Identification and characteristics of the structural gene for the *Drosophila* eye colour mutant *sepia*, encoding PDA synthase, a member of the Omega class glutathione S-transferases

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The eye colour mutant *sepia* (*se¹*) is defective in PDA {6-acetyl-2-amino-3,7,8,9-tetrahydro-4*H*-pyrimido[4,5-*b*]-[1,4]diazepin-4-one or pyrimidodiazepine} synthase involved in the conversion of 6-PTP (2-amino-4-oxo-6-pyruvoyl-5,6,7,8-tetrahydropteridine; also known as 6-pyruvoyltetrahydropterin) into PDA, a key intermediate in drosopterin biosynthesis. However, the identity of the gene encoding this enzyme, as well as its molecular properties, have not yet been established. Here, we identify and characterize the gene encoding PDA synthase and show that it is the structural gene for *sepia*. Based on previously reported information [Wiederrecht, Paton and Brown (1984) J. Biol. Chem. **259**, 2195–2200; Wiederrecht and Brown (1984) J. Biol. Chem. **259**, 14121–14127; Andres (1945) Drosoph. Inf. Serv. **19**, 45; Ingham, Pinchin, Howard and Ish-Horowicz (1985) Genetics **111**, 463–486; Howard, Ingham and Rushlow (1988) Genes Dev. **2**, 1037–1046], we isolated five candidate genes predicted to encode GSTs (glutathione S-transferases) from the presumed sepia locus (region 66D5 on chromosome *3L*). All cloned and expressed candidates exhibited relatively high thiol transferase and dehydro-ascorbate reductase activities and low activity towards 1-chloro-2,4-dinitrobenzene, characteristic of Omega class GSTs, whereas only CG6781 catalysed the synthesis of PDA *in vitro*. The molecular mass of recombinant CG6781 was estimated to be 28 kDa by SDS/PAGE and 56 kDa by gel filtration, indicating that it is a homodimer under native conditions. Sequencing of the genomic region spanning CG6781 revealed that the *se¹* allele has a frameshift mutation from 'AAGAA' to 'GTG' at nt 190–194, and that this generates a premature stop codon. Expression of the CG6781 open reading frame in an *se¹* background rescued the eye colour defect as well as PDA synthase activity and drosopterins content. The extent of rescue was dependent on the dosage of transgenic CG6781. In conclusion, we have discovered a new catalytic activity for an Omega class GST and that CG6781 is the structural gene for *sepia* which encodes PDA synthase.

Key words: *Drosophila*, drosopterin, eye colour mutant, glutathione S-transferase, pyrimidodiazepine synthase, *sepia*.

INTRODUCTION

The eye colour pigments of *Drosophila melanogaster* have been the subject of many investigations since the discovery of the first eye colour mutant. Two classes of pigments, the brown 'ommochromes' and red 'drosopterins', are responsible for the typical eye colour of *Drosophila*. Drosopterins consist of at least five compounds that can be separated by TLC [1] and all are made up of a PDA {6-acetyl-2-amino-3,7,8,9-tetrahydro-4*H*-pyrimido-[4,5-*b*]-[1,4]diazepin-4-one or pyrimidodiazepine} portion and a pteridine portion [2–4].

The first step in drosopterin biosynthesis is the formation of H_2NTP [2-amino-4-oxo-6-(D-*erythro*-1′,2′,3′-trihydroxypropyl)-7,8-dihydropteridine triphosphate; also known as 7,8-dihydroneopterin triphosphate] from GTP by GTP cyclohydrolase I, encoded by the *Punch* gene [5,6]. H_2NTP is then converted into 6-PTP (2-amino-4-oxo-6-pyruvoyl-5,6,7,8-tetrahydropteridine; also known as 6-pyruvoyltetrahydropterin) by PTP synthase, the product of the *purple* gene [7–10]. A better understanding of the biosynthetic pathway was achieved in 1981 with the discovery of a new compound [11] that promotes the incorporation of ^{14}C-

