A Single Locus Encodes Both Phenylalanine Hydroxylase and Tryptophan Hydroxylase Activities in Drosophila

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We have used a full-length clone encoding rabbit tryptophan hydroxylase (TRH) to isolate the Drosophila homologue (DTPH). Southern analysis of Drosophila genomic DNA reveals a pattern indicative of a single gene. The single transcript is expressed in adult head and body mRNA but is also detected in mRNA from early embryos. The embryonic transcript is ubiquitously expressed and appears to concentrate in yolk granules. In situ hybridization of TRH-homologous antisense RNA probe to sectioned tissue from third instar larvae demonstrated the presence of this transcript in fat body and cuticular tissue. Developmental immunoblot analysis using antibodies raised against a β-galactosidase-Drosophila fusion protein revealed a 45-kDa embryonic protein also detected in female abdomens and a 50-kDa protein found in larval and adult stages. Immunocytochemical analysis of the Drosophila protein in the larval central nervous system showed that it appeared to be present in both serotonin- and catecholamine-containing neurons. A nonfusion protein generated in Escherichia coli hydroxylates both tryptophan and phenylalanine. We propose that there are only two aromatic amino acid hydroxylase genes in Drosophila: one encoding tyrosine hydroxylase, DTH, and DTPH, a gene encoding both tryptophan and phenylalanine hydroxylase activities.

Numerous studies in both vertebrates and invertebrates have implicated the biogenic amines in synaptical modulation and behavioral plasticity. The genes and the encoded proteins for tyrosine hydroxylase (tyrosine 3-monoxygenase, EC 1.14.16.2, TH) and tryptophan hydroxylase (tryptophan 5-monoxygenase, EC 1.14.16.4, TRH), the rate-limiting enzymes in the synthesis of dopamine and serotonin, respectively, have thus far been characterized primarily in mammalian species (for example, rabbit TRH, Grenett et al., 1987; rat TRH, Daron et al., 1988; rat TH, Grima et al., 1985). These two enzymes belong to a gene family of which phenylalanine hydroxylase (phenylalanine 4-monoxygenase, EC 1.14.16.2, PAH) is the third member. Current molecular data from amino acid sequence comparisons suggest that TH diverged from the ancestral gene before TRH and PAH diverged from each other (Kennuda et al., 1986). These enzymes hydroxylate their respective amino acid substrates by similar mechanisms (Kaufman and Fisher, 1974), and characterization of the genes encoding these enzymes suggests that they arose from a single ancestral gene. Comparison of the deduced amino acid sequences of the hydroxylases suggests that the amino-terminal third of the protein contains the regulatory and substrate specificity domain, and the rest of the protein is responsible for the hydroxylase activity (Ledley et al., 1985).

We have initiated a molecular genetic analysis of this gene family in Drosophila. The Drosophila central nervous system contains catecholamine and serotonin; however, the function of these neuroactive molecules is not understood. The Drosophila gene encoding TH has been cloned using a molecular probe for the vertebrate gene (DTH; Neckameyer and Quinn, 1989). The high degree of conservation between Drosophila TH and its vertebrate counterparts suggests that studies to characterize the regulation of TH in Drosophila may be useful in understanding vertebrate TH as well.

In this paper we have used a full-length clone encoding rabbit TRH (Grenett et al., 1987) to isolate Drosophila cDNA clones by reduced stringency hybridization. We present evidence to suggest that Drosophila contains only two aromatic amino acid hydroxylase genes: DTH, and a gene encoding both tryptophan and phenylalanine hydroxylase activities. This gene will be referred to as DTPH.

