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Molecules and Cells

Analysis of Two Promoters that Control the Expression of the *GTP cyclohydrolase I* Gene in *Drosophila melanogaster*

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GTP cyclohydrolase I (GTPCH) is a key enzyme in the *de novo* synthesis of tetrahydrobiopterin. Previously, the *Drosophila melanogaster GTPCH* gene has been shown to be expressed from two different promoters (P1 and P2). In our study, the 5′-flanking DNA regions required for P1 and P2 promoter activities were characterized using transient expression assay. The DNA regions between -98 and +31, and between -73 and +35 are required for efficient P1 and P2 promoter activities, respectively. The regions between -98 and -56 and between -73 and -41 may contain critical elements required for the expression of *GTPCH* in *Drosophila*. By aligning the nucleotide sequences in the P1 and P2 promoter regions of the *Drosophila melanogaster* and *Drosophila virilis GTPCH* genes, several conserved elements including palindromic sequences in the regions critical for P1 and P2 promoter activities were identified. Western blot analysis of transgenic flies transformed using P1 or P2 promoter-lacZ fusion plasmids further revealed that P1 promoter expression is restricted to the late pupae and adult developmental stages but that the P2 promoter driven expression of *GTPCH* is constitutive throughout fly development. In addition, X-gal staining of the embryos and imaginal discs of transgenic flies suggests that the P2 promoter is active from stage 13 of embryo and is generally active in most regions of the imaginal discs at the larval stages.

INTRODUCTION

Tetrahydrobiopterin (BH_4) is an essential cofactor required for the catalytic activity of aromatic amino acid hydroxylases, which are key enzymes in the biosynthesis of several neurotransmitters such as dopamine, noradrenaline, adrenaline, and serotonin (Kaufman, 1993). In addition, BH_4 is an important cofactor for glycerol ether mono-oxygenases (Kaufman et al., 1990) and also various isoforms of nitric oxide (NO) synthase, which synthesizes NO from L-arginine (Marletta et al., 1998). NO in turn regulates a huge number of biological processes, including immune modulation, neurotransmission, vasodilation, and programmed cell death. Hence, the regulation of the cellular concentration of BH_4 is critical for a variety of cellular processes.

The *de novo* synthesis of BH_4 requires three enzymatic reactions, involving GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase, and sepiaterin reductase (Smith, 1987; Takikawa et al., 1986). The conversion of GTP to 2,4 dihydroneopterin triphosphate, catalyzed by GTP cyclohydrolase I (GTPCH), is the first and rate-limiting reaction in the *de novo* synthesis of BH_4. Thus, the proper regulation of GTPCH is very important for the control of BH_4 level in the cell, and mutations in the *GTPCH* gene can cause BH_4 deficiency and lead to severe clinical diseases including hyperphenylalaninemia and dopa-responsive dystonia (Hirano et al., 1998; Ichinose et al., 1994; Thony and Blau, 1997). Mammalian GTPCH is regulated by a variety of mechanisms and is sensitive to end-product feedback inhibition by BH_4. This feedback inhibition is mediated through the formation of a complex between GTPCH with GTPCH feedback regulatory protein (GFRP) and BH_4 (Harada et al., 1993: Milstien et al., 1996). GTPCH is also regulated at the transcriptional level by extra-cellular signal molecules, including interferon-γ, interleukin1β, tumor necrosis factor α, lipopolysaccharide, epidermal growth factor, and agents that elevate the intracellular cAMP concentration (D'Sa et al., 1996; Frank et al., 1998; Geller et al., 2000; Hirayama et al., 2001; Pluss et al., 1996; Vann et al., 2000; Ziegler et al., 1993). Several transcription factors such as CREB, ATF2, c-Jun, Sp1, and Sp3 have been shown to be involved in the regulation of mammalian GTPCH expression (Al Sarraj et al., 2005; Chandran et al., 2008). In addition, GTPCH is positively regulated via post-translation modification. It has been further reported that stimulation of GTPCH activity in mammalian cell culture occurs through phosphorylation (Hesslinger et al., 1998; Lapize et al., 1998).

