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Characterization of *Drosophila Tyramine β-Hydroxylase* Gene and Isolation of Mutant Flies Lacking Octopamine

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Octopamine is likely to be an important neuroactive molecule in invertebrates. Here we report the molecular cloning of the *Drosophila melanogaster* gene, which encodes tyramine β-hydroxylase (TBH), the enzyme that catalyzes the last step in octopamine biosynthesis. The deduced amino acid sequence of the encoded protein exhibits 39% identity to the evolutionarily related mammalian dopamine β-hydroxylase enzyme. We generated a polyclonal antibody against the protein product of *Tβh* gene, and we demonstrate that the TBH expression pattern is remarkably similar to the previously described octopamine immunoreactivity in *Drosophila*. We further report the creation of null mutations at the *Tβh* locus, which result in complete absence of TBH protein and blockage of the octopamine biosynthesis. *Tβh*-null flies are octopamine-less but survive to adulthood. They are normal in external morphology, but the females are sterile, because although they mate, they retain fully developed eggs. Finally, we demonstrate that this defect in egg laying is associated with the octopamine deficit, because females that have retained eggs initiate egg laying when transferred onto octopamine-supplemented food.

Key words: cloning of Tyramine β-hydroxylase*; TBH-immunocytochemistry;* Dopamine β-hydroxylase*; octopamine-null mutant; egg-laying behavior;* Drosophila *neurogenetics*

Octopamine is likely to be an important neuroactive molecule in many invertebrates, because physiological studies, carried out primarily in arthropods, have provided considerable evidence about its role as a neurotransmitter, a neuromodulator, or a neurohormone (for review, see Evans, 1980, 1985, 1992; David and Coulon, 1985). Octopamine appears to regulate diverse physiological functions in different organisms such as neuromuscular transmission in locusts (Hoyle, 1984; Malamud et al., 1988), light production in fireflies (Nathanson, 1979), production of submissive postures in lobsters (Livingstone et al., 1980), feeding and sting response in honey bees (Braun and Bicker, 1992) (for review, see Bicker and Menzel, 1989; Burrell and Smith, 1995), and induction of lipid and carbohydrate metabolism at times of stress (for review, see Orchard et al., 1993). Because of the structural similarities between noradrenaline and octopamine and an absence of noradrenaline from several arthropod species, octopamine is at times considered to be the arthropod noradrenaline. Although studied primarily in invertebrates, octopamine also is found in vertebrates, but its functions there have not been well studied (for review, see David and Coulon, 1985).

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We were interested in developing molecular–genetic tools to study the role of octopamine in the fruit fly *Drosophila melanogaster*, because one could conceive strategies to manipulate genetically the level of octopamine and study its effects on specific behaviors of the organism. In principle, it should be possible to create flies that are congenitally devoid of any octopamine or flies in which the presence of octopamine is conditional. *Drosophila* is known to contain octopamine (O'Dell et al., 1987) and recently, octopamine immunoreactivity has been described in specific neurons and neuropil areas of the nervous system and also in neuronal terminals innervating the larval body wall muscles (Monastirioti et al., 1995). Pharmacological studies in *Drosophila* have suggested involvement of octopamine in behaviors such as phototaxis and learning as well as in certain aspects of development (Dudai et al., 1987). High-affinity octopamine binding sites have been described in studies with *Drosophila* head homogenates (Dudai and Zvi, 1984), and a gene encoding a putative octopamine/tyramine receptor has been cloned and its pharmacological properties have been investigated (Arakawa et al., 1990; Saudou et al., 1990; Robb et al., 1994). Mutation in the *inactive* locus causes hypoactivity, and the mutant flies are reported to have 15% of normal octopamine (O'Dell et al., 1987; O'Dell and Burnett, 1988). Despite all of these studies, at present there is no well defined function assigned to octopamine in *Drosophila.*

To manipulate the levels of octopamine genetically, we decided to first identify a gene encoding one of the enzymes in the biosynthetic pathway of octopamine. Molecular identification of such a gene allows determination of the cytogenetic location of the gene on salivary chromosomes, which can facilitate isolation of mutations in that genetic locus. Octopamine biosynthesis requires tyrosine decarboxylase activity to convert tyrosine to tyramine and tyramine β-hydroxylase (TBH) activity to convert tyramine to octopamine. Livingstone and Tempel (1983) have

demonstrated that these two enzymatic activities exist in *Drosophila melanogaster* and that the tyrosine decarboxylase activity is distinct from the *Ddc*-encoded dopa decarboxylase activity. No other information existed on the two enzymes in *Drosophila*. However, studies on biochemical properties of a partially purified TBH from the thoracic nervous system of lobster *Homarus americanus* had demonstrated that it was remarkably similar to dopamine β-hydroxylase (DBH), which converts dopamine to noradrenaline in mammals (Wallace, 1976). The salient features of this similarity are (1) both enzymes recognize either tyramine or dopamine (the precursors of octopamine and noradrenaline, respectively) as substrates, although with different affinities, (2) both enzymes require oxygen and ascorbic acid as cosubstrates, and (3) both enzymes bind copper and are inhibited by metal chelators (Wallace, 1976). These similar properties make it highly likely that the enzymes TBH and DBH, which catalyze the final hydroxylation step in the production of octopamine and noradrenaline, are functionally homologous (Wallace, 1976) and are probably related evolutionarily. Because DBH amino acid sequences from several mammalian species were available (Lamouroux et al., 1987; McCafferty and Angeletti, 1987; McMahon et al., 1990), we decided to attempt to clone the *Drosophila Tyramine β-hydroxylase* (*Tβh*) gene by using the possible evolutionary homology between the TBH and DBH amino acid sequences.

In this study, we describe cloning of a *Drosophila* gene that has homology to DBH, and we provide immunocytochemical, biochemical–genetic, and behavioral–genetic evidence confirming this gene as the *Drosophila melanogaster Tβh* gene. We report on creation of null mutations at the *Tβh* locus and analysis of the phenotype associated with the octopamine deficit. We show that these octopamine-less flies survive to adulthood and are normal in external morphology and that the males are fertile. Interestingly, mutant females are sterile, because although they mate, they retain fully developed normal oocytes. Finally, we demonstrate that the egg-retention defect is associated with the octopamine deficit, because mated gravid females that have retained oocytes initiate egg laying when transferred onto octopamine-supplemented food.

MATERIALS AND METHODS

PCR. Degenerate oligonucleotides were designed for the regions of homology among the three mammalian DBH proteins shown in Figure 1. The primer sequences were:

5′-ACNACNTAT/CTGGTGT/CTAT/CAT-3′,
5′-GGA/GTTA/GTGA/GTAA/GTGNACT/CTC-3′,
5′-GGNGAA/GATGGAA/GAAT/CGC-3′,
5′-AAN-ACC/TTCCATA/GTGA/GTGNAC-3′,

where n = A/G/C/T. PCR reactions were done in a total volume of 20 μl, and the incubation mixture contained 1 μg genomic DNA as template, 5 μM of each primer, 1.5 mM $MgCl_2$, 200 μM each of deoxynucleotidyl triphosphates, and 2 U of *Taq* polymerase (Perkin-Elmer Cetus, Emeryville, CA). For reamplification reactions, 0.5 μl of the first PCR reaction was used as template. Conditions for the PCR amplification were as follows: initial denaturation at 94°C for 2 min followed by 5 cycles at 94°C for 1 min, 42°C for 2 min, and 72°C for 2 min, and 25 cycles at 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min.

PCR products were gel purified, treated with the Klenow fragment of DNA polymerase I, and then phosphorylated with T4 kinase (enzymes were purchased from New England Biolabs, Beverly, MA). The products then were subcloned into the *Sma*I site of BlueScript-KS (Stratagene, La Jolla, CA), and their sequences were determined.

Screening of cDNA libraries and sequencing. cDNA clones were isolated by standard methods from a λgt11 recombinant fly head cDNA library (Itoh et al., 1985). The plasmid clone containing the 120 bp PCR product was used as a hybridization probe labeled by the random primer method. The entire insert from the positive clones was subcloned into the *Eco*RI site of Bluescript-SK (pDmDBH). DNA fragments from pDmDBH then were subcloned into polylinker sites of pBluescript-SK, and sequencing of both strands was performed. The sequence also was confirmed from double-stranded overlapping deletions covering the entire 3 kb length of pDmDBH.

RNA isolation and Northern analysis. Total RNA was isolated from adult heads and bodies of Canton-S flies using the guanidium hydrochloride method (Davis et al., 1986); 12 μg from each sample RNA was separated on 1.2% formaldehyde/agarose gel and transferred to Biotrans nylon membrane. Antisense RNA probe was synthesized with T7 polymerase from a subclone (pPXDBH) carrying a 0.87 kb *Pst–Xho* fragment from the cDNA clone (Fig. 2*B*). The filter was hybridized overnight at 65°C under conditions described in Yao et al. (1992). Subsequently, the filter was hybridized with a random-primed DNA probe of pRP49 (Kongsuwan et al., 1985) as a control.

RNA in situ *hybridization. In situ* detection of RNA in whole-mount larval CNS was as described in Ebens et al. (1993) using digoxygenin-labeled DNA probe synthesized for the *Pst–Xho* fragment of the cDNA clone (Fig. 2*B*) according to the manufacturer's protocols (Boehringer Mannheim, Indianapolis, IN).