MATERIALS AND METHODS

Southern Hybridization and Screening of Drosophila cDNA Libraries—4 μg of wild-type Canton S Drosophila genomic DNA was digested with various restriction enzymes (New England Biolabs), electrophoresed through a 0.7% agarose gel, and transferred to nitrocellulose filter paper (Southern, 1975). Low stringency conditions (6 × SSPE, 50 °C) were as described in Neckameyer and Quinn (1989). The probe (pTRH779) was a 2,441-base pair cDNA containing the complete coding region for rabbit tryptophan hydroxylase, the gift of Savio Woo of Baylor College of Medicine (Ledley et al., 1987). Low stringency conditions for screening the λgt11 recombinant head-specific Drosophila cDNA library (Itoh et al., 1985), similar to those used for the Southern analysis, were as described in Neckameyer and Quinn (1989). Higher stringency hybridization of the rabbit TRH-homologous Drosophila cDNA clones was done at 42 °C in 50% formamide, 3 × SSPE, 50 mm Tris-HCl (pH 7.6), 1 × Denhardt's, 1 mm EDTA, and 20 μg/ml salmon sperm DNA. The filters were then washed at 65 °C in 2 × SSPE, 0.2% SDS.

A cDNA clone isolated from this library, λC12, was used to probe a cDNA library containing sequences from 0–3-h embryos (Pole et al., 1986), using higher stringency hybridization conditions of 50% formamide, 5 × SSPE, 20 μg/ml salmon sperm DNA, 0.5% SDS, 20 mm Tris-HCl (pH 7.6), 1 mm EDTA at 42 °C followed by washes in 0.2 × SSPE, 0.2% SDS at the same temperature.

DNA Sequencing—DNA fragments from cDNA clones isolated from the λgt11 library were subcloned into the polylinker regions of

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M13mp18 and M13mp19 (Messing, 1983; Yanisch-Perron et al., 1985) and their sequence determined by the method of Sanger et al. (1977) using the Sequenase enzyme (U. S. Biochemical Corp.). The sequence of \( \alpha DTPH.1 \) was multiply confirmed using M13 subclones of both polarities. The 3' region of the \( \alpha DTPH.1 \) cDNA clone, including the 3' untranslated region, which was multiply sequenced on one strand. Double-stranded deletions of a representative embryonic cDNA clone were prepared as described by Henikoff (1987) and were sequenced as described above. Computer analysis was done using the Wisconsin GCG programs.

**RNA Isolation, Blotting, and Hybridization**—Polyadenylated RNA was isolated from 0-6 h embryos, and adult heads and bodies of wild-type Canton S flies using the phenol-chloroform method. 2 \( \mu \)g of each sample was separated on formaldehyde, 1% agarose gels, blotted, hybridized, and washed using the high stringency conditions described in Nussenzweig and Chinn (1989).

**In Situ Hybridization to Polytenic Chromosomes**—Salivary gland chromosomes from third instar Canton S larvae were dissected and fixed according to the procedure of Engels et al. (1986). Biotinylation of probes, hybridization, and washing conditions were identical to those described.

**RNA in Situ Hybridization to Tissues**—For hybridization to whole embryos, antisense digoxigenin-labeled RNA probes from DTPH (cloned into SK+), Stratagene) and DTH (cloned into pgEM1, Promega) were made exactly according to the manufacturer's protocols (Boehringer Mannheim). In situ hybridization was according to Tague and Tague (1980). The embryo extraction procedure was 2 times instead of the 4 times as described by Tague et al. (1980). After hybridization and washing of hybridized tissues were exactly as described. Under the hybridization conditions used, the probes did not cross-react with each other.

**Preparation of Antibody against the Drosophila Tryptophan Hydroxylase Protein**—The 1.45-kb cDNA insert from \( \alpha DTPH.1 \) was subcloned into the \( \beta \)-galactosidase fusion vector pWR590-1 (gift of Li-He Guo and Ray Wu; Guo and Wu, 1984). Isolation of an in-frame fusion protein was confirmed by DNA sequencing of the \( \beta \)-galactosidase-DTPH junction as well as by induction of the fusion protein by incubation of the cells in 1 mM isopropyl 1-thio-\( \beta \)-D-galactopyranoside (Sigma), blotting the cell extract onto nitrocellulose, and visualizing the ~110-kDa fusion product with an antibody to \( \beta \)-galactosidase (Promega). Isolation and purification of the fusion protein were done as described by Tague et al. (1988).