Drosophila melanogaster (*D. melanogaster*) GTPCH has a near 80% homology with its mammalian counterparts in terms of amino acid sequence. This high degree of conservation affords extensive genetic and molecular analysis of *Drosophila* GTPCH to better understand the mechanisms underlying its regulation (Mackay and O'Donnell, 1983; Mackay et al., 1985; Reynolds and O'Donnell, 1988). *Drosophila* GTPCH is encoded by the gene *Punch* (*Pu*), which has been shown to pro-

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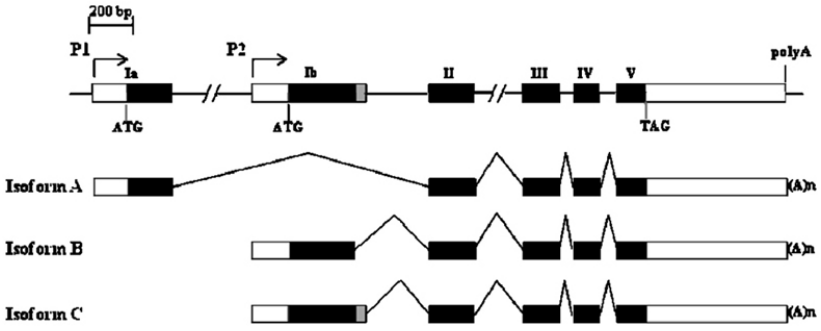

Fig. 1. Schematic representation of the organization of the *Drosophila melanogaster GTPCH* gene and the transcripts for its isoforms. The transcription start sites derived from the two promoters (P1 and P2) of the *Drosophila melanogaster GTPCH* gene are indicated by arrows and the exons (I-V) are shown by boxes. The first exons for isoform A and isoforms B and C are indicated by Ia and Ib, respectively. The open boxes indicate the 5′- or 3′-untranslated regions of the exons. The translation start codons and stop codon are indicated under the boxes. The transcripts for isoforms A, B, and C are shown below the *GTPCH* gene structure and the introns are shown by lines connecting the exons. The transcripts for isoform A and isoforms B and C are produced from two different promoters, P1 and P2, respectively. The transcripts for the B and C isoforms are produced from the differential use of a 5′-splice site within the first intron, which is shown by gray box.

duce at least three different transcripts (A, B, and C) via the activity of alternative promoters and through alternative splicing (Fig. 1) (McLean et al., 1993). The GTPCH isoforms derived from transcript A (1.70 kb), B (1.75 kb), and C (1.80 kb) have identical catalytic core domain, but different N-terminal sequences. The variation in the N-terminal sequences of *Drosophila* GTPCH isoforms confers differential enzymatic activities and regulatory properties (Funderburk et al., 2006). The N-terminal extensions of isoform A, and isoforms B and C, while sharing no similarity in sequence, are involved in facilitating end-product feedback inhibition by BH_4. However, the N-terminal extensions of isoforms B and C provide an additional level of regulation in that they possess negative regulatory domains, which serve as substrates for protein kinase A and C (Funderburk et al., 2006). The phosphorylation of residues within these negative regulatory domains may alleviate the negative regulation imposed on the enzyme (Funderburk et al., 2006).

Drosophila GTPCH isoform A and isoforms B and C are derived from two different promoters (P1 and P2) and are expressed in different tissues and at different times during development, unlike the mammalian GTPCH isoforms which appear to be expressed in the same cells (McLean et al., 1993). The transcripts for isoform A (1.70 kb), which are responsible for the production of eye pigment, are more abundant in the adult heads than in any other tissue during developmental stages. The transcripts for isoform B (1.75 kb) are more abundant than those of isoform A in the larvae and embryos. However, the expression profile of isoform C has not yet been characterized. The differential expression of *Drosophila* GTPCH isoforms, containing various regulatory regions in their N-terminal sequences, may reflect a diversity of BH_4 regulation in cells that is dependent on the tissue type and developmental stages.

In our present study, we wished to better understand the regulation of *GTPCH* gene expression and thus used transient assays to further characterize the two promoters, P1 and P2, which are required for the expression of all three isoforms. We also cloned the promoter regions for *Drosophila virilis* (*D. virilis*) *GTPCH* and performed sequence comparisons of these regions between *D. melanogaster* and *D. virilis* to elucidate any potential *cis*-regulatory elements. In addition, we established transgenic flies harboring GTPCH promoter-lacZ fusion plasmids to perform P1 and P2 promoter analysis.

