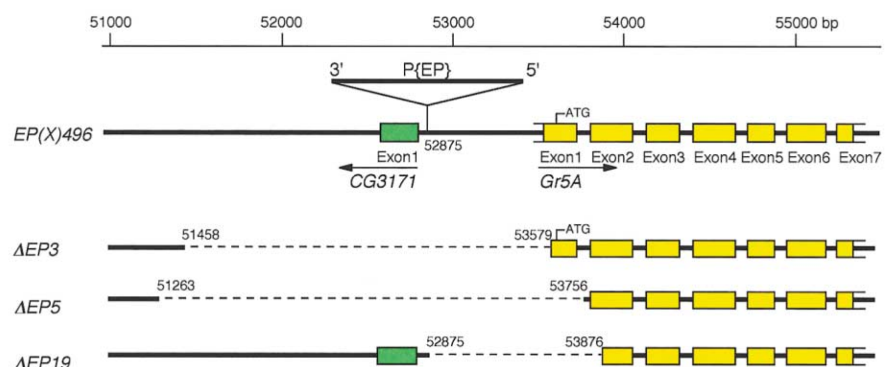
$Tre^{\Delta EP19}$ (GenBank accession number AB066612) is distinct from $Tre^{\Delta EP3}$ or $Tre^{\Delta EP5}$ since the 3′ end of the P element has been precisely excised out, leaving the *CG3171* gene structure intact. $Tre^{\Delta EP19}$ uncovers a 1.0 kbp sequence in the direction of *Gr5a* that includes the promoter, the 5′ leader, and a 253 bp sequence of the entire exon 1, intron 1, and part of exon 2. Therefore, only the *Gr5a* gene structure is specifically and most severely disrupted in $Tre^{\Delta EP19}$. Taking the genomic analyses together, disruption of *Gr5a* but not *CG3171* is associated with *Tre* mutations.

The expression of *CG3171* and *Gr5a* mRNAs in wild-type and *Tre* mutant flies

The transcripts of *CG3171* and *Gr5a* were then investigated in the P excised mutants. RT-PCR with total RNAs isolated from the head amplified a *CG3171* mRNA sequence in Tre^{+} and Tre^{01} flies (Figure 3a). No amplification was observed with $Tre^{\Delta EP3}$ or $Tre^{\Delta EP5}$ templates. With $Tre^{\Delta EP1}$, $Tre^{\Delta EP13}$, and $Tre^{\Delta EP18}$ templates, the RT-PCR also failed to amplify the fragment. However, there was another class of mutations in which the expression of *CG3171* mRNA was observed. $Tre^{\Delta EP7}$, $Tre^{\Delta EP11}$, $Tre^{\Delta EP12}$, $Tre^{\Delta EP14}$, and $Tre^{\Delta EP19}$ belonged to this class. Among these, $Tre^{\Delta EP19}$ was predicted to express *CG3171* from the intact genomic structure. In the Northern blot analysis, the antisense RNA probe of *CG3171* labeled a 1.6 kbp mRNA from wild-type heads (Figure 3b). The 1.6 kbp band was not detectable in $Tre^{\Delta EP3}$ or $Tre^{\Delta EP5}$, but was present in $Tre^{\Delta EP19}$, as in the RT-PCR analysis. *CG3171* mRNA was further analyzed in various wild-type tissues. Figure 3c shows a Northern blot with adult head, thorax, abdomen, and appendages, showing that *CG3171* mRNA is expressed in various tissues. The mRNA was normally expressed in the labella, the head, and the appendages of a mutant *poxn* [10] in which chemosensory neurons are transformed to mechanosensory neurons (Figure 3e,f). The low tissue specificity of *CG3171* is inconsistent with the claim of Ishimoto et al. that *CG3171* mRNA was identified from the differential screen of cDNA libraries between wild-type and *poxn* mutant tissues [4]. A *CG3171* cDNA clone LD12308 (GenBank accession number AA438512) was isolated from an embryonic library. We also observed that *CG3171* mRNA is expressed throughout embryo to adult stages (data not shown). A different line of evidence supporting *CG3171* as a developmental gene was provided by Toba et al. [11]. They showed that developmental phenotypes were induced in various tissues of the transformants carrying a misexpression construct inserted adjacent to *CG3171* locus when the expression was activated by Gal4 drivers.