The protein solution was dialyzed at room temperature with several changes against 50 mM Tris-HCl (pH 8.0), 10% glycerol, 0.5 M NaCl and spun at 8000 rpm for 10 min at 4 °C in an HB4 rotor (Sorvall). Protein concentration was determined using a modified Bradford assay with BSA as standard (Bio-Rad). The supernatant was electrophoresed onto 1.5-mm-thick 7.5% acrylamide-SDS preparative gels, and the fusion protein product was cut out and stored at -70 °C. The gel slice was pulverized by several passages through a 20-gauge needle, resuspended in the above buffer, and aliquots were tested on Southern dot blots according to the manufacturer's suggestions. The immune response of the antibody was determined by a homologous assay of antibody to the DTPH protein was induced by the addition of isopropyl 1-thio-\( \beta \)-D-galactopyranoside to 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2 mM at an AW of 0.7 followed by 2
1987). A pattern suggestive of a single gene was revealed (Fig. 1B). This same probe was used to screen (again, at reduced stringency) a cDNA library made from adult head mRNA. The largest clone isolated in this screen, λcDTPH.1, was hybridized under more stringent conditions to the same genomic Southern blot. λcDTPH.1 recognized the same genomic bands as those recognized by rabbit TRH, confirming that this cDNA arose from the same locus (Fig. 1B).

Other (smaller) rabbit TRH-homologous Drosophila cDNA clones were isolated in this screen, and all recognized this same pattern of restriction fragments. The relative simplicity of the pattern revealed by hybridization of the TRH-homologous probes to the genomic Southern blot suggests the majority of the TRH-homologous sequences reside in a single restriction fragment (the 4.5-kb EcoRI, the 5-kb PstI, and the -14-kb PvuII fragments). BamHI and Sac1 cut Drosophila genomic DNA less frequently, and the resulting TRH-homologous fragment is larger (greater than 15 kb). At the reduced stringency conditions for Southern hybridization (Fig. 1B), a weakly hybridizing pattern of fragments can be detected. These correspond to DTH-homologous sequences. No other bands are seen.

The insert from λcDTPH.1 was sequenced in its entirety and was found to be 1,449 nucleotide base pairs in length, not including a 13-nucleotide poly(A) tail (Fig. 2). There is a single large open reading frame from nucleotides 1 to 1,367, but the ATG at nucleotides 10-12 most likely codes for the initiator methionine. The immediate upstream sequence, G A A A (AUG) agrees well with the Drosophila initiation site consensus C/A A A (AUG) (Cavener, 1987). Assuming that this ATG does code for the initiator methionine, the deduced molecular mass of the predicted 453-residue protein is 51,539, which is very similar to those of the TRH and PAH proteins deduced from the sequences of the mammalian genes. A poly(A) tail is found 81 base pairs from the TAG stop codon at nucleotides 1,368-1,370, and the polyadenylation signal AATAAA is found 16 nucleotides upstream of the poly(A) tail (underlined in Fig. 2). The embryonic cDNA library was constructed from the Oregon R strain of D. melanogaster and the adult head cDNA library from Canton S. We presume that the nucleotide substitutions arise from differences between the two strains and that the cDNAs represent the same transcript.

In vitro transcription followed by in vitro translation of the DTPH head cDNA with a rabbit reticulocyte system shows that the DTPH cDNA is capable of directing the synthesis of
a 50-kDa protein (data not shown), the size expected for translation of the cDNA. The size of this protein is essentially identical to those of the vertebrate TRH proteins. Since the embryonic cDNA clones lack the initiator AUG, they cannot successfully be translated in vitro.