MATERIALS AND METHODS

Construction of luciferase expression plasmids

pT-luc, was used as a parental plasmid for the construction of the promoter-luciferase fusion constructs (Lee et al., 1997). DNA fragments of about 1.7 and 2.1 kb, spanning -1580 to +84 and -1990 to +146 with respect to the transcription start site of the *D. melanogaster GTPCH* P1 and P2 promoter, respectively, were cloned into the *Bam*HI site of pT-luc. The resulting recombinant plasmids were designated as P1-BS-luc and P2-Sal-luc, respectively. Each deletion plasmid was constructed by PCR fragment or restriction digestion.

DNA transfection and luciferase assay

The dimethyldiotadecylammonium bromide-mediated transfection of *D. melanogaster* Schneider Line 2 was performed using 24-well tissue culture plates as described previously (Han, 1996). Each transfection mixture contained 5 ng of the appropriate reporter plasmid and 100 ng of pcopia LTR-lacZ internal control plasmid. Three days after transfection, cell extracts were prepared by adding 20 μl of Cell Lysis Reagent (Promega, USA) to each well after removal of the media by aspiration. The 24-well plates were agitated gently on a rotary shaker for 5 min, and cell extracts were transferred to microcentrifuge tubes. After centrifugation for 5 min, 2 μl and 1 μl of the supernatant were subjected to a β-galactosidase assay and a luciferase assay, respectively. The β-galactosidase assay was performed using a coloriemetric method as described previously (Han and Manley, 1993). The luciferase assay was performed with a luminometer (Turner Designs, USA). Luciferase activities were normalized by determining the luciferase/β-galactosidase activity ratios and by averaging the values from duplicate experiments. Each transfection was repeated 3-8 times and the average values and standard deviations were calculated

Isolation of *D. virilis GTPCH* gene

A *D. virilis* genomic library was obtained from Dr. J. Tamkun (University of California at Santa Cruz) and screened under reduced stringency conditions using *D. melanogaster* GTPCH cDNA as a probe. Hybridizations under reduced stringency were performed by first prehybridizing overnight at 37°C in 40% formamide, 5× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt's (1× Denhardt's: 0.2 μg/ml each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 50 μg/ml denatured herring sperm DNA. This was followed by hybridizing overnight under the same conditions except that probes radiolabeled with ^{32}P were added to the prehybridization buffer. After hybridization, the filters were washed three times in 2× SSC, 0.1% SDS for 20 min each at 50°C.

Transgenic flies

The transgenic flies carrying the P1 or P2 promoter-lacZ fusion plasmids were generated by P-element-mediated germ line transformation. To construct the transformation plasmids, the 5′-flanking regions corresponding to -530 and +84 in the P1 promoter and -389 and +146 in the P2 promoter were amplified by PCR and cloned into the transformation vector, pCaSpeR-AUG-βgal after digestion with *Eco*RI and *Bam*HI (Thummel et al., 1988). The w^{1118} strain was microinjected with these transformation plasmids. The resulting transgenic lines were generated and maintained as described previously (Kim et al., 2008).

Western blot analysis

Equal amounts of the fly protein homogenate (20 μg of protein per sample) prepared from tissues at various stages of development were boiled for 5 min in sample buffer (62.5 mM Tris-Cl; pH 6.8; 2% SDS; 10% glycerol; 2% β-mercaptoethanol; and 10 μg/ml bromophenol blue), resolved using 8% SDS-PAGE, and electroblotted onto a nitrocellulose membrane. The primary antibody, anti-β galactosidase antibody (Promega, USA) or anti-H3 antibody (Abcam, UK), was used at dilution of 1:5,000, and the immunolabeling was detected with a peroxidase-conjugated goat anti-rabbit antibody (Sigma, USA) and visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA).

X-gal staining of *Drosophila* embryos and imaginal discs

X-gal staining of *Drosophila* embryos and imaginal discs was carried out essentially as described previously (Hazelrigg, 2000). Briefly, the embryos were collected at different stages, dechlorinated in 50% bleach for 1 min, and washed with NaCl-Triton solution (0.7% NaCl/0.04% Triton X-100). The embryos were then fixed for 20 min in 50% heptanes/50% fixative (0.1 M sodium phosphate, pH 7.2/4% formaldehyde) and washed with distilled water. This was followed by rehydration with NaCl-Triton solution for 5 min and staining with X-gal solution (10 mM sodium phosphate, pH 7.2/150 mM NaCl/1 mM $MgCl_2$/3.1 mM $K_4[Fe(CN)_6]$/$K_3[Fe(CN)_6]$/0.3% Triton X-100/0.2% X-gal) at 37°C overnight. The embryos were then devitellinized with 50% heptanes and 50% methanol by vortexing for 20 s, washed with ethanol, mounted, and then photographed. For X-gal staining of imaginal discs, third instar larvae were dissected, fixed for 20 min in PBS solution containing 0.75% glutaraldehyde, and washed with PBS. X-gal staining was then performed as described for the embryos.