Protein purification and preparation of antibodies. The 1.2 kb *Sal–Xho* fragment of the DmDBH cDNA (Fig. 2*B*) was inserted into the *Xho*I site of the bacterial expression vector pET15b (Novagen, Madison, WI) in frame with the His^6 tag and under the bacteriophage T7 promoter present in the vector. The resulting plasmid was introduced into the *Escherichia coli* BL21(DE3) strain (Studier and Moffatt, 1986); cultures of the transformed cells were grown to OD_{600} = 0.5, induced with 1 mM isopropyl β-D-thiogalactopyranoside, then grown for 3 more hr and harvested. Pellets were resuspended in 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8, 7.5 mM β-mercaptoethanol, and cells were lysed completely by 2 × 30 sec sonication. After 30 min centrifugation at 10,000 rpm, the lysate was adsorbed to a nickel chelate affinity resin (Qiagen, Chatsworth, CA) column, and the protein was eluted with a pH gradient in the above buffer. The purified protein was renatured at 4°C by dialysis against a buffer containing (in mM): NaCl 500, Tris 20, pH 7.4, PMSF 0.5, containing 6, 4, 2, 1, 0.5, and 0 M urea, and it was stored at −70°C. Rats were immunized subcutaneously at multiple sites with 25 μg of purified protein in incomplete Freund's adjuvant every 2 weeks.

For specific antibody purification, 60 μg of purified protein was subjected to Western blotting; the nitrocellulose band was fragmented, washed, and incubated in a 1:5 dilution of anti-TBH serum for 48 hr at 4°C. The specific immunoglobulins were eluted from the fragments at 4°C with 500 μl of elution buffer (100 mM glycine/HCl, pH 2.6, 0.2% Tween 20, 100 mM NaCl, and 100 μg/ml BSA) for 10 min. The eluate was neutralized immediately with 25 μl of 2 M Tris/HCl, pH 8, and was kept aliquoted at −70°C.

Immunoblot analysis of wild-type and Tβh *mutants.* Crude homogenates of head and body tissue were used for the immunoblots. Purified anti-TBH IgG was used at 1:30 dilution for the primary antibody incubations. The enhanced chemiluminescence system (Amersham, Arlington Heights, IL) was used for detection of the primary antibody according to the manufacturer's instructions. Horseradish peroxidase-conjugated goat anti-rat secondary antibody was used at a final concentration of 1:1500.

Immunohistochemistry. Third instar larval brains were chosen for these studies for the following reasons: (1) the staining can be done in whole mounts; (2) the pattern of immunoreactivity is simple, which makes comparisons possible; and (3) the staining was reproducible. In contrast, the adult staining had to be done in sections and was variable in our hands, and thus, comparisons were difficult.

Third instar larval brains were dissected in PBS containing (in mM): NaCl 137, KCl 3, KH_2PO_4 1.8, Na_2HPO_4 10, pH 7.5, and fixed in ice-cold Bouin's fixative containing (in ml): saturated aqueous picric acid solution 75, formaldehyde 25, glacial acetic acid 5 for 1 hr at room temperature. After fixation, samples were washed in PBS (three times, 15 min each) and in PBT (PBS containing 0.3% Triton X-100 and 0.1% BSA) (two times, 15 min each). Washes were followed by preincubation with 5% normal goat serum for 1 hr at room temperature and overnight incubation with purified anti-TBH antibody (1:25 in PBT) at 4°C. The samples then were washed in PBT (six times, 15 min each) and incubated overnight at 4°C in goat anti-rat FITC-conjugated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) diluted at 1:100 in PBT. After several washes in PBT and a final wash in PBS, the samples were mounted in mounting media (80% glycerol, 2% *N*-propylgalate in PBS). For each genotype, a minimum of four samples were immunoprocessed together; the staining intensity was similar between samples of the same

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Bovine  PFHIPLDPEG TLELSWNISY AQETIYFQLL VRELKAGVLF GMSDRGELEN
 Human  PYHIPLDPEG SLELSWNVSY TQEAIHFQLL VRRLKAGVLF GMSDRGELEN
   Rat  PYHIPLDPEG TLELSWNVSY DQEIIHFQLQ VQGPRAGVLF GMSDRGEMEN 100
                                                    ————

Bovine  ADLVVLWTDR DGAYFGDAWS DQKGQVHLDS QQDYQLLRAQ RTPEGLYLLF
 Human  ADLVVLWTDG DTAYFADAWS DQKGQIHLDP QQDYQLLQVQ RTPEGLTLLF
   Rat  ADLVMLWTDG DRTYFADAWS DQKGQIHLDT HQDYQLLQAQ RVSNSLSLLF 150
        —> #3

Bovine  KRPFGTCDPN DYLIEDGTVH LVYGFLEEPL RSLESINTSG LHTGLQRVQL
 Human  KRPFGTCDPK DYLIEDGTVH LVYGILEEPF RSLEAINGSG LQMGLQRVQL
   Rat  KRPFVTCDPK DYVIEDDTVH LVYGILEEPF QSLEAINTSG LHTGLQQVQL 200

                                                 •          **
Bovine  LKPSIPKPAL PADTCTMEIR APDVLIPGQQ TTYWCYVTEL PDGFPRHHIV
 Human  LKPNIPEPEL PSDACTMEVQ APNIQIPSQE TTYWCYIKEL PKGFSRHHII
   Rat  LKPEVSTPAM PADVQTMDIR APDVLIPSTE TTYWCYITEL PLHFPRHHII 250
                                          ————————> #1
                        **
Bovine  MYEPIVTEGN EALVHHMEVF QCAAEFRDHP HFSGPCDSKM KPQRLNFCRH
 Human  KYEPIVTKGN EALVHHMEVF QCAPEMDSVP HFSGPCDSKM KPDRLNYCRH
   Rat  MYEAIVTEGN EALVHHMEVF QCTNESEAFP MFNGPCDSKM KPDRLNYCRH 300
                   #4 <————————

                                              ***
Bovine  VLAAWALGAK AFYYPEEAGL AFGGPGSSRF LRLEVHYHNP LVITGRRDSS
 Human  VLAAWALGAK AFYYPEEAGL AFGGPGSSRY LRLEVHYHNP LVIEGRNDSS
   Rat  VLAAWALGAK AFYYPEEAGV PLGSSGSSRF LRLEVHYHNP RNIQGRRDSS 350
                                          #2 <————————
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Figure 1. Alignment using LINEUP program of a portion of DBH amino acid sequence from three mammalian species (bovine, human, rat) in the histidine-rich regions. The numbers to the *right* correspond to amino acid coordinates of the rat DBH. *Asterisks* indicate the paired histidines or the H-X-H residues; *dot* indicates Tyr^{236} of the rat sequence, conserved in all three species. The regions selected for primer design are *underlined* with *arrows* indicating the 5′−3′ direction of the primer.

genotype in a given batch. Each genotype was examined at least three times. Samples were observed on a Bio-Rad MRC600 confocal microscope (Hercules, CA). Samples were viewed under identical conditions with respect to laser level and gain. Images were photographed from the computer screen using similar exposures.

Chromatography. Brains of adult male flies were dissected and fixed immediately and homogenized in ice-cold 0.1 M perchloric acid solution. Samples were centrifuged for 15 min in an Eppendorf at 4°C, and supernatants were kept at −70°C until used for the HPLC analysis. Chromatographic separations were achieved as in Linn et al. (1994), using a Vydac reverse-phase C-18 HS-54–15 HPLC column (15 cm × 4.6 mm inner diameter, 3 μm particles) (Hesperia, CA). The mobile phase contained 70 mM monobasic sodium phosphate, 0.5 μM EDTA, 0.1 mM l-octanesulfonate (sodium salt), 8% methanol, and 2% acetonitrile, and was adjusted to pH 5.5. The mobile phase was run isocratic at 0.85 ml/min. The first electrode was set at a potential of 0.38 V and the second electrode at 0.73 V. Using these potential settings, dopamine and serotonin were detected selectively on channel 1, whereas octopamine, *N*-acetyl octopamine, and tyramine were detected on channel 2. Identification of compounds was based on comparison of retention times with external standards, which were run at the beginning and end of each daily series. Standards were prepared in 0.1 N perchloric acid solution daily. Peak identification also was determined on the basis of changes in retention time or peak area as a function of systematic change in chromatographic conditions, including pH, percentage organics, or applied channel voltage (Linn et al., 1994). Chemicals were purchased from Sigma (St. Louis, MO) except *N*-acetyloctopamine, which was provided by Research Biochemicals (Natick, MA) incorporated as a part of the Chemical Synthesis Program of the National Institute of Mental Health Contract 278-90-0007.

Fly stocks and genetics. The following strains were obtained from the Bloomington, Indiana, *Drosophila* stock center: (1) *p845 (sn[Pw$^+$-lacW], w^{1118})*, (2) *Df(1)sn^{C128}/FM6*, (3) *Df(1)sn^{C128}/C(1)DX y f; Dp (1;2)sn^{+72d}/bw^D*, (4) *y2 sc w sn^{x2} B/Df(1)sxl*, (5) *Cy/Sp; ry^{506} Sb P[ry+Δ2–3]/TM6 Ubx* (see Lindsley and Zimm, 1992). The genetic schemes used to generate local transpositions took advantage of the p845 element, which is a transposition in the *sn* locus. Briefly, virgin *p845 sn* females were crossed to P transposase-bearing males. Male or virgin female progeny were crossed en masse to females or males carrying X chromosome balancer *y^2 sc w sn^{x2} Bar*. Progeny were screened for female or male flies (depending on the F1 cross), which were revertant for the singed phenotype. To establish individual lines, *sn* revertants were mated singly to balancer-carrying flies. Lines that carry the P insertion in the X chromosome were determined based on the cosegregation of the yellow and white markers, and they were maintained. Mutations at the *Tβh* locus that disrupt the gene were found based on loss of TBH protein band in immunoblot analysis of the X-linked lines.