Phosphorylation of the hydroxylase enzymes by several kinases has been implicated in their regulation in vivo. A serine residue in the mammalian enzymes believed to be phosphorylated by the Ca\(^{2+}\)/calmodulin-dependent protein kinase type II (Ehret et al., 1989) is conserved in DTPH (the residue is denoted in Fig. 2 by the arrow).

Chromosomal Localization—In situ hybridization of the DTPH cDNA to third instar larval salivary gland polytene chromosomes was performed to confirm the presence of a single site for this gene, as well as to initiate a screen for possible mutants. The gene was localized to 66A on the left arm of the third chromosome, proximal to the DTH locus at 65B (data not shown). No other sites were detected. To date, no previously isolated mutant strains map in this region which would qualify as potential TRH mutations.

Northern Analysis—The insert from \(\lambda\)DTPH.1 was hybridized to poly(A\(^+\)) RNA blots and revealed a single transcript of about 1.75 kb which was enriched in adult heads and present in adult bodies, as expected for a gene encoding TRH. However, this transcript was also detected (although at a lower level) in polyadenylated RNA isolated from 0–6-h embryos, a time point at which no serotonin-containing neurons are present (Fig. 3). Hybridization of this same Northern filter to a Drosophila TH probe showed that DTH transcripts are not expressed in 0–6-h embryos. The ~300-base-pair difference in length between the transcript and the cDNAs is probably composed of 5'‐untranslated sequences, which is comparable to the size of the 5'‐untranslated region of DTH. Unlike the Drosophila TH transcript, the DTPH transcript does not appear to contain a long 3'‐untranslated region. The importance of that sequence (if any) is not clear.

Homology with Other Hydroxylases—Alignment of the deduced DTPH, Drosophila TH, rat PAH, and rat TRH proteins (not shown) shows that there is greater identity between DTPH and rat PAH and rat TRH than between DTPH and Drosophila TH at both the nucleotide and amino acid levels, implying, as has been suggested by Grenett et al. (1987), that TH diverged from the ancestral gene early in the evolution of this family. There is 50% identity between the deduced amino acid sequences of DTPH and rabbit TRH and 58.7% identity between DTPH and rat PAH. In comparison, there is a 46% identity on the amino acid level between DTH and DTPH. Although there is somewhat more identity between DTPH and rat PAH than between DTPH and rabbit TRH, there do exist residues in common between the Drosophila protein and tryptophan hydroxylase which are not found in phenylalanine hydroxylase. These residues are found throughout the proteins, including both the regulatory and hydroxylase domains.

RNA in Situ Hybridizations—RNA in situ hybridization experiments were undertaken to characterize the distribution of the DTPH and DTH transcripts further. DTPH and DTH cDNAs were cloned into transcription vectors, linearized, and antisense RNA probes were made using digoxigenin-labeled nucleotide. As expected, there was no detectable expression of DTH in 0–6-h embryos, consistent with the Northern data (Fig. 3). However, DTPH transcripts were strongly detected in 0–6-h embryos (and less strongly, but still clearly present, in 0–40-min embryos; data not shown). The expression was ubiquitous throughout the embryo; however, the signal appeared to concentrate in yolk granules. Third instar larvae were fixed, embedded in paraffin, sectioned, and hybridized to tritium-labeled DTPH or DTH antisense RNA probes (Fig. 4). Not unexpectedly, since dopamine is an intermediate in the formation of melanin, the majority of DTH expression was detected in cuticular tissue. Note the intense staining of mouthparts by the DTH probe in Fig. 4D. The majority of DTPH expression was localized to fat body (the Drosophila equivalent of liver tissue), as well as to mouthparts, although the intensity of cuticular expression was significantly less for DTPH than DTH.