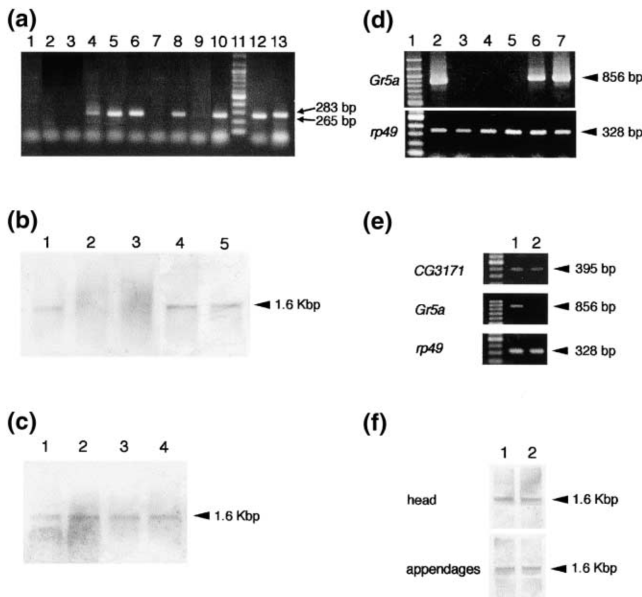
We then analyzed *Gr5a* mRNA in wild-type and mutant

Figure 2



Schematic diagram of the deletions in the genome of the P excised *Tre* mutants. The numbers indicated are loci in the genomic scaffold (GenBank accession number AE003435). The gene organization of *Gr5a*, the partial gene organization of *CG3171*, and the insertion of a P{EP} construct [9] in the parent *EP(X)496* are shown to scale. The P{EP} size is not shown to scale. Exons of *CG3171* and *Gr5a* are shown in green and yellow, respectively. The 5′ leader and the 3′ untranslated region of *Gr5a* have not yet been determined. Deleted regions are indicated by dotted lines.

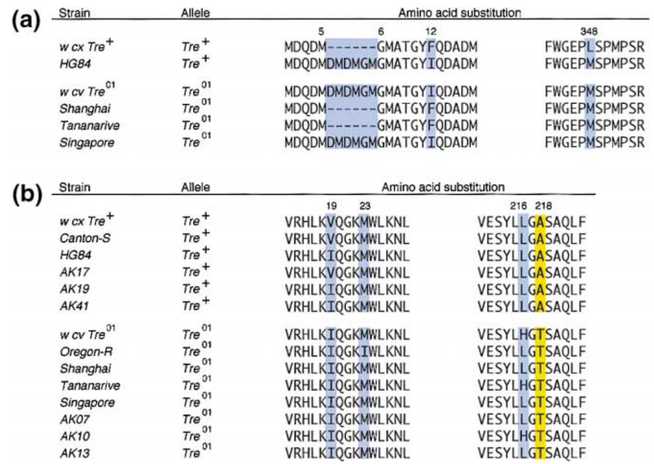
Figure 3



Analysis of *CG3171* and *Gr5a* mRNAs in wild-type and *Tre* mutants. **(a)** RT-PCR analysis of *CG3171* mRNA from P excised *Tre* mutants compared with wild-type *Tre*$^{+}$ and spontaneous mutant *Tre*^{01} flies. A 265 bp fragment between exon 1 and 2 is expected to be amplified from wild-type mRNA. A 283 bp fragment is amplified for *w cv Tre*^{01} due to the 18 bp insertion in exon 2. The numbers refer to the following: 1, *w Tre*$^{\Delta EP1}$; 2, *w Tre*$^{\Delta EP3}$; 3, *w Tre*$^{\Delta EP5}$; 4, *w Tre*$^{\Delta EP7}$; 5, *w Tre*$^{\Delta EP11}$; 6, *w Tre*$^{\Delta EP12}$; 7, *w Tre*$^{\Delta EP13}$; 8, *w Tre*$^{\Delta EP14}$; 9, *w Tre*$^{\Delta EP18}$; 10, *w Tre*$^{\Delta EP19}$; 11, 100 bp ladder; 12, *w cx Tre*$^{+}$; and 13, *w cv Tre*^{01}. **(b)** Northern blot analysis with DIG-labeled *CG3171* probe against poly(A)$^{+}$ RNAs from the heads of adult flies from the following: 1, *EP(X)496*; 2, *w Tre*$^{\Delta EP3}$; 3, *w Tre*$^{\Delta EP5}$; 4, *w Tre*$^{\Delta EP19}$; and 5, *Canton-S*. A single band of about 1.6 kbp in size is labeled in lanes 1, 4, and 5. **(c)** Northern blot analysis with various adult tissues of *w cx Tre*$^{+}$. The numbers refer to the following: 1, head minus proboscis; 2, thorax; 3, abdomen; and 4, appendages. See explanations in (b). **(d)** RT-PCR analysis of *Gr5a* (upper lanes) and *rp49* mRNAs (lower lanes) of the following: 1, 100 bp ladder; 2, *EP(X)496*; 3, *w Tre*$^{\Delta EP3}$; 4, *w Tre*$^{\Delta EP5}$; 5, *w Tre*$^{\Delta EP19}$; 6, *w cx Tre*$^{+}$; and 7, *w cv Tre*^{01}. An 856 bp *Gr5a* sequence between the 5′ leader and Exon 4 is amplified in lanes 2, 6, and 7. The control 328 bp *rp49* sequence is amplified in all lanes. **(e)** The expression of *CG3171* (upper lanes), *Gr5a* (middle lanes), or *rp49* (lower lanes) in the labella analyzed by RT-PCR. The numbers 1 and 2 refer to *w cx Tre*$^{+}$ and *poxn*, respectively. A 395 bp *CG3171* sequence between Exon 1 and 3 is amplified. See explanations in (d) for *Gr5a* and *rp49*. **(f)** Northern blot with *CG3171* probe against poly(A)$^{+}$ RNAs from wild-type and *poxn* heads or appendages. The numbers 1 and 2 refer to *w cx Tre*$^{+}$ and *poxn*, respectively. See explanations in (b).