Excisions of the MF372 transposon were done as follows: virgin females from the insertion line were crossed en masse to males of the stock carrying the P transposase. Male progeny of this cross were mated individually to females from the X balancer stock, *FM7a*, and male progeny of the F1 cross were used in immunoblot analysis as above.

Fertility assay at 25°C. Single virgin females, 1–2 d old, were mated to three Canton-S males in unyeasted test tubes containing standard food, and they were allowed to lay eggs for 6 d. At day 6, parents were discarded and progeny were counted at day 17. The number of progeny per female was calculated by dividing the total number of progeny produced by the total number of females assayed (including those that laid no eggs).

Octopamine feeding. Single $T\beta H^{M18}$ females were mated with three Canton-S males in unyeasted test tubes containing standard food. After 5–6 d, the mated females were transferred to food supplemented with different concentrations of octopamine or other biogenic amines used in this study. For initial tests, instant fly food (Carolina Biochemicals) was

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1     GAATTCCGCTAACGG GACAAAAGTCGCGCG CGACTCGTAAGCTTG TTTACTTTGCCATTA TAAATCCGGCGGGCG CTTGTTTGTGTTTGT GTTGATCAGCAGCCA
106   TAGCGAATTAATTTC AAAGTGTTTGGCTAT TAGTGATTAGCGATA AACGATATCCGAGAA CAATAATTCCGCCAC CGATCTGCCGGCCGC GCAATCTCAAATCTC
211   AAAATGCTTAAAATG CCGCTGCAGCTGAGC AGTCAGGATGGCATT TGGCCAGCCCGATCC GCCAGGCGACTCCAT CACCACCACCAACTG GCTTATCATCATCAC

1       M  L  K  M  P  L  Q  L  S  S  Q  D  G  I  W  P  A  R  S  A  R  R  L  H  H  H  H  Q  L  A  Y  H  H  H
316   AAGCAGCAGCAGCAG CAGCAGGCGAAACAG AAACAAAAGCAAAAT GGAGTGCAGCAAGGA CGTTCGCCGACATTT ATGCCAGTGATGCTG CTCCTCCTAATGGCC
35    K  Q  Q  Q  Q  Q  Q  A  K  Q  K  Q  K  Q  N  G  V  Q  Q  G  R  S  P  T  F  M  P  V  M  L  L  L  L  M  A
421   ACACTGCTTACGCGC CCGCTGAGCGCCTTT TCCAACCGCTTATCC GACACAAAGCTGCAC GAGATCTACCTGGAC GACAAGGAGATTAAG CTGAGCTGGATGGTC
70    T  L  L  T  R  P  L  S  A  F  S  N  R  L  S  D  T  K  L  H  E  I  Y  L  D  D  K  E  I  K  L  S  W  M  V
526   GACTGGTACAAGCAG GAGGTGCTCTTCCAC TTGCAGAATGCTTTC AACGAACAGCACCGC TGGTTCTATCTGGGT TTCTCCAAGCGCGGC GGCCTGGCGGATGCG
105   D  W  Y  K  Q  E  V  L  F  H  L  Q  N  A  F  N  E  Q  H  R  W  F  Y  L  G  F  S  K  R  G  G  L  A  D  A
631   GATATTTGCTTCTTT GAGAATCAGAATGGA TTCTTCAATGCGGTA ACCGATACGTACACC AGTCCGGATGGACAG TGGGTGAGACGGGAC TACCAGCAGGACTGT
140   D  I  C  F  F  E  N  Q  N  G  F  F  N  A  V  T  D  T  Y  T  S  P  D  G  Q  W  V  R  R  D  Y  Q  Q  D  C
736   GAGGTCTTCAAGATG GATGAGTTCACGTTG GCGTTTAGGCGCAAG TTTGACACCTGCGAC CCTTTGGATCTGCGA CTCCATGAGGGCACA ATGTACGTGGTTTGG
175   E  V  F  K  M  D  E  F  T  L  A  F  R  R  K  F  D  T  C  D  P  L  D  L  R  L  H  E  G  T  M  Y  V  V  W
841   GCCCGTGGTGAAACG GAACTGGCCCTGGAG GATCACCAGTTCGCT CTGCCCAATGTGACG GCACCGCACGAGGCG GGTGTTAAGATGCTA CAGCTACTACGGGCC
210   A  R  G  E  T  E  L  A  L  E  D  H  Q  F  A  L  P  N* V* T*  A  P  H  E  A  G  V  K  M  L  Q  L  L  R  A
946   GACAAGATACTTATA CCCGAATCCGAGTTG GATCACATGGAGATC ACACTGCAGGAGGCG CCAATTCCCAGTCAG GAGACCACGTACTGG TGTCACGTTCAGCGA
245   D  K  I  L  I  P  E  S  E  L  D  H  M  E  I  T  L  Q  E  A  P  I  P  S  Q  E  T  T  Y* W  C  H  V  Q  R
1051  CTGGAGGGCAATCTC CGGCGTCGCCATCAC ATCGTTCAGTTCGAG CCGCTTATCCGAACG CCGGGCATCGTGCAT CACATGGAAGTGTTT CACTGCGAGGCCGGT
280   L  E  G  N  L  R  R  R  H* H*  I  V  Q  F  E  P  L  I  R  T  P  G  I  V  H* H*  M  E  V  F  H  C  E  A  G
1156  GAGCACGAGGAGATT CCCCTGTATAACGGC GACTGTGAACAGTTG CCGCCACGGGCGAAG ATCTGCTCAAAAGTG ATGGTCCTGTGGGCC ATGGGCGCTGGCACC
315   E  H  E  E  I  P  L  Y  N  G  D  C  E  Q  L  P  P  R  A  K  I  C  S  K  V  M  V  L  W  A  M  G  A  G  T
1261  TTTACCTATCCTCCG GAAGCCGGTCTACCA ATCGGCGGACCCGGC TTCAATCCGTACGTT CGACTGGAGGTACAT TTCAATAATCCGGAG AAGCAGTCGGGCTTG
350   F  T  Y  P  P  E  A  G  L  P  I  G  G  P  G  F  N  P  Y  V  R  L  E  V  H  F  N  N  P  E  K  Q  S  G  L
1366  GTGGACAACTCCGGC TTTCGCATCAAGATG TCGAAGCACTGCGTC AGTATGACGGCCGTT ATGGAACTGGGCCTG GAGTACACCGACAAA ATGGCCATTCCGCCT
385   V  D  N  S  G  F  R  I  K  M  S  K  H  C  V  S  M  T  A  V  M  E  L  G  L  E  Y  T  D  K  M  A  I  P  P
1471  GGCCAAACCGCATTC CCGCTGAGCGGCTAT TGTGTGGCGGACTGC ACACGAGCCGCTCTG CCGGCGACGGGCATC ATCATCTTTGGCTCT CAGCTGCATACGCAT
420   G  Q  T  A  F  P  L  S  G  Y  C  V  A  D  C  T  R  A  A  L  P  A  T  G  I  I  I  F  G  S  Q  L  H* T* H*
1576  CTGCGTGGCGTTCGC GTCCTAACCCGACAC TTTCGCGGCGAACAG GAGCTGCGCGAGGTG AACCGCGATGACTAC TACTCGAATCACTTC CAGGAGATGCGCACC
455   L  R  G  V  R  V  L  T  R  H  F  R  G  E  Q  E  L  R  E  V  N  R  D  D  Y  Y  S  N  H  F  Q  E  M  R  T
1681  CTGCACTACAAGCCG CGTGTCCTGCCCGGC GACGCTTTGGTAACC ACTTGCTACTACAAC ACCAAGGATGACAAG ACCGCCCTCGGCGGA TTCTCCATCAGCGAT
490   L  H  Y  K  P  R  V  L  P  G  D  A  L  V  T  T  C  Y  Y  N  T  K  D  D  K  T  A  L  G  G  F  S  I  S  D
1786  GAGATGTGCGTCAAC TATATCCACTACTAT CCGGCCACCAAACTG GAGGTCTGCAAGAGT TCCGTTTCCGAGGAG ACGCTCGAGAATTAC TTTATTTACATGAAG
525   E  M  C  V  N  Y  I  H  Y  Y  P  A  T  K  L  E  V  C  K  S  S  V  S  E  E  T  L  E  N  Y  F  I  Y  M  K
1891  CGCACGGAGCATCAG CATGGCGTGCATTTG AATGGAGCCAGGTCG TCCAATTACCGGAGC ATCGAATGGACCCAG CCGCGTATCGATCAG CTGTACACCATGTAC
560   R  T  E  H  Q  H  G  V  H  L  N  G  A  R  S  S  N  Y  R  S  I  E  W  T  Q  P  R  I  D  Q  L  Y  T  M  Y
1996  ATGCAGGAGCCGCTG AGCATGCAGTGCAAC AGGTCCGATGGCACT CGCTTCGAGGGGCGG TCTAGCTGGGAGGGC GTGGCTGCGACGCCC GTACAAATTCGCATA
595   M  Q  E  P  L  S  M  Q  C  N* R* S* D  G  T  R  F  E  G  R  S  S  W  E  G  V  A  A  T  P  V  Q  I  R  I
2101  CCCATTCACCGCAAA CTGTGCCCCAACTAC AATCCGCTGTGGCTG AAGCCATTGGAGAAG GGCGATTGCGATTTG CTGGGGGAGTGCATC TATTAGGGCGCCGT