RESULTS AND DISCUSSION

Promoter analysis of the *D. melanogaster GTPCH* gene using transient expression assays

Three different GTPCH transcripts (A, B, and C) of *D. melanogaster* are each expressed from two distinct promoters, P1 and P2, which are located about 2.8 kb apart. All of these transcripts are composed of 5 exons and each has a distinct first exon (Fig. 1) (McLean et al., 1993). The transcripts for the B and C isoforms are produced from the differential use of a 5′-splice site within the first intron. The A and B transcripts have been shown to be expressed in different tissues and at different stages of development, suggesting the differential regulation of the P1 and P2 promoters (McLean et al., 1993).

To further understand the regulatory mechanisms controlling the P1 and P2 promoters, we identified the promoter region required for the expression of *D. melanogaster GTPCH* transcripts using a transient expression assay. For this experiment, we constructed luciferase expression plasmids containing serial deletions of the 5′-flanking region of the GTPCH transcripts. Each deletion was fused to a luciferase reporter gene and co-transfected with the pcopia LTR-lacZ plasmid as an internal control into *D. melanogaster* Schneider Line 2 using previously described method (Lee et al., 1997). The promoter activity for each of the deletion constructs was determined from the luciferase levels in the transfected cells. As shown in Fig. 2A, the P1 promoter activity is not severely affected by a deletion from -1580 to -96 with respect to the transcription start point (tsp). However, a deletion from -96 to -56 reduced this expression to about 10%. A 3′-deletion of the 5′-flanking region from +84 to +31 had no significant effects on promoter activity. These results suggest that a 127 bp long DNA segment between -96 and +31 is required for efficient expression of the *Drosophila GTPCH* transcript A and that a 41 bp DNA segment between -96 and -56 may contain the critical element required for this expression.

Figure 2B shows the result obtained from the analysis of the P2 promoter. The deletion from -1990 to -77 or from +146 to +35 had no significant effects on promoter activity, but a further deletion from -77 to -61 or -45 reduced expression to about 45% or 20%, of the control levels, respectively. These results suggest that a 112 bp long DNA segment between -77 and +35 is required for efficient expression of *Drosophila* GTPCH transcripts B and C, and that the 33 bp DNA segment between -77 and -45 may contain the critical element for their expression.

Comparison of the *GTPCH* promoter sequences of *D. melanogaster* and *D. virilis*

To identify the *cis*-regulatory elements that are important for the *GTPCH* promoter activities, several approaches were possible in *Drosophila*. One of these involves comparing promoter sequences of *D. melanogaster* with those of closely related species such as *D. virilis* and thereby identifying highly conserved sequences. This approach has proven applicability in *Drosophila* and has previously contributed to the identification of several *cis*-regulatory elements (Choi et al., 2000; Mismer et al., 1988). In using this approach, we isolated and characterized genomic clones encoding *D. virilis GTPCH.* We then determined the entire coding sequence and also the transcription start sites by 5′-RACE. We found that the *Drosophila virilis GTPCH* gene has the same exon and intron structure as *D. melanogaster GTPCH* (data not shown) and thus has the potential gene structure to encode three alternative transcripts.

From an alignment of the nucleotide sequences in the 5′-flanking region of the *D. melanogaster* and *D. virilis GTPCH* genes, we identified highly conserved sequences as shown in Figs. 3A and 3B. The nucleotide sequences in the P1 promoter regions between -50 and +9 are almost identical between these two species, suggesting important roles for these elements in *GTPCH* expression. The conserved region between -50 and +9 includes other important elements such as a TATA box sequence and a transcription initiator. However, a highly conserved nucleotide sequence was not evident in the region between -96 and -56, which we had identified to be critical for promoter activity in our transient expression assay. However, palindromic sequences consisting of two 6- and 8-bp half sites separated by 1 bp were found to be at similar positions in this region, about 86 bp upstream of the transcription start site in *D. melanogaster* and *D. virilis*, respectively. These two palindromic sequences have no considerable homology except that both half sites contain a CGA sequence.