Developmental Immunoblot Analysis—Antibodies against the DTPH protein were generated to aid in further characterization of this locus. A β-galactosidase-DTPH fusion protein was expressed in E. coli, and the denatured antigen from acrylamide gels was injected into New Zealand White rabbits. After two boosts of antigen within a 2-month period, serum from all three injected rabbits recognized a single 50-kDa species on immunoblots in Drosophila head extracts as well as the fusion protein band used as antigen. These proteins
were not recognized with any preimmune serum (data not shown).

Developmental immunoblot analysis showed a single protein comigrating at 50 kDa in extracts from all larval and pupal stages and adult heads (Fig. 5). Surprisingly, a 45-kDa species was recognized in embryonic extracts. The 50-kDa band first appears in 12–18-h embryos, just as the level of the 45-kDa protein begins to decrease. The level of the 50-kDa species is maintained through the adult stage and increases somewhat in third instar larvae, late pupae, and adult head.

The 45-kDa species is also detected in female but not in male abdomens.

**Immunocytochemistry**—Antiserum from one of the three rabbits proved useful for immunocytochemistry. The central nervous system was dissected from all larval stages and incubated with a 1:50 dilution of affinity-purified antibody. At all of these developmental stages, distinct neurons were detected in the ventral ganglion and the brain lobes (Fig. 6).

Preimmune serum from this rabbit did not show any specific staining. The pattern of DTPH-immunoreactive neurons was more complicated than expected on the basis of known serotonin neurons. The pattern in the ventral ganglion was analyzable because of its segmental nature. Three segmental subpatterns were readily identified: 1) pairs of ventral lateral neurons (Fig. 6B, also detected in the focal plane of 6A); 2) dorsolateral neurons in the abdominal neurones (Fig. 6C); and 3) medial unpaired neurons (Fig. 6A). The pairs of ventral lateral neurons were in locations and in a pattern similar to the previously defined serotonin neurons (Vallés and White, 1988). Simultaneous labeling experiments with serotonin antibodies confirmed that the DTPH immunoreactivity localized to serotonin-immunoreactive neurons (data not shown).

DTPH immunofluorescence was also detected in the ring gland (Fig. 6C) and in processes in the ventral ganglion (Fig. 6B), which appeared identical to those seen with antibodies against serotonin (Vallés and White, 1986). No staining above background was seen in gut, malpighian tubules, cuticle, or imaginal discs (data not shown); however, background staining with preimmune serum is quite high in these tissues and may obscure a real signal.

The DTPH-immunoreactive dorsolateral neurons and the unpaired medial neurons were in the same location as the neuronal sets detected by glyoxylic acid-induced histofluorescence (Budnik and White, 1988), suggesting that the catecholamine-containing neurons are also DTPH-immunoreactive. Immunocytochemical studies done with an antibody raised in rabbits against mammalian TH (Pel-Freez) and with the DTPH antibody revealed that the TH-immunoreactive pattern is a subset of the pattern seen with the DTPH antibody. This is consistent with the localization of the DTPH transcript to dopamine-containing tissues.

Cells corresponding in position to the thoracic dorsolateral catecholamine neurons were not detected. However, these cells were only observed in some samples with glyoxylic acid-induced histofluorescence and were not observed with the less sensitive dopamine or TH immunocytochemistry (Budnik et al., 1986; Budnik and White, 1988).

The DTPH antibody also recognizes yolk granules in 0–6-h embryos (data not shown). This is consistent with its localization in immunoblots (Fig. 5) to embryonic stages and to female but not male abdomens.