630   P  I  H  R  K  L  C  P  N  Y  N  P  L  W  L  K  P  L  E  K  G  D  C  D  L  L  G  E  C  I  Y
2206  ACATTAGGCATTAGG CGAATAGGCGAATGG GGGCGTGGCATAGCA CACACTCACACTCAC ACCCACACCCACACA CACACACACATACAG CACATACAATACACG
2311  CGCACGGCATGGATA ATATGAGAATTTCGA TTTCAACAGTCGCCT ATGCAAAAGCTAAAC TCATTCACGTCTATT TTGAAAGCTTAACTC ATGAATACTTTTGAA
2416  TATTTAGCAAAAAAA AGCTTGAAAACAGAT CTAAAAACATTTAAA AAAAGGTTATTTTAC TCCTATTTTGGTTAG TTAGTTCTTACTAAC AGTAACTAACTGATA
2521  GCCTAAATGTTTCTG TTGACTTTACTTTGA AATTCGCAACAGAAA TCAGAGGAATCACTT TTTTTTGTTTTGTT TTTTACGCCTTATGG AAGTTTGACTGTAGT
2626  CGCTTTGTATTTTAC CCTTTATGTGCATGG TCATTAGCATTATCA ACGCGTTTGTATGTG TGCGTGTGTGTGTGT GTGTGTGTGTGTAAG GGCAACAGGGCAAAA
2731  CAAATGTTTTCACTC TTTAAATAACTAATG CATTTACCAATCCTG CCCATGTTGTTCACC CTTTTTACTTTCACT TTCACTTTGCTTTCG CCTTTGACTTTGTTT
2836  GTGCCCTTTGCCCTT GTTGTTTTGGCCAGC CTTTGAAATTTGCCG ACTGACGGAATTC
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B

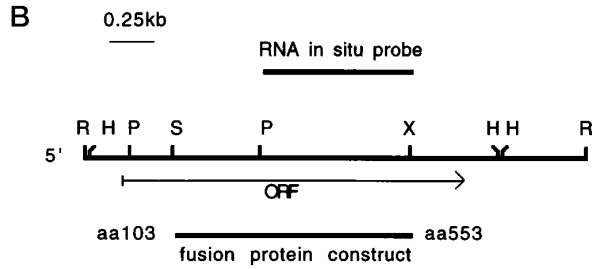

Figure 2. *A*, Nucleotide and deduced amino acid sequences of *Drosophila Tβh* cDNA (EMBL Data Library number Z70316). Amino acid residues in *bold* indicate paired histidines or the H-X-H sites. Residues in *bold* with an *asterisk* correspond to those amino acids of the bovine DBH protein modified by mechanism-based inhibitors (see text, Fig. 3). Residues in *bold italics* correspond to consensus for potential N-glycosylation sites, and *underlined* tetrads of amino acids correspond to potential phosphorylation sites. The stop codon is *underlined*. *B*, Restriction map of the *Drosophila Tβh* cDNA. Restriction sites: *H*, *Hin*dIII; *P*, *Pst*I; *R*, *Eco*RI; *S*, *Sal*I; *X*, *Xho*I. The open reading frame region of the cDNA is indicated by the *arrow below* the restriction map. The fragments used for the RNA *in situ* probe and the fusion protein construct are represented with *solid lines above* and *below* the restriction map, respectively. The first and last amino acid included in the fusion protein construct are indicated at the end of the corresponding line.

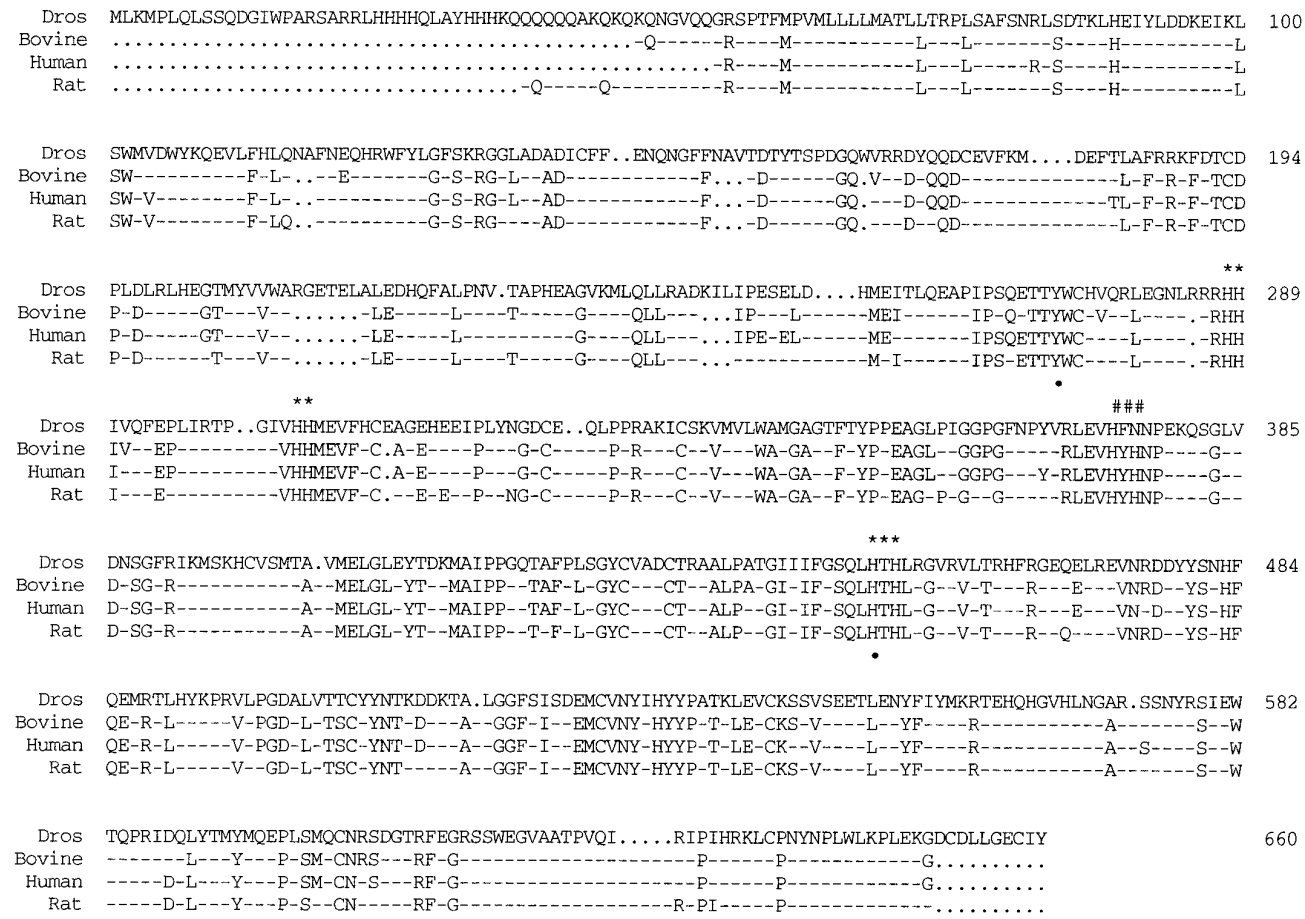

Figure 3. Alignment using LINEUP program of *Drosophila* TBH protein sequence to the DBH protein from three mammalian species. The *top line* corresponds to the *Drosophila* TBH amino acids 1–660. Only the identical amino acids are shown, whereas gaps are marked with *dots* and nonconserved residues are marked with *dashed lines*. *Asterisks* indicate conserved His-His or His-X-His residues, and # indicates an His-X-His site of the mammalian DBH not conserved in the *Drosophila* TBH. The residues Tyr^{273} and His^{452} (TBH coordinates), which correspond to those modified by mechanism-based inhibitors (DeWolf et al., 1988, 1989), are indicated with a *dot below* them.

used; in subsequent tests, standard sucrose-inactivated yeast/agar fly food was used. Initial tests were done with 50, 25, 10, and 4 mg/ml of octopamine to determine the optimal concentration. In all subsequent tests, a concentration of 10 mg/ml was used. Dopamine, norepinephrine, and tyramine were used to supplement the food at concentrations of 20 and 10 mg/ml. At a minimum, 10 flies were tested for all the drugs used. Flies were removed on day 6 after transfer and progeny counted at day 17 after transfer. The number of progeny produced was divided by the total number of females tested.