The highly conserved sequences in the P2 promoter region are found in three regions, -71~-41, -37~-29 and in the vicinity of the transcription start site (-5~+10). The region between -71

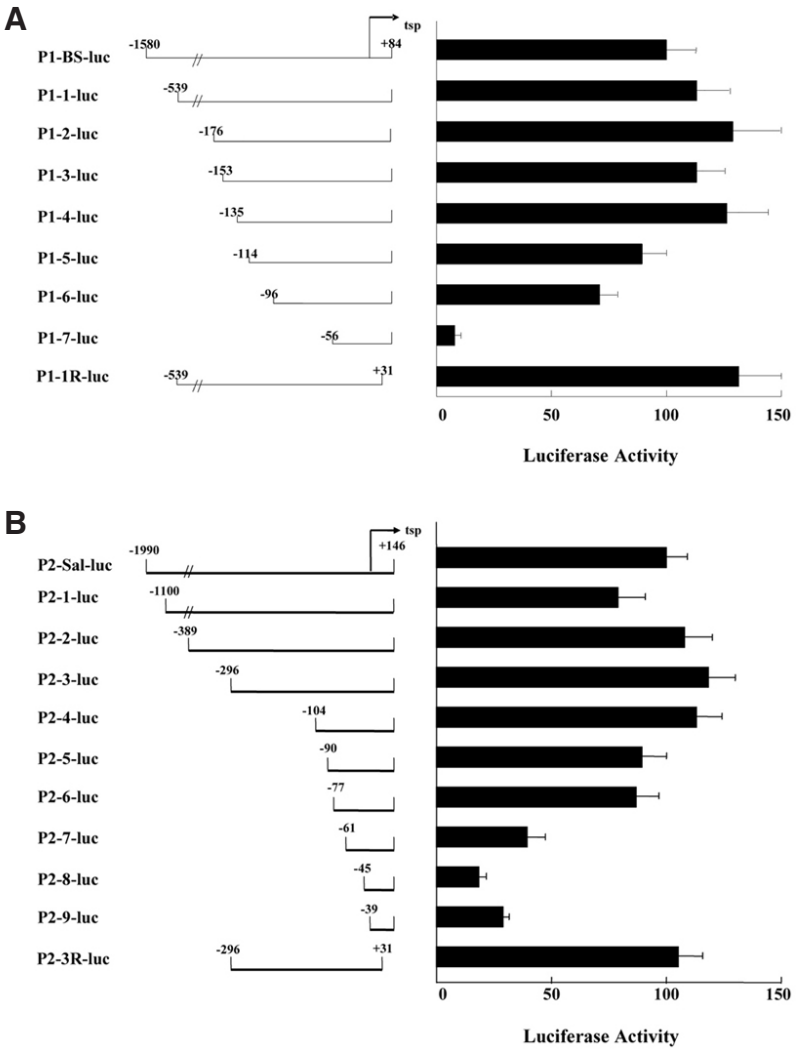

Fig. 2. Deletion analysis of the P1 and P2 promoter regions of the *Drosophila melanogaster GTPCH* gene. The promoter regions used for the construction of the promoter-luciferase fusion plasmids are indicated by the lines. The numbers on these lines indicate the distances in nucleotides from the tsp. The relative luciferase activities of the cells transfected with these promoter-luciferase fusion plasmids are shown in the right panel. The standard deviations about the mean are indicated by error bars. (A) Deletion analysis of the P1 promoter. The luciferase activities in cells transfected by each plasmid were calculated as a percentage of the activity in cells transfected with P1-BS-luc (100%). (B) Deletion analysis of the P2 promoter. The luciferase activities in cells transfected by each plasmid were calculated as a percentage of the activity in cells transfected with P2-Sal-luc (100%).

and -41 is included in the region -77~-45, which was identified to be critical for promoter activity. Interestingly, the regions between -71 and -41 of the P2 promoters contains the conserved palindromic sequences consisting of two 8-bp half sites separated by 9 bp and 8 bp in *D. melanogaster* and *D. virilis*, respectively. These palindromic elements could thus be a candidate binding site for transcription factors that usually form multimers. In addition, the P2 promoter of *D. virilis GTPCH* also lack a typical TATA box sequence as the P2 promoter of *D. melanogaster GTPCH* does. However, both species have a fully conserved 9-bp sequence (GTTCTTATT) centered at position -34, which may thus have an important function with respect to general transcription factors such as TFIID and TFIIB. In *Drosophila*, many TATA-less genes have been found to be house-keeping genes, which show constitutive expression. The TATA-less P2 promoter of *D. melanogaster GTPCH* may therefore be involved in constitutive expression of GTPCH isoforms B and C, since transcript B was detected throughout development. In addition, the P1 promoter may function in tissue-specific expression of GTPCH isoform A in the adult fly head.