flies. RT-PCR was successful with poly(A)$^{+}$ RNA prepared from 300 heads and 100 labella (Figure 3d,e). Figure 3d shows that an 856 bp *Gr5a* mRNA fragment was not identified in the deletions *Tre*$^{\Delta EP3}$, *Tre*$^{\Delta EP5}$, and *Tre*$^{\Delta EP19}$, while *Gr5a* mRNA is transcribed in wild-type, *EP(X)496*, and *Tre*^{01} heads. It is therefore suggested that intact mRNAs are not transcribed in those mutants. Figure 3e compares *Gr5a* mRNAs from wild-type and *poxn* labella by the RT-PCR. The amounts of the template RNAs were adjusted by an internal control reaction for the ribosomal protein RP49. *Gr5a* mRNA was identified in the wild-type labella, as observed in the heads. In the *poxn* labella, however, it was either absent or present in severely decreased amounts, suggesting that *Gr5a* is predominantly expressed in the gustatory sensory neurons in the labella. The expression analyses supported that *Gr5a* is expressed in the gustatory neurons and that the mutation of *Gr5a* is associated with the *Tre* phenotype.

Figure 4



Predicted amino acid polymorphism in the coding sequences of **(a)** *CG3171* and **(b)** *Gr5a* among laboratory and wild isofemale strains. Residue numbers are shown above the amino acid sequences. Changes in amino acids are indicated in colors. An Ala218Thr polymorphism in *Gr5a* is highlighted in yellow.

Polymorphism of *CG3171* and *Gr5a* in the spontaneous mutation *Tre*^{01}

Both *CG3171* and *Gr5a* mRNAs were identified in the flies carrying the spontaneous mutation *Tre*^{01} (Figure 3a,d). We therefore investigated the nucleotide polymorphisms leading to amino acid changes. A 1.5 kbp genomic sequence downstream of the start codon of *CG3171* was analyzed in *w cx Tre*$^{+}$, *w cv Tre*^{01}, and four isofemale wild strains. The gustatory phenotype was also investigated for the wild strains as described. One strain (*HG84*) was *Tre*$^{+}$, and the other three strains were *Tre*^{01}. Sequence analysis revealed a total of 12 single-nucleotide polymorphisms (SNPs) and an 18 bp insertion/deletion in exons. In addition, a total of 18 SNPs and two oligonucleotide insertions/deletions were also identified in the introns (GenBank accession numbers AB066613–8). The insertion of the 18 bp oligonucleotide ATGGATATGGA TATGGGA, leading to an insertion of six amino acids in the N-terminal region, was identified in *w cv Tre*^{01} and two wild strains, *HG84*(*Tre*$^{+}$) and *Singapore*(*Tre*^{01}) (Figure 4a). In these three strains, an SNP in exon 2 leading to a Phe12Ile substitution was also identified. A second

nonsynonymous SNP in exon 7 leading to a Leu348Met substitution was identified in all strains, except in *w cx Tre*$^+$. None of the three sites, however, were linked to the gustatory dimorphism.