RESULTS

Cloning and sequencing of a *Dopamine β-hydroxylase* (*DβH*)-like *Drosophila* gene

As a first step toward identifying the *Tyramine β-hydroxylase* gene of *Drosophila*, we cloned *Drosophila* sequences with homology to mammalian *Dopamine β-hydroxylase genes* using PCR technology. We used degenerate oligonucleotide primers designed for the amino acid regions implicated in the function of the protein such as copper-binding sites and the putative active center (discussed in McMahon et al., 1990). In these regions, the amino acid sequences of the human, rat, and bovine DBH are 100% identical (Fig. 1). A low-stringency PCR reaction using genomic DNA as a template and primers 1 and 2 yielded an amplified 328 bp product. When the 328 bp product was used as a template along with primers 1 and 4, a 120 bp band was amplified. This was the expected size band, assuming spacing conservation between the corresponding amino acid residues of primers 1 and 4 in mammalian and *Drosophila* proteins and absence of intronic sequence in the *Drosophila* genomic DNA. A 120 bp band also was produced when we used primers 3 and 4 in the initial PCR reaction and in a follow-up reaction, primers 1 and 4. The 120 bp product was subcloned, and 10 independent clones were sequenced. Only two of these, which contained the same insert in opposite orientations, were flanked by sequences corresponding to primers 1 and 4 at either ends, and these also included an open reading frame of about 40 amino acids. A protein data bank search revealed these to be most homologous to a 40 amino acid region of known mammalian DBH sequences.

Armed with this 120 bp probe, we screened a *Drosophila* head cDNA library (Itoh et al., 1985) and isolated one cDNA clone out of 2×10^5 clones. This cDNA was sequenced completely from both strands (Fig. 2) and found to be 2895 nucleotides in length and with a long open reading frame (ORF) from nucleotide 172 to 2193. The first ATG codon of the ORF at position 214 is likely to encode the initiator methionine, because the sequence preceding it exhibits a perfect match with the *Drosophila* initiation codon consensus C/A A A A/C (ATG) (Cavener, 1987). The stop codon at position 2194–2196 is followed by stop codons in all three frames, and a 702 nucleotide long 3′ untranslated region, yet polyA tail is not included. The deduced translation product of this

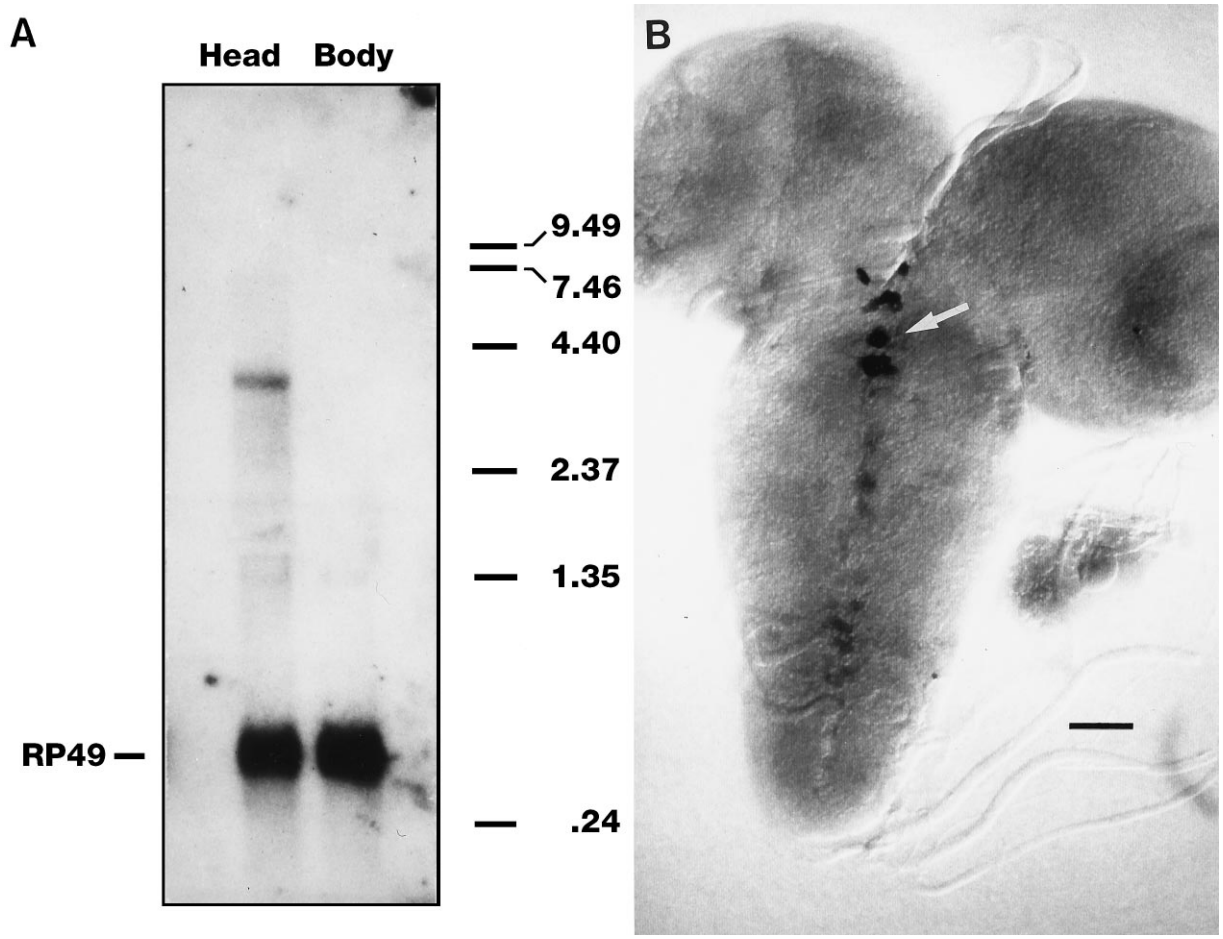

Figure 4. Tβh transcript analysis. *A*, Total Canton-S RNA (12 μg/lane) from adult head and body tissue was hybridized with a ^{32}P-labeled RNA probe corresponding to the *Pst–Xho* fragment of *Tβh* cDNA and subsequently with a ^{32}P-labeled DNA probe of RP49. The size of the transcript was determined relative to RNA markers (RBL) shown on the *right*. *B*, Cellular distribution of the *Tβh* transcript. Confocal image of whole-mount third instar larval CNS hybridized with a digoxigenin-labeled DNA probe corresponding to the *Pst–Xho* fragment of the *Tβh* cDNA. Anterior is to the top. The CNS is composed of the paired brain lobes and a fused ventral ganglion composed of the subesophageal ganglion, thoracic ganglion, and abdominal ganglion (see legend to Fig. 6*C*). The *in situ* hybridization signal as detected with an alkaline phosphatase-conjugated antidigoxigenin antibody was present in the ventral ganglion in a cluster of cells in the subesophageal ganglion (*white arrow*) and in cells along the midline of the thoracic and abdominal ganglia. Scale bar, 50 μm.

ORF, assuming translational initiation at ATG^{214}, is a 660 amino acid polypeptide, with a predicted molecular mass of ~76,000.

Two potential N-glycosylation sites, Asn-X-Ser/Thr (Hubbard and Ivatt, 1981) (Fig. 2*A*) were found at Asn^{227} and Asn^{604}. In addition, four potential calmodulin-dependent protein kinase phosphorylation sites, Arg-X-Y-Ser/Thr (Pearson et al., 1985) (Fig. 2*A*) were found at Ser^{77}, Thr^{58}, Thr^{214}, and Thr^{462}. Finally, among the 31 histidine residues found in the deduced amino acid sequence, there are two paired histidines at positions 288/289 and 304/305 and one His-X-His at position 452–454 (Fig. 2*A*). Such closely spaced histidines have been reported in binding sites for copper in copper-binding proteins (Sigel, 1981), and they also have been found in the mammalian DBH proteins (Lamouroux et al., 1987; McMahon et al., 1990).

Sequence comparisons

Comparison of the deduced amino acid sequence with protein sequences in data bases using the GENIFRO experimental BLAST Network Service (Altschul and Lipman, 1990) revealed highest score of homology to the bovine, human, and rat DBH proteins. Figure 3 shows alignment of the *Drosophila* amino acid sequence with mammalian sequences using multiple sequence alignment program, LINEUP. The overall identity between the *Drosophila* protein and the mammalian DBH proteins is 39%, and the similarity is 59% with inserted gaps. However, certain regions exhibit greater conservation; e.g., residues 404–519 and 325–392 (*Drosophila* protein coordinates) show 55% and 47% identity and 68% and 63% similarity, respectively.