Analysis of *Drosophila* GTPCH expression in the transgenic flies by Western blot

To determine the differential expression patterns of *Drosophila* GTPCH isoforms *in vivo*, we established transgenic flies transformed by the P1 or P2 promoter-lacZ fusion plasmids. The 5′-flanking regions between -530 and +84 of the P1 promoter and -389 and +146 of the P2 promoter were amplified by PCR and inserted into the transformation vector, pCaSpeR-AUG-βgal. The w^{1118} strain was then microinjected with these constructs and 5 and 7 transgenic lines transformed with P1 promoter- and P2 promoter-lacZ fusion plasmids, respectively, were obtained. These transgenic lines were subsequently analyzed and compared using developmental Western blotting with anti-β-galactosidase antibody. All of the transgenic lines transformed with the same plasmid gave essentially same expression pattern for LacZ, even though these levels of LacZ expression differed among the lines. The typical results we obtained from the developmental Western blot analyses of the transgenic lines transformed with P1 promoter- and P2 promoter-lacZ fusion plasmids are shown in Figs. 4A and 4B, respectively. These data revealed that the P1 promoter expression is restricted to the late pupae and adult stages (Fig. 4A). In addition, the P1 promoter is more active in the adult head than the adult body. On the other hand, the P2 promoter activity was detected after the 6 hour embryo stage and increased until the 24 h embryo stage (Fig. 4B). However, P2 promoter activity was barely detectable during the larval stage. During the pupae and adult

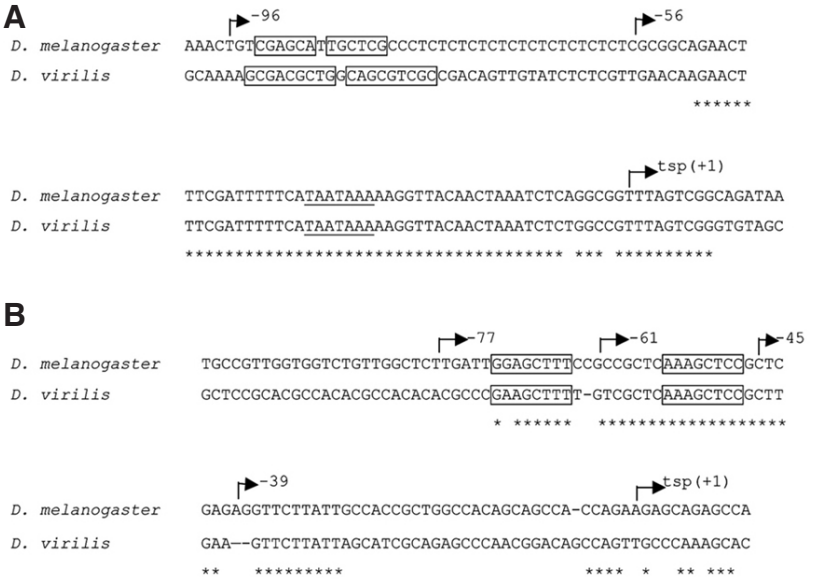

Fig. 3. The conserved nucleotide sequences in the 5′-flanking regions of *D. melanogaster* and *D. virilis GTPCH* genes. The nucleotide sequences in P1 (A) and P2 (B) promoter of *D. melanogaster* and *D. virilis GTPCH* genes are aligned. Regions representing palindromic sequences are boxed and identical nucleotide sequences are indicated by asterisks. The numbers indicate distances in nucleotides from the transcription start point (tsp).