**Enzyme Assays**—To demonstrate that DTPH directed the hydroxylation of both phenylalanine and tryptophan, the adult head DTPH cDNA, which can direct the synthesis of a 50-kDa protein species in vitro, was placed under the control of an inducible T7 promoter and introduced into E. coli. The 50-kDa species is presumably a single subunit of a multimeric enzyme; however, the recombinant human PAH subunit produced in E. coli is capable of hydroxylating phenylalanine to tyrosine in the presence of exogenous cofactor (Ledley et al., 1987). E. coli protein extracts containing the induced DTPH protein were assayed for the ability to hydroxylate both phenylalanine and tryptophan (Fig. 7). Similarly induced E. coli containing the pT7-7 plasmid alone was incapable of hydroxylating either substrate, consistent with the fact that these bacteria do not contain iron-dependent aromatic amino acid hydroxylases. Therefore, all activity assayed must result from the induced DTPH 50-kDa species. Although it is frequently difficult to generate a recombinant bacterial protein with enzymatic activity (see, for example, Katarova et al., 1990), the crude extract containing the induced protein was clearly able to hydroxylate both tryptophan and phenylalanine in assays similar to those used for the mammalian enzymes. Incubation of the reaction mixtures in tubes open to the air proved essential for activity. This is consistent with the dependence on molecular oxygen for hydroxylation by the vertebrate enzymes.

Phenylalanine hydroxylase activity of the DTPH protein was decreased by the inclusion of tryptophan in the reaction mixtures (Fig. 7A). As would be expected, tryptophan hydroxylase activity was also decreased by the inclusion of phenylalanine (Fig. 7B), suggesting that the two substrates compete for binding to the DTPH protein.
Fig. 7. Enzymatic assays of induced DTPH protein in E. coli. The cDNA insert from xDPH.l was placed under the control of an inducible T7 promoter and transformed into E. coli. Increasing amounts of crude extract showed increasing tryptophan hydroxylase activity, which could be competed out by the inclusion of L-phenylalanine. B, the same extract also directed the production of tyrosine from phenylalanine, which could be competed out by the presence of L-tryptophan in the reaction mixture (A). Relative fluorescence from nonsubstrate, noncofactor, and plasmid controls was used as baseline. This figure shows the results of two independent assays from a single extract.

Reactions incubated without either pterin cofactor or without either L-phenylalanine or L-tryptophan were also incapable of producing the expected product, demonstrating that the hydroxylase was specific to the exogenous substrate and similar to the mammalian aromatic amino acid hydroxylases, was absolutely dependent on the pterin cofactor for activity. As mentioned above, reactions incubated with no DTPH protein showed no activity, demonstrating that both phenylalanine and tryptophan hydroxylase activities extrapolate to zero. The protein, which pelleted with the insoluble material, was clearly active. The soluble fraction was too dilute to yield significant activity.

In some preparations, increasing activity was seen for both hydroxylation reactions with increasing amounts of previously saturating substrate (not shown) because of the variability of induction. Additionally, the protein pelleted with insoluble material, making uniform resuspension more difficult. Under the most optimal conditions, a band could be seen on a Coomassie-stained gel which was specifically recognized by the DTPH antibody. We estimate this level of induction to yield no more than 5% DTPH protein. Under less optimal conditions, no Coomassie-stained gel band could be detected by the DTPH antibody, demonstrating that both TRH and PAH activities. This is in contrast to vertebrates, in which independent loci encode PAH and TRH. The vertebrate PAH and TRH genes share extensive identity (rabbit tryptophan hydroxylase shares 230 out of 434 amino acid residues with human phenylalanine hydroxylase; Grenett et al., 1987) and are more similar to each other than to TRH.