Similarly, *Gr5a* polymorphisms were also analyzed for the 1.7 kbp genomic region in the following six strains: *w cx Tre*$^+$, *Canton-S* (*Tre*$^+$), *HG84* (*Tre*$^+$), *w cv Tre*^{01}, *Oregon-R* (*Tre*^{01}), and *Singapore* (*Tre*^{01}) (Figure 4b). There were a total of 25 SNPs within the exons of the *Gr5a* gene. Nineteen SNPs and two single-nucleotide insertions/deletions were also found in the introns (GenBank accessions numbers AB066619–24). Among the exon polymorphisms, we identified four nonsynonymous SNPs. The SNPs Met23Ile and Leu216His were identified only in *Oregon-R* (*Tre*^{01}) and *w cv Tre*^{01}, respectively. Val19Ile was identified in all three *Tre*^{01} strains but also in *HG84* (*Tre*$^+$). The only SNP that cosegregated with the *Tre* phenotype was Ala218Thr. For statistical comparison, we extended the analysis with eight additional wild strains, *Shanghai*, *Tananarive*, and six *AK* isofemale strains, of which three strains were *Tre*$^+$ and five strains were *Tre*^{01} (Accessions AB066625–40). Neither Val19Ile, Met23Ile, nor Leu216His cosegregated with the gustatory phenotype. Ala218Thr, which cosegregated with the *Tre* phenotype in the previous analysis, again cosegregated in the additional eight strains without exceptions. Assuming that the allelic frequencies of *Tre*$^+$ and *Tre*^{01} are 0.25 and 0.75, as was observed in a collection of *AK* strains, the probability of cosegregation between Ala218Thr and the *Tre*/*Tre*^{01} gustatory dimorphism occurring by chance among the 14 strains is about 0.01%. We therefore concluded that Ala218Thr is significantly correlated with the gustatory *Tre* phenotype. The Ala218 is located in the predicted second intracellular loop domain of GR5a. Since the second or the third loop is known to be critically important in the activation of G protein both by rhodopsin [12, 13] and by mGluR1 [14], GR5a may activate G protein through a similar mechanism, and the Ala218 may be involved in the activation process. Although functional characterization of *Gr5a* has yet to be performed, we propose that *Gr5a* encodes a gustatory sugar receptor controlled by the locus *Tre*.

Gustatory receptors and the sweet taste response in flies

By Clustal W analysis, *Gr5a* is shown to be most closely related phylogenetically to *Drosophila* candidate gustatory receptor gene *Gr61a* and a gene cluster of *Gr64a–f* on the third chromosome [6, 7]. Since *Tre* affects the taste response to a limited subset of sugars (Morita et al., unpublished data), some of these receptors may also be involved in the sweet taste response to different subsets of sugars. Physiological studies showed that the input from sugar-sensitive neurons in the labellar, tarsal, and other gustatory organs controls the proboscis extension reflex and the feeding behavior [15]. Scott et al. [6] and Dunipace et al. [7] showed that GRs are expressed in gustatory neurons in distinct subsets of gustatory organs. Future studies on the localization and the projection of gustatory neurons expressing GR5a and the related GR members may provide clues to understanding the neuronal mechanism underlying feeding behavior in flies.

Materials and methods

Fly stocks, mutagenesis, and screening procedure

Homozygous *w cx Tre*$^+$ carrying *Tre*$^+$ and recessive markers *w* and *cx* was used as a wild-type control for the feeding preference test. Homozygous *w cv Tre*^{01} carrying a spontaneous mutation *Tre*^{01} and recessive markers *w* and *cv* was also used. Both *cx* and *cv* are located near the gene *Tre*. Wild-type isogenic strains *Canton-S* and *Oregon-R* were obtained from Indiana Stock Center. *HG84* (Africa), *Shanghai*, *Tananarive*, *Singapore*, and a set of *AK* (Akayu, Japan) strains are isofemale wild strains collected from various locations. All wild strains were isogenized for the X chromosome by crossing single males to an X chromosome balancer. Homozygous *poxn* males were used in experiments to analyze the expression of *CG3171* and *Gr5a*. To induce mutations, *EP(X)496*, which has a single insertion of P{EP}construct at 5A13 on the salivary X chromosome, was used as a parental strain. Males were crossed to virgin females of a transposase source *C(1)DX, y^1 f^1; ry^{506} P{$ry^{+t7.2}$=Δ2-3}99B* to induce imprecise excisions in the germline cells of the F1 progenies. The P excisions were identified by the w^{+mC} marker in the construct in subsequent generations. About 350 P excised, white-eyed males were obtained from a 6,000 F2 screen. P excised males were then subjected to a feeding preference test as described below. Candidate males showing low preference to trehalose were singly mated to virgin X chromosome balancer females to establish strains. A total of 22 P excised *Tre* mutations were recovered. They were given allele names of ΔEP followed by a number.