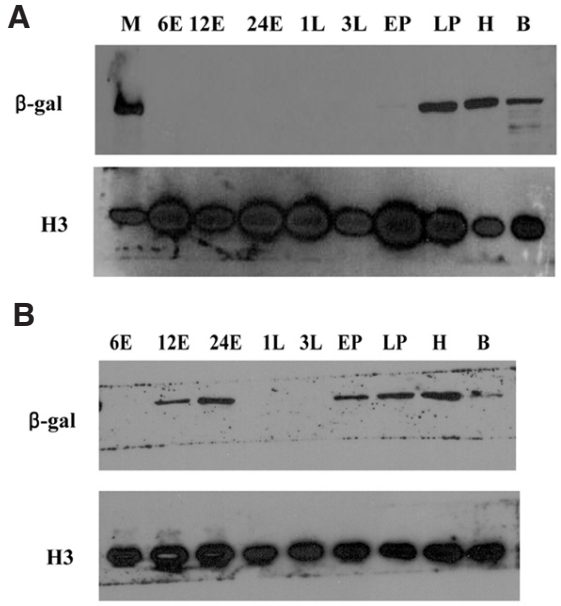

Fig. 4. Expression of the promoter-lacZ transgenes at various stages of *Drosophila* development. Protein extracts of transgenic flies transformed with P1 (A) or P2 (B) promoter-lacZ fusion plasmids were prepared at various stages of development and subjected to Western blot analysis using antibodies against β-galactosidase. The indicated stages are defined as follows: lane 6E, 0-6 h embryo; lane 12E, 6-12 h embryo; lane 24E, 12-24 h embryo; lane 1L, first instar larvae; lane 3L, third instar larvae; lane EP, early pupae; lane LP, late pupae; lane H, adult heads; lane B, adult bodies. As an internal control for GTPCH protein expression, Western blot analysis using an antibody against histone 3 (H3) was performed. The positive controls for β-galactosidase and H3 are shown in lane M.

stages, the P2 promoter is again active and promotes higher expression in adult head compared with the adult body. These results suggest that the P2 promoter is constitutively active throughout development whereas the P1 promoter is active only after the late pupae stage.

These results are consistent with the previous data obtained from Northern blotting analysis, which indicated the *Drosophila* adult head-specific activity of the P1 promoter and constitutive expression driven by P2 promoter (McLean et al., 1993). Hence, in the early stages of fly development, GTPCH isoforms B and C but not A may be expressed. All three isoforms may be highly expressed in the adult head, where a high concentration of BH_4 may be needed for the production of eye pigment and diverse neurotransmitters.

X-gal staining of embryos and imaginal discs from the transgenic flies

To enable a more detailed analysis of the GTPCH expression that is differentially derived from the P1 and P2 promoters in *D. melanogaster*, embryos and imaginal discs from transgenic flies were examined by X-gal staining. Transgenic embryos at different stages (1-16) of embryo in the P1 and P2 transgenic flies were collected and stained. Figure 5 shows these staining results for stages 10 to 16. As shown in Fig. 5A, the embryos of the P1 transgenic flies did not positively stain with X-gal, suggesting that the P1 promoter is not active throughout the development of the embryo. However, the P2 promoter appears to be active from stage 13, in which the differentiation of the central nervous system and peripheral nervous system start to occur (Fig. 5B). These results may suggest that GTPCH isoforms B and C are necessary for proper development of the nervous system. The staining of the imaginal discs at the larval stage showed that P1 promoter is not active in the imaginal discs (Fig. 6A), whereas the P2 promoter is generally active in most regions of the imaginal discs (Fig. 6B). However, in the brain disc, P2 promoter activity was detectable only in the ventral ganglion, which develops the central nervous system.

BH_4 is an essential cofactor required for catalytic activity of diverse enzymes in the cell and its biosynthesis is critically dependent upon GTPCH expression. Hence, the expression of GTPCH would be required in all tissues in the fly. A higher expression of GTPCH is in fact needed in tissues such as the eye and nervous system, where high levels of BH_4 are required for the production of eye pigment and neurotransmitters. In our present experiment, we have shown that GTPCH expression in