had fully expected to find a complex Southern pattern under low stringency conditions with the rabbit TRH probe. However, we observed a simple pattern indicative of a single gene. This implied that either the Drosophila PAH and TRH genes had diverged further from each other relative to the vertebrate enzymes, and the rabbit TRH probe recognized only one of the two genes, or that there was only one gene encoding both enzymes. The pattern of restriction fragments in Drosophila genomic DNA recognized by the full-length rabbit TRH cDNA is the same pattern recognized by several rabbit TRH-homologous clones isolated from Drosophila cDNA libraries. A more weakly hybridizing pattern of fragments could be detected after hybridization of the probes at reduced stringency. These bands correspond to those recognized by both mammalian and Drosophila TRH probes. This suggests that only two aromatic amino acid hydroxylase genes exist in Drosophila: one corresponding to TRH, and the other corresponding to TRH and PAH. While screening the Drosophila cDNA libraries for rabbit TRH-homologous sequences, duplications that were hybridized under more stringent conditions with DTH. Those clones with strong homology to the vertebrate TRH gene hybridized, although more weakly, to DTH, indicating that the hybridization conditions were sufficiently reduced to detect cross-hybridization to a less similar species. A single locus was detected after in situ hybridization of the DTPH probe to salivary gland chromosomes, and a single transcript was detected in both early embryos and adults on Northern blots. Nucleic acid and amino acid comparison indicated that DTPH is highly homologous to both mammalian PAH and TRH and less so to TRH. These data are consistent only with either a single locus encoding both PAH and TRH or two separate but contiguous genes. The simple genomic Southern pattern refutes the latter possibility. Additionally, there is only a single cDNA clone with homology to tryptophan hydroxylase. Since Drosophila must require TRH and PAH activities, we propose that this single locus provides both these functions.

The unexpected transcript detected in poly(A) RNA from 0-6-h embryos could be specific for PAH function and would explain the presence of this transcript at a stage in development prior to the presence of defined serotoninergic neurons. The 0-6-h transcript most likely encodes the 45-kDa embryonic protein that is sequestered in yolk granules and detected in female but not in male abdomens. Using an antibody to dopa decarboxylase, Konrad and Marsh (1987) detected no dopa decarboxylase expression in Drosophila ovaries in 0-14-h embryos. Vallés and White (1988) did not detect the presence of serotonin either in ovaries or 0-16-h embryos. It is therefore highly likely the RNA and protein expression detected in early embryogenesis reflects a PAH component of this locus. The cDNAs isolated from a 0-3-h embryonic library are essentially identical to those isolated from head tissue, suggesting that the 45-kDa species does not arise through alternative splicing but more likely through post-translational modifications. Assuming that the 45-kDa embryonic species largely (or exclusively) functions as a phenylalanine hydroxylase, one could speculate that the substrate preferences of the 45- and 50-kDa DTPH species might be determined by changes in the amino-terminal regulatory domain or by differential phosphorylation of potential sites. It should also be noted that the hydroxylase enzymes in vertebrates are multimeric and that the differences between the 45- and 50-kDa species could form a conformational change in the holoenzyme resulting in altered substrate specificities. The presence of the DTPH transcript in dopamine-con-
taining tissues such as mouthparts, which do not contain serotonin, suggests that this transcript is present in these tissues to encode a protein that can hydroxylate phenylalanine to tyrosine. The DTPH transcript is detected in fat body, the Drosophila equivalent of mammalian liver (a tissue in which PAH is highly expressed). DTH, under identical hybridization conditions, does not cross-hybridize with the DTPH transcript in fat body (Fig. 4). This transcript must also encode a protein capable of hydroxylating tryptophan to 5-hydroxytryptophan as the first step in the synthesis of serotonin. (Drosophila synthesizes serotonin through a 5-hydroxytryptophan intermediate; Livingstone and Tempel, 1983.) The level of expression of this transcript in the nervous system is expected to be significantly reduced relative to that found in other tissues since tryptophan hydroxylase is the rate-limiting enzyme in the synthesis of serotonin, which is specifically localized to a small population of cells within the nervous system (Valles and White, 1988). DTH expression in the nervous system is similarly reduced relative to that found in cuticular tissue.