Several amino acid residues have been reported to be important for the function of DBH protein. These residues are conserved between the mammalian and the *Drosophila* proteins, indicating an evolutionary relationship. One stretch of six conserved amino acids (270–275) and another of seven (448–454) include Tyr^{273} and His^{452}, respectively. These are residues that have been identified as putative active sites for the bovine DBH using mechanism-based inhibitors (DeWolf et al., 1988, 1989). In addition, the two paired histidines (288–289 and 306–307) and the His-X-His residue (453–455), which are likely to be involved in copper binding, also are conserved. However, a second His-X-His residue present in the mammalian proteins is not conserved in the *Drosophila* protein (374–376). It also should be pointed out that

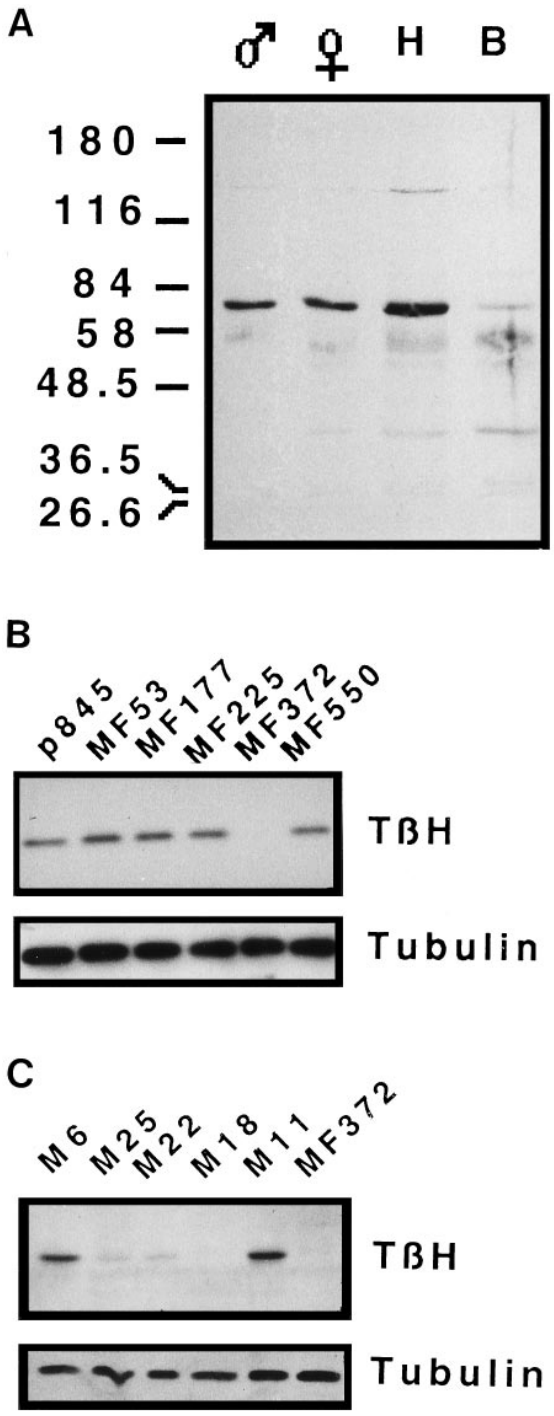

Figure 5. Immunoblot analysis of wild-type and mutant lines. Affinity-purified anti-TBH antiserum was used as the primary antibody; signal was visualized by chemiluminescent detection. *A*, Immunoblot analysis of head and body homogenates from Canton-S male and female flies. Protein (30 μg) from each sample was analyzed by SDS-PAGE on a 7.5% gel. *B*, TBH immunodetection in a sample of lines produced by the P element mutagenesis. Protein, equivalent of one head homogenate, from the original p845 line (insert in the *sn* locus) and five independent new insertion lines was loaded. To control for sample loading, the blot was stripped and reprobed using an anti-Tubulin antibody. A total of 250 lines were screened by this method. Note the lack of signal in MF372 lane. *C*, TBH immunodetection in a sample of lines in which the transposon in MF372 was excised. The procedure was the same as in *B*. A total of 67 lines were screened. Note the lack of signal in M18 and MF372 lanes and normal signal in M6 and M11 lanes.

the spacing between the above regions is almost the same in the *Drosophila* and the mammalian proteins. Finally, the two glycosylation sites also are conserved.

Northern analysis and RNA *in situ*

Total RNA from adult head and body tissues was hybridized with a riboprobe created from the antisense strand of the 870 bp *Pst–Xho* fragment of the cDNA (Fig. 2*B*). A single transcript of ~3.4 kb, which appeared to be head enriched, was detected in the adult heads (Fig. 4*A*). The absence of signal in the body does not imply that there is no transcript in the body, but just that it is at a much lower concentration than in the head.

We studied the *in situ* distribution of the transcript in whole-mount third instar larval CNSs, using a digoxigenin-labeled DNA probe synthesized for the *Pst–Xho* fragment (Fig. 2*B*). A discrete expression pattern consisting of a small population of the larval CNS cells localized exclusively in the midline of the ventral ganglion was revealed (Fig. 4*B*). The pattern comprises a large cluster of cells at the midline of the subesophageal ganglion and unpaired cells in the midline of the thoracic and abdominal ganglia. In contrast to the ventral ganglion, the brain lobes were devoid of any signal. In the larval CNS, cell-specific transcript localization pattern shows a remarkable correlation with the previously described octopamine-immunoreactive neuronal pattern (Monastirioti et al., 1995). This strongly supports the identification of the gene we have isolated as the gene that encodes TBH. We will, therefore, refer to this gene as *Tβh* and its protein product as TBH.

TBH immunoreactivity resembles octopamine immunoreactivity

Polyclonal anti-TBH serum was raised in rats against a bacterially expressed purified internal part of the protein (*Sal–Xho* fragment) (Fig. 2*B*) and was affinity purified as described in Materials and Methods. In immunoblots, the affinity-purified antibody revealed a single band corresponding to the predicted 76 kDa protein. Comparison of protein extracts from adult heads and bodies shows that TBH is enriched in the head, but it is also present in the body at lower levels (Fig. 5*A*).

Immunocytochemical analysis of the larval CNS using the affinity-purified antibody showed that the protein is detected in cell bodies of the ventral ganglion in a pattern that correlates with the RNA expression pattern. A large group of intensely stained cells is detected in the ventral midline of the subesophageal ganglion, and two to three cells are stained in the midline of each of the thoracic and abdominal neuromeres (Fig. 6*A*). In addition, pairs of paramedial cells (*arrows* in Fig. 6*A*) were detected in the three thoracic neuromeres and in the first abdominal neuromere. Intense staining also was detected in the neuropil. Immunoreactive fibers travel along both sides of the midline and extend from the ventral ganglion to the central brain lobes. Transverse fibers also are detected between the brain hemispheres extending to the center of each lobe where they form an immunoreactive focus (Fig. 6*A*).

The overall cellular and neuropil expression of TBH in the larval CNS shows a striking similarity to the octopamine immunoreactivity pattern (Monastirioti et al., 1995, their Fig. 1). However, more cell bodies in the ventral midline of the abdominal neuromeres seem to be TBH-immunoreactive than were observed to be octopamine-immunoreactive. It is possible that octopamine levels in the extra cells were below detectable levels or that the TBH is expressed in additional cells. Because of specific and

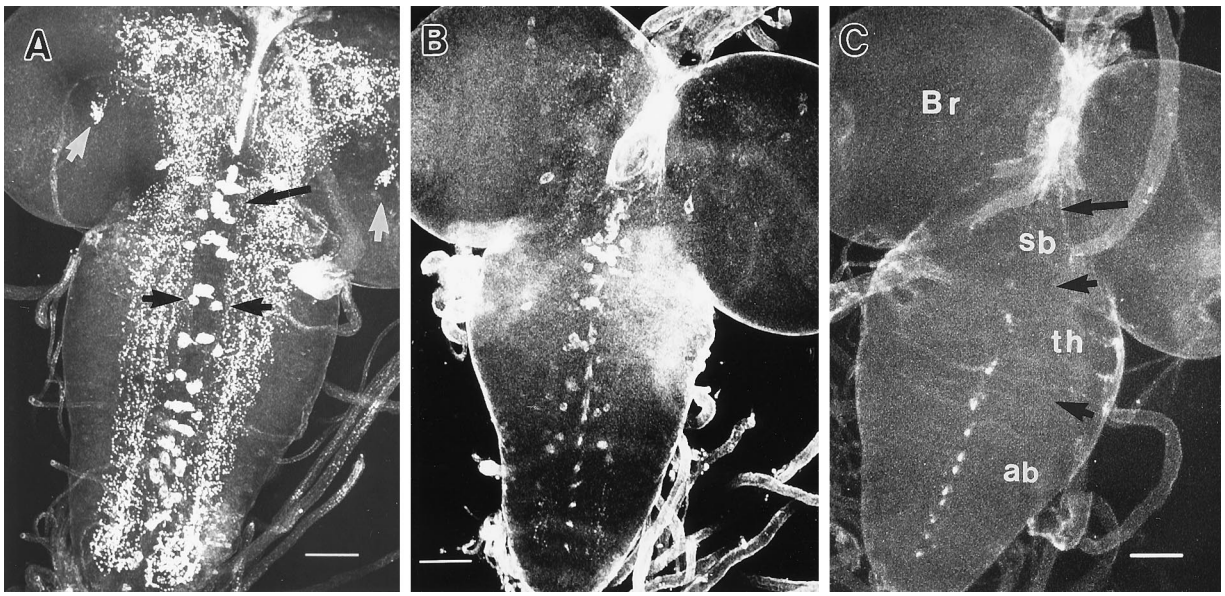

Figure 6. TBH immunoreactivity in representative samples of larval CNSs from wild-type (Canton-S) and *Tβh* mutant strains. Third instar larval CNSs were immunoreacted with affinity-purified anti-TBH antiserum primary antibody and an anti-rat FITC-conjugated secondary antibody. These images were collected as Z series at the same laser level and gain settings. Care was taken to photograph and develop the negatives using similar conditions, but the images from the mutant brains were developed longer to show the residual signal. Each image represents a superimposition of 8 to 10 confocal sections taken at a Z step of 2.16 μm. In all images, the anterior is to the top. *A*, Confocal image of wild-type larval CNS. Note the intense immunoreactivity in the neuropil and the characteristic foci (*white arrows*), one in each brain lobe. In the ventral ganglion, note the cells in the subesophageal region (*black arrow*) and along the midline in the thoracic and abdominal ganglia and the paramedial cells (*black arrowheads*). *B*, Confocal image of a larval CNS of the $T\beta h^{MF372}$ mutant. Note the near absence of the neuropil staining and the reduced signal in the TBH-immunoreactive cells compared with the wild type. (Because of the low TBH signal, the background signal is high). *C*, Confocal image of a larval CNS from the $T\beta h^{nM18}$ excision mutant. Note the complete absence of immunoreactivity from both the neuropil and the TBH-expressing cells. *Black arrow* points to the subesophageal ganglion; *black arrowheads* delineate thoracic ganglia. (We believe that the dotted pattern along the midline of the ventral ganglion is caused by nonspecific staining, because we have observed such staining in dorsal midline using other antibodies, especially when the antibody concentration is high. This pattern is confined to the dorsal-most sections, which are not included in the wild-type CNS representation in *A*. *Br*, Brain lobes; *sb*, subesophageal ganglion; *th*: thoracic ganglion; *ab*: abdominal ganglion. Scale bar, 50 μm in *A–C*.

incompatible fixation conditions necessary for each antigen [formaldehyde for TBH, high percentage (6.25%) of glutaraldehyde for octopamine], we have been unable to detect both octopamine and TBH antigens simultaneously.