labelled H_2NTP into drosopterin [12,13]. This compound was shown by Brown and co-workers [11] and Jacobson et al. [14] to be PDA, also known as 6-acetylhomopterin (Scheme 1).
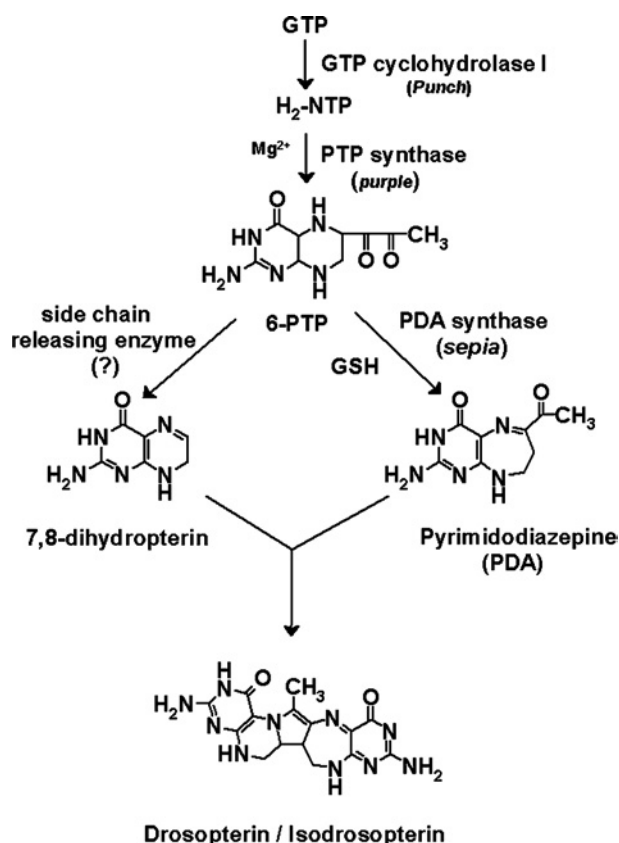
Wiederrecht and Brown [15] first identified PDA synthase activity and partially purified the enzyme from *Drosophila* heads. From an analysis of various eye colour mutants, they showed that at least three enzymes are involved in the conversion of H_2NTP into PDA [13]: one, called sepiapterin synthase A, present in limited amounts in *purple* [7,8]; a second, named PDA synthase, that is missing in *sepia*; and a third deficient in *clot*. Since addition of partially purified PDA synthase to heated extracts of *sepia* increased PDA synthesis to the wild-type level, they suggested that *sepia⁺* encodes the structural gene for PDA synthase. While *sepia* and *clot* have similar eye colour phenotypes, the 'leaky' ability of *clot* extracts to support the synthesis of PDA led to the suggestion that *clot* is involved indirectly in the conversion of H_2NTP into PDA. The *purple* gene product, later purified and characterized as 6-PTP synthase [9], is required for the Mg^{2+}-dependent conversion of the H_2NTP into 6-PTP [16]. This is converted into PDA by partially purified PDA synthase in an *in vitro* reaction that requires the presence of GSH at physiological

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Scheme 1   Proposed pathway of biosynthesis of drosopterins in *D. melanogaster*

concentrations and an additional thiol compound such as 2-mer-captoethanol. As 2-mercaptoethanol is no longer required under anaerobic conditions, it was proposed that, since PDA has two more electrons than 6-PTP, the reducing power required for the conversion of 6-PTP into PDA is supplied by GSH [13].

Recently, the structural gene for *clot* was identified in the course of a genetic analysis aimed at recovering P-element-induced mutations in the 25E region of chromosome 2 [17]. The predicted secondary structure, together with protein-domain database searches, indicated that the *clot* gene encodes a protein related to the glutaredoxin family of the thioredoxin-like enzyme superfamily.

In the present paper, we report identification and characteristics of the structural gene for PDA synthase, based on molecular and biochemical analyses. We have also rescued the phenotype of the se^1 mutant by transgenic expression of CG6781, thus confirming that CG6781 is the structural gene for *sepia*.

EXPERIMENTAL

Drosophila strains

Wild-type *D. melanogaster Oregon-R* was used to prepare enzyme fractions. Breeding populations of flies were maintained at $25 \pm 1\,°C$ on a standard yeast medium with propionic acid added as a mould inhibitor. Adult (0–2 days old) heads were separated from bodies by sieving and stored at $-80\,°C$. se^1 flies were obtained from the Bloomington Stock Center (Department of Biology, Indiana University, Bloomington, IN, U.S.A.; BL 1668); the mutant is described in FlyBase (http://flybase.bio.Indiana.edu/).

Cloning and expression of the putative gene

The coding sequences of candidate genes and the *clot* gene were amplified by RT (reverse transcriptase)–PCR. Total RNA was extracted from 0–2-day-old adult wild-type flies with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions, and PCR was carried out under the following conditions: one cycle of 94 °C for 4 min; 30 cycles of 94 °C for 1 min, annealing for each gene for 1 min, 72 °C for 1 min; and 1 cycle of 72 °C for 7 min using gene-specific primer sets. The sequences and annealing temperatures of primer sets used in the PCR reactions are shown in Table 1. PCR products were subcloned into the multiple cloning site of pET 15b (Novagen) and transformed into BL21 (DE3) cells. To express the proteins, transformants were cultured at 37 °C in 100 ml of LB (Luria–Bertani) medium containing 50 μg/ml ampicillin, and protein expression was induced by adding 0.5 mM isopropyl β-D-thiogalactoside at an absorbance (A) of 0.5. After 3 h, the cells were collected by centrifugation at 5000 g for 10 min and suspended in 4 ml of ice-cold Binding Buffer (pET system manual; Novagen). After sonication, the supernatant containing the soluble enzyme was collected by centrifugation at 10 000 g for 20 min at 4 °C, and purified with His · Bind Resin (Novagen) according to the manufacturer's instructions.

Assay of PDA synthase

PDA synthase activity was assayed spectrophotometrically by separating the reaction product, PDA, using reversed-phase HPLC. The standard reaction mixture (0.1 ml) contained 75 μM H_2NTP, 5 mM GSH, 70 mM 2-mercaptoethanol, 10 mM MgCl$_2$, 4 units of PTP synthase, 40 μg of each purified enzyme and 100 mM Pipes buffer (pH 7.5). After incubation at 30 °C for 2 h in the dark, the reaction was stopped by boiling for 5 min and the mixture was then centrifuged to remove precipitated proteins.

Table 1   Primers used for cloning

Restriction enzymes used were NdeI and BamHI for CG6781, CG6776 and CG6662, and NdeI and XhoI for CG6673A, CG6673B and Clot. Temp$_a$ is the annealing temperature used in the corresponding PCR reaction. Underlining indicates the recognition sequences for restriction endonucleases mentioned. The boldface letters indicate start and stop codons in each primer.

Clone	Forward primer (5′–3′)	Reverse primer (5′–3′)	Temp$_a$ (°C)
CG6781	CAT**ATG**AGTAACGGCAGGCATTTGGCAAAAG	GGATCC**TCA**GGCATCCTTGACCAGCAG	66
CG6673A	CAT**ATG**GCCCTGCCGCAAAAGCACT	CTCGAG**CTA**TGGTGTACCCTTGAAGGCAATGTC	66
CG6673B	CAT**ATG**GCCCTGCCGCAAAAGCACT	CTCGAG**TTA**CAAGGGCTGGAAGGCTACATCGTA	66
CG6776	CAT**ATG**AGTTCTGGTAAACATTTGGCCAAAGGTTC	GGATCC**CTA**AGCCAGCAGATCGTAGTTTGC	65
CG6662	CAT**ATG**AGCAATACTCAGCACTTAACTATTGGCTC	GGATCC**CTA**CCCCAATTTGACACGTTTG	61
Clot	CAT**ATG**GTGGTCACACACAACGTCAAGGGATAC	CTCGAG**TTA**ATCCTCATCCTCGAACATCATCTCG	63

A portion (0.07 ml) of each mixture was subjected to HPLC with a reversed-phase C_{18} column (Inertsil ODS-3: 4.6 mm × 250 mm; GL Sciences) connected to a photodiode array detector (Waters 996) and a scanning fluorescence detector (Waters 474). The material was eluted isocratically with