Additional evidence that DTPH does encode TRH activity comes from the immunocytochemical data. Previously identified serotonin-immunoreactive cells are also DTPH-immunoreactive, but the pattern of serotonin-containing neurons includes both the dopamine- and serotonin-containing neurons. It is not likely that the DTPH immunoreactivity in the dopamine-containing neurons is caused by cross-reactivity against TH, as such cross-reactivity was not observed on immunoblots, nor can the antibody immunoprecipitate in vitro translated DTH. The DTPH transcript is expressed in dopamine-containing tissue, so one could expect to see DTPH protein in these tissues, as well. If 5-hydroxytryptophan (and therefore serotonin) is generated in Drosophila by an evolutionarily diverged hydroxylase, it is unlikely that we would be able to detect serotonin-containing neurons with the DTPH antibody.

In vertebrates, TPH and PAH can hydroxylate each other’s substrates (Ichiyama et al., 1976; Renson et al., 1962). That the 50-kDa protein is capable of PAH activity, at least in vitro, is suggested by the PAH activity profile presented by Geltosky and Mitchell (1980). They assayed PAH activity in Drosophila and found activity in larval instars with a peak in pupal stages. At these stages of the Drosophila life cycle, the fly would require both the synthesis of serotonin in the nervous system and tyrosine as part of the cuticular metabolism.

The DTPH protein induced in E. coli is capable of hydroxylating both tryptophan and phenylalanine. As mentioned earlier, the mammalian PAH and TPH enzymes are able to hydroxylate both substrates, as well. However, in vertebrates there are clearly two distinct enzymes with nonoverlapping tissue distribution. In Drosophila, there is a single gene product which must be regulated to prevent the hydroxylation of an inappropriate substrate in the different tissues.

These considerations suggest that the activity of the same protein must be regulated differentially in serotonergic and dopaminergic neurons. Given the similarity of the amino acids substrates, the fact that these enzymes share the same pterin cofactor, and that both serotonergic and dopaminergic neurons contain the enzyme dopa decarboxylase, what regulatory controls prevent the production of serotonin in dopaminergic neurons? One such regulatory control may involve end product inhibition by tyrosine. In serotonergic neurons, there is no TH activity to hydroxylate tyrosine to l-dopa, and there may be negative feedback from the build-up of tyrosine to help force the reaction kinetics to the production of 5-hydroxytryptophan. In dopaminergic neurons, tyrosine is hydroxylated by TH, and removal of the product may help stimulate the phenylalanine hydroxylase reaction. It is also conceivable that other factors such as specific kinases, proteolytic enzymes, and/or phosphatases may help regulate the hydroxylation reactions.

The cloning of a Drosophila PAH homologue has recently been reported (Morales et al., 1990). We agree on much of the nucleotide sequence data, but they report a substantially different (and less similar with either PAH or TRH) deduced amino-terminal protein sequence (within the first 120 residues). The differences cannot be accounted for by alternative splicing. Additionally, little characterization of the gene or gene product(s) was reported, and their deduced amino acid sequence was compared with only vertebrate TH and PAH and not with vertebrate TRH or with Drosophila TH.

We suggest that the ancestral hydroxylase gene most strongly resembles PAH in structure and function and that the other hydroxylases evolved and were refined for use in the nervous system (and, for both DTPH and DTH, cuticular tissue). The estimated evolutionary distance between PAH and TRH is ~600 million years (Grenett et al., 1987), approximately the same as that between vertebrates and invertebrates. We propose that in Drosophila, and presumably in other invertebrate species, the ancestral hydroxylase gene has diverged to form TH and a gene encoding both TRH and PAH functions. In all mammalian species examined thus far, separate enzymes exist for the hydroxylation of tyrosine, tryptophan, and phenylalanine. Comparative enzymatic studies of the Drosophila aromatic amino acid hydroxylases with their vertebrate counterparts will enable us to determine which regions of the proteins are involved in which activities (substrate recognition, hydroxylation), and will aid in characterizing those factors regulating the hydroxylation reactions. Given the neuronal localization of TRH and the non-neuronal distribution of PAH in vertebrates, understanding the regulation of the Drosophila counterpart(s) may increase our understanding of the evolution of the biogenic amines in the nervous system.

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