Generation of hypomorphic and null *Tβh* mutants

In situ hybridization of the cDNA clone to third instar larval polytene chromosomes and preliminary molecular characterization of the genomic DNA (data not shown) localize the gene to 7D1–2 region on the X chromosome between the *sn* and *l(1)mys* loci (Fig. 7).

Our strategy to generate *Tβh* mutants did not presuppose any specific phenotypic consequence. We used P element-associated "local transposon jumps" methodology (Tower et al., 1993; Zhang and Spradling, 1993) as a mutagen and screened the putatively mutagenized chromosomes by assaying for TBH in immunoblots (Dolph et al., 1993). When P elements are mobilized, there is a high incidence of local transposon hops that can create insertions and possible disruption in the neighboring genes (Cooley et al., 1988). A fertile P element insertion line, p845, which carries an insertion of the synthetic transposon PlacW (Bier et al., 1989) in the 5′ end of the *sn* gene, causing a singed phenotype, was available (K. O'Hare, personal communication) (Fig. 7). We mobilized this P element using the Δ2–3 transposase and isolated new insertions that are accompanied by reversion at the *sn* locus (details of the mutagenesis to be described elsewhere). Approximately 250 lines carrying new insertions on the X chromosome were screened for TBH protein in immunoblots. A representative immunoblot (Fig. 5*B*) presents the results from six lines, including line MF372 in which TBH protein was not detected. Southern analysis showed that the P transposon has been inserted in the genomic region of the *Tβh* gene, 3′ to a genomic *Eco*RI fragment containing the ATG (data not shown).

TBH immunoreactivity in the third instar larval MF372 CNS was much reduced in the cell bodies, although the pattern was similar to the wild type, and neuropil was devoid of any signal (Fig. 6*B*). This suggested that MF372 transposon insertion in the *Tβh* locus has caused a hypomorphic mutation, $T\beta h^{MF372}$.

To generate null *Tβh* mutants, excisions of the P transposon insertion in $T\beta h^{MF372}$ were created by using the Δ2–3 transposase.

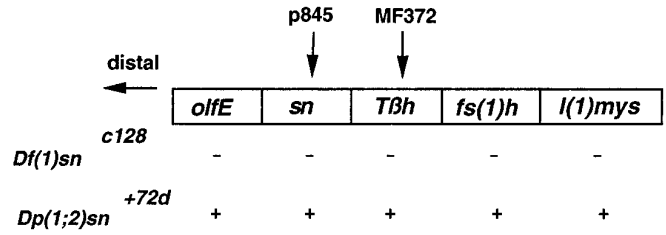

Figure 7. Genetic map of the 7D1–2 region of the X chromosome. Four known loci besides *Tβh* are represented by *boxes*. The *signs below* each box indicate the loci uncovered by $Df(1)sn^{C128}$ (−) or covered by the $Dp(1;2)sn^{+72d}$ (+), two of the chromosomal rearrangements corresponding to the area. *Arrows* point to the position of p845 and MF372 transposon insertions. *olfE*, Olfactory E; *sn*, singed; *Tβh*, *Tyramine β-hydroxylase*; *fs(1)h*, female sterile homeotic; *l(1)mys*, lethal (1) myospheroid.

Table 1. Amine concentrations (pg/brain)

	Octopamine	Tyramine	*N*-acetyl-octopamine	Dopamine	Serotonin
Canton-S	263.3	7.6	23.3	80.7	66.2
$T\beta h^{nM18}$	<0.5	79.5	<0.5	80.3	65.2
$T\beta h^{MF372}$	7.6	60.6	<0.5	104.1	63.8
$T\beta h^{rM6}$	275.3	11.8	18.0	94.3	53.8
$T\beta h^{rM11}$	150.5	11.9	15.7	85.2	80.8

Numbers represent the mean value of three independent experiments.

Individual lines from 62 independent excision chromosomes were established and screened for disruption of the *Tβh* locus by assaying protein levels in immunoblots. Excision events can be grouped in three classes with a distinct phenotypic outcome in each case. First, many excisions are precise and restore the gene and the phenotype. In these cases, wild-type TBH signal is observed (51/67), as in lines M6 and M11 (Fig. 5*C*). Second, in some excision events, the element is excised only partially, which may result either in the same phenotype or in one distinct from the original insertion. Examples are lines M22 and M25, which show reduced TBH signal (Fig. 5*C*). Finally, some imprecise excisions also delete flanking genomic sequences resulting in disruption of the gene, creating protein nulls such as line M18 (Fig. 5*C*). Larval CNSs from lines M18, the putative protein null, and M6, the revertant to wild type, then were checked immunocytochemically. In contrast to the initial MF372 insertion line, TBH immunoreactivity was not detected in the larval CNS of the M18 line (Fig. 6*C*), whereas staining in the M6 line was indistinguishable from the wild type (data not shown). These data suggest that M18 represents a null mutation, $T\beta h^{nM18}$, and that M6 is a revertant ($T\beta h^{rM6}$) of the $T\beta h^{MF372}$ hypomorphic mutation.

Tβh-null mutants are devoid of octopamine and accumulate tyramine

Null mutations of the *Tβh* gene should block conversion of tyramine to octopamine and, thus, eliminate octopamine from the fly brain while perhaps accumulating tyramine. To assess levels of octopamine, its precursor tyramine, and its metabolite *N*-acetyloctopamine, brain extracts of mutants, null $T\beta h^{nM18}$, hypomorph $T\beta h^{MF372}$ and control flies, Canton-S, and revertants $T\beta h^{rM6}$ and $T\beta h^{rM11}$ were analyzed by HPLC with electrochemical detection (HPLC-ECD) (Table 1). For comparison, we also measured dopamine and serotonin in the same extracts. In the wild-type (Canton-S) brains, the amount of octopamine measured was ~263 pg/brain; the values in the revertants were comparable at 275 and 150 pg/brain. However, an octopamine peak was not detected in the null mutant $T\beta h^{nM18}$, and very little octopamine; ~7.6 pg/brain was found in the hypomorph $T\beta h^{MF372}$. Interestingly, a six- to eightfold increase in tyramine was observed in the null $T\beta h^{nM18}$ and the hypomorph $T\beta h^{MF372}$ (Table 1) compared with the normal level of ~8 pg/brain. In addition, the metabolite *N*-acetyloctopamine was not detected (<0.5 pg) in the mutant lines. No significant differences in dopamine and serotonin levels were detected. Norepinephrine was not detected in either wild-type or mutant extracts (data not shown).

The HPLC results are consistent with the biochemical expectation that loss of tyramine hydroxylation in *Tβh*-null animals will result in absence of octopamine and accumulation of tyramine.

Tβh-null females retain eggs

Mutant *Tβh* flies survive to adulthood; their external appearance is normal, and they do not exhibit any obvious defects. Under optimal growing conditions, their viability is comparable to their heterozygous siblings, but under unfavorable, crowded conditions, their viability is reduced. Mutant males are fertile, but the mutant females are sterile. These females appear to mate normally; they produce fully developed ovarioles that become abnormally large within a few days of eclosion as eggs are retained.

To further investigate the sterility and egg retention observed in the mutant females, and to ascertain that the defects were a direct consequence of the genetic lesion at the *Tβh* locus, individual females of different genotypes were assayed for number of progeny produced over a defined time. The data are tabulated in Table 2, the genetic limits of Deficiency ($Df(1)sn^{C128}$) and Duplication ($Dp(1;2)sn^{72d+}$) chromosomes that uncover and cover *Tβh* are explained in Figure 7. The following observations support association of the egg-retention defect with the lesion at the *Tβh* locus. (1) During a 6 d assay period, $T\beta h^{nM18}$-null mutant females or $T\beta h^{nM18}$/*Deficiency* females did not produce any progeny. (2) Flies of the genotype $T\beta h^{nM18}$/*Deficiency*/*Duplication* were fully fertile. (3) Females homozygous for the insertion chromosome $T\beta h^{MF372}$ showed near normal fertility, but $T\beta h^{MF372}$/*Deficiency* females showed very low fecundity. The decrease in fertility of the $T\beta h^{MF372}$/*Deficiency*-bearing females suggests a gene–dosage effect on the expression of the phenotype. (4) Females carrying the revertant chromosomes $T\beta h^{rM6}$ and $T\beta h^{rM11}$ are fully fertile. These revertants are important controls, because they have wild-type TBH protein levels and normal fertility.