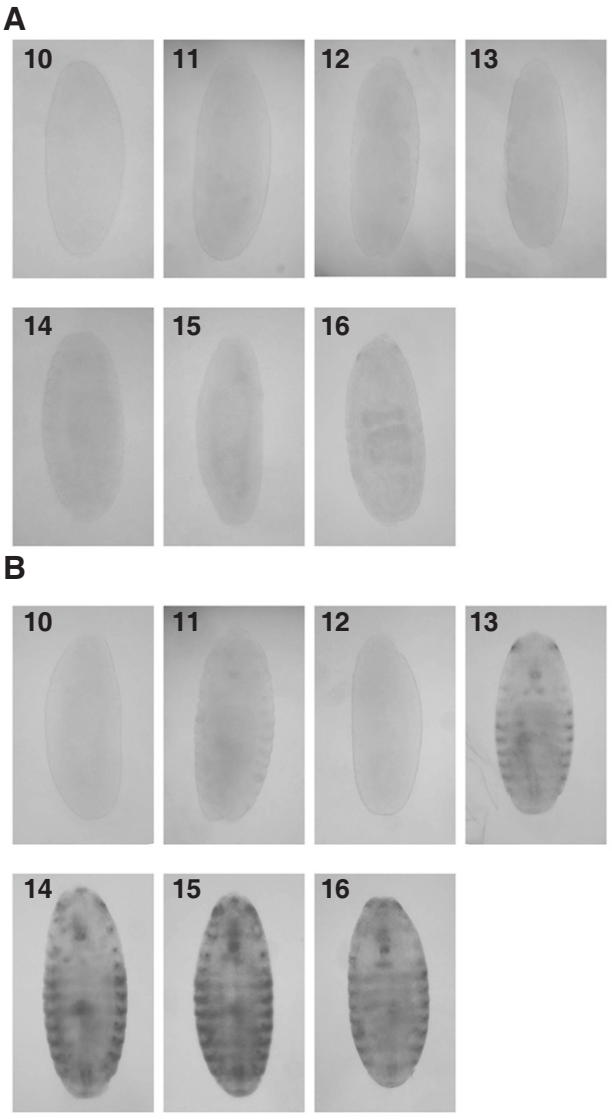

Fig. 5. X-gal staining of embryos in transgenic *Drosophila*. The embryos at stage 10-16 of the transgenic flies transformed with the P1 (A) or P2 (B) promoter-lacZ fusion plasmids were collected and then stained with X-gal.

the embryonic and larval stages of *Drosophila* development is derived mainly from its P2 promoter, whereas in pupae and adult flies this expression is driven from both the P1 and P2 promoters. Moreover, the P2 promoter appears to promote the constitutive expression of GTPCH throughout fly development, whilst the P1 promoter promotes the more specific expression of GTPCH in adult brain. GTPCH levels are thus high in the adult brain, in which high level of BH_4 are required, via the combined activity of its two promoters. Further characterization of the transcription factors involved in P1 and P2 promoter activities, and the functional differences between the GTPCH isoforms will give us a better understanding of the regulation of the *GTPCH* gene in *D. melanogaster*.

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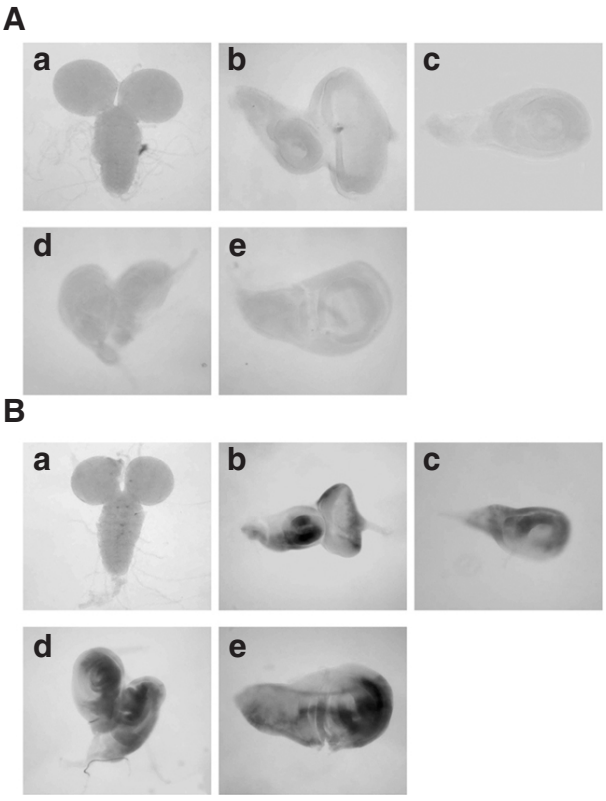

Fig. 6. X-gal staining of the imaginal discs in transgenic *Drosophila*. The imaginal discs at larval stage of transgenic flies transformed with the P1 (A) or P2 (B) promoter-lacZ fusion plasmids were stained with X-gal. a, brain discs; b, eye discs; c, haltere discs; d, leg discs; e, wing discs.

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