Feeding preference test

Food-deprived flies were introduced into a micro test plate and allowed to feed for 1 hr by providing them with the choice between 20 mM trehalose and 2 mM sucrose solutions. The solutions were mixed with 1% agar and a blue food dye (0.125 mg/ml brilliant blue FCF; $C_{37}H_{34}O_9NaS$) for the trehalose solution or a red food dye (0.5 mg/ml acid red 27; $C_{20}H_{11}O_{10}N_2Na_3S_3$) for the sucrose solution. After feeding, flies were anesthetized and observed under the dissection microscope. They were categorized into four groups according to the abdominal colors: blue (group 1), red (group 2), purple (group 3), and no staining (group 4). The preference index (PI) of 20 mM trehalose against 2 mM sucrose was calculated as the sum of the number of flies in group 1 and half the number of flies in group 3 divided by the total number of flies in groups 1, 2, and 3. (For an example of the feeding test, see Figure S1 in the Supplementary material available with this article online.)

Northern blot analysis

Poly(A)$^+$ RNA was extracted from adult heads or other tissues by an mRNA isolation kit (Roche). Electrophoresis was performed on a 1% formaldehyde gel by loading 2 μg of poly(A)$^+$ RNA per lane and blotting it onto a nylon membrane (Amersham Pharmacia). The antisense RNA probe was prepared from the cDNA sequence of LD12308 and labeled by a digoxigenin labeling kit (Roche). Hybridization was carried out at 65°C for 16 hr, and the signals were detected with anti-digoxigenin-alkali phosphatase complex (Roche).

RT-PCR

Total or poly(A)$^+$ RNAs isolated from the adult heads were used as templates for the RT reaction of *CG3171* and *Gr5a* mRNAs with an oligo-dT primer. The reaction mixtures were then used for PCR with a set of gene-specific primers. For the expression analysis in the *poxn* flies, poly(A)$^+$ RNA from 100 labellar tissues were also analyzed. The *CG3171*-specific primers were TTTTGTTACTGTGCTGCGCGGAG (exon 1, sense), CAAAGACACAGGCACTAATGGC (exon 2, antisense),

and GAAGGAGCAGAAGAGCAGGTC (exon 3, antisense). The *Gr5a*-specific primers were CTGTTTTATTCCTCATCACTGGCC (exon 1, sense) and TGTCCATGTAACTCCAGCCGAAGGT (exon 4, antisense). The exon 1 primer sequence locates in the 5′ leader, 103 bp upstream of the predicted start codon. A control RT-PCR was carried out for the ribosomal protein RP49 with primers GACCATCCGCCCAGCATACAG and AATCTCCTTGCGCTTCTTGGAGGAG.

Cloning and sequencing analysis

An embryonic cDNA clone LD12308 (GenBank accession number AA438512) for *CG3171* and the bacteriophage P1 genomic clones DS07265 and DS07361 covering the genomic region 5A9–5B1 (Berkely *Drosophila* Genome Project, http://www.fruitfly.org/) were obtained from Research Genetics. We determined the entire cDNA sequence of LD12308. The sequence was identical to the predicted mRNA of *CG3171* (GenBank accession number AE003435) except that the 5′ leader starts 17 bp upstream and that the 3′ untranslated region of 136 bp follows after the stop codon. Genomic or cDNA sequences were either directly sequenced or cloned into vectors after PCR amplification. They were analyzed with conventional methods using 3′ dye-labeled dideoxynucleotide terminators followed by primer extension reactions and were then analyzed using the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). With a set of primers, GAGCACTGATTCCA CACACGGGCATT and ATCAAGTATTTATGTAGCGGGCGGAAAGT, an approximately 3 kbp flanking genomic sequence that harbors the insertion site of *EP(X)496* and partially spans the *CG3171* and *Gr5a* gene loci in the wild-type genome is amplified by PCR. Fragments shorter than 3 kbp were amplified by the PCR in the P excised deletions *w* $Tre^{\Delta EP3}$, *w* $Tre^{\Delta EP5}$, and *w* $Tre^{\Delta EP19}$ and were subjected to the sequence analysis.

Supplemental material

Supplemental material including an additional figure showing an example of the feeding test are available at http://images.cellpress.com/supmat/supmatin.htm.

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