Octopamine feeding induces egg-laying in *Tβh* mutant females

To check whether the egg-retention defect is caused by octopamine deficit, 6-d-old mated mutant $T\beta h^{nM18}$ females were transferred to food supplemented with different concentrations of

Table 2. Fertility of females at 25°C

Female genotype	No. tested	% Fertile females	# Progeny/female −OA	# Progeny/female +OA
w $T\beta h^{nM18}$	16	0	0	12
w $T\beta h^{nM18}/Df(1)sn^{C128}$; bw^{D}	10	0	nd	nd
w $T\beta hn^{M18}/Df(1)sn^{C128}$; $Dp(1;2)\ sn^{l+72dJ}$	39	100	55	nd
w $T\beta hr^{M6}$	37	100	44	nd
w $T\beta hr^{M11}$	25	100	52	nd
w $T\beta h^{MF372}$	34	100	36	nd
w $T\beta h^{MF372}/Df(1)sn^{C128}$	27	70	4	nd

Individual females were tested for fertility, and number of progeny per female was calculated as explained in Materials and Methods. −*OA*, no octopamine supplement; +*OA*, octopamine supplement; *nd*, not done.

octopamine and allowed to lay eggs for 6 d, and progeny counted on day 17 from the transfer. Females transferred to 4–10 mg/ml octopamine produced low but significant numbers of progeny (average 12 progeny per fly). Higher concentrations (25–50 mg/ml) of octopamine induced egg-laying, but the mutant females died within a few hours. No progeny was produced when food was supplemented with 10 mg/ml of tyramine or dopamine. However, norepinephrine had the same effect as octopamine at 10 mg/ml. Thus, we can conclude that the sterility of *Tβh* mutant females is a direct consequence of octopamine deficit resulting from the disruption of the *Tβh* locus.

DISCUSSION

The experiments described in this paper represent the initial step toward our ultimate goal to understand the role of octopamine in insect behavior and physiology. We undertook the molecular identification of the *Drosophila* gene that encodes TBH, the enzyme that catalyzes the final step in the synthesis of octopamine, using an approach based on the supposition that TBH is related evolutionarily to mammalian DBH. TBH, the protein product of the gene we identified, showed ~39% overall identity to the mammalian DBH protein. The functional similarity between these two proteins was underscored further by the high conservation around Tyr^{273} and His^{452} (TBH sequence), the two residues that have been identified as putative active sites of the bovine DBH (DeWolf et al., 1988, 1989), and by the conservation of the paired histidine residues that are important for copper binding. An antibody generated against TBH demonstrated that TBH expression in the nervous system closely resembles the octopamine immunoreactivity (Monastirioti et al., 1995). Thus, the immunocytochemical localization was consistent with the notion that TBH, as part of the octopamine biosynthetic machinery, should be localized in the same cells as octopamine. TBH immunoreactivity is detected in both neuronal cell bodies and in neuronal processes.

A genetic strategy was developed to create mutations at the *Tβh* locus that did not depend on "a priori" knowledge of the phenotype other than a decrease or absence of TBH protein. A P transposon insert in the *Tβh* locus was recovered initially ($T\beta h^{MF372}$) using local transposon hops (Tower et al., 1993; Zhang and Spradling, 1993), and precise and imprecise excisions of this insert were subsequently induced. Based on immunoblot and immunocytochemical analysis, we had the following genotypes on the same parental chromosome: (1) $T\beta h^{MF372}$, a hypomorphic mutation that reduces TBH level dramatically, (2) $T\beta h^{rM6}$, a revertant that restores the TBH levels completely, and (3) $T\beta h^{nM18}$, a null mutation that results in flies devoid of any TBH protein.

We measured the levels of octopamine, tyramine, *N*-acetyloctopamine, dopamine, and serotonin in brain extracts of mutant and wild-type flies. Several conclusions regarding the *Tβh*-null flies can be drawn from this analysis. First, *Tβh*-null flies have octopamine levels below detection capability, but the relatively low wild-type levels of tyramine are increased by approximately eightfold. Second, *N*-acetyloctopamine, a metabolite of octopamine, also is below the level of detection. Third, dopamine and serotonin are not affected significantly. Therefore, the two major factors that could impact the behavior and physiology of *Tβh*-null mutants are the absence of octopamine and increased levels of tyramine. Although low levels of tyramine are present in insect nervous systems, at present its function is not known. However, pharmacological studies using crude membrane preparations from *Drosophila* have suggested tyramine as a partial agonist for octopamine receptors (Uzzan and Dudai, 1982). In fact, in the case of a cloned *Drosophila* octopamine/tyramine receptor, tyramine has been shown to be more potent than octopamine when assayed in binding studies or as an inhibitor of adenylate cyclase activity in stably transfected mammalian cells (Saudou et al., 1990; Robb et al., 1994). Although the endogenous ligand for this receptor in *Drosophila* has not been identified, its existence and pharmacological properties suggest that a severalfold increase in the tyramine level could have a physiological effect. Moreover, because TBH is transported in neuronal processes, in the null mutants, TBH-containing vesicles must accumulate tyramine, which in all likelihood will be released at the synapse.

Flies devoid of octopamine eclose, and the eclosed adults, in general, appear normal. They are able to walk, fly, and mate. The viability of these flies suggests that octopamine is not essential in any vital physiological function. However, we cannot rule out that an increased level of tyramine may substitute functionally for octopamine in this genotype. The reduced viability of *Tβh* nulls under crowded conditions suggests that in the wild, these flies would be severely handicapped.

Homozygous *Tβh*-null females retain fully developed eggs. This functional deficit is correlated directly with the null mutation at the *Tβh* locus for the following reasons. (1) Both homozygous females carrying the null mutation $T\beta h^{nM18}$, and hemizygous females $T\beta h^{nM18}/Df(1)sn^{C128}$ produce no progeny, whereas 100% of the examined females having the above hemizygous phenotype as well as the duplication chromosome $Dp(1;2)sn^{72d}$ produce progeny. (2) Revertant females, $T\beta h^{rM6}$ and $T\beta h^{rM11}$, produce normal numbers of progeny. (3) Homozygous females carrying the hypomorphic mutation $T\beta h^{MF372}$ do produce progeny, but hemizygous $T\beta h^{MF372}/Df(1)sn^{C128}$ females show impaired fertility, because they produce reduced numbers of progeny.

The cause of the reduced fertility appears to be retention of eggs in the females, although some role in the late stage of egg maturation cannot be ruled out. This retention could result from absence of octopaminergic input in some process essential in transit of the egg from the ovarioles into the ovipositor via the oviduct and/or extrusion of the egg by the ovipositor. An obvious possibility is that octopaminergic input is important in myogenic contractions; however, that octopamine may have a role in some other as yet undefined physiological process necessary for generating a viable fertilized egg cannot be discounted. Studies in other insects, locusts for example, have shown that myogenic contractions of the visceral muscles of the oviduct and ovipositor, which appear to be under neurohormonal control, are responsible for moving eggs along to the ovipositor in an orderly fashion and for egg extrusion (Lange and Orchard, 1984; Lange et al., 1984). Furthermore, it would appear that different regions of the oviduct will exhibit distinct contractile patterns depending on whether eggs are pushed, retained, or extruded. Octopamine has been implicated in the modulation of oviductal visceral muscle by two lines of evidence: innervation of the oviductal muscle by octopaminergic median unpaired neurons (Kalogianni and Pflüger, 1992), and physiological evidence that octopamine modulates activity of the oviductal muscle (Kalogianni and Theophilidis, 1993). To our knowledge, there is no physiological evidence of the effect of octopamine on the ovipositor; however, modulation of the sting response by octopamine has been demonstrated in the honey bee, *Apis mellifera* (Burrell and Smith, 1995).

The egg retention observed in $T\beta h^{nM18}$ females can be con-

nected directly with the absence of octopamine, because feeding octopamine to females is sufficient to induce egg deposition. Because feeding tyramine has no effect, one can conclude that at least the receptors involved in this behavior are octopamine-specific and tyramine is not a potent agonist. On the other hand, noradrenaline is an agonist, because it too can induce egg laying. Indeed, earlier pharmacological studies support the idea that noradrenaline binds to octopamine receptors and causes at least stimulatory effects in adenylate cyclase activity, similar to that of octopamine (Uzzan and Dudai, 1982). However, noradrenaline cannot be detected by HPLC either in wild-type or *Tβh* mutant brain preparations (C.E. Linn, unpublished observations). Additional analysis is necessary before we know whether octopaminergic processes innervate the relevant muscle or octopamine acts as a neurohormone.

Identification of the *Drosophila Tβh* gene and creation of null mutations in the *Tβh* locus constitute an important step in developing a molecular genetic approach to understand octopamine-regulated processes. These approaches will allow characterization of subtle mutant phenotypes and facilitate assignment of defined neurons with specific behaviors, and are likely to help in sorting out receptor functions. Progress in understanding octopamine-mediated processes and the molecular reagents generated in the fruit fly have potential to catalyze studies in other invertebrate systems better suited for physiological studies. Moreover, biochemical characterization of TBH may help develop strategies in insect pest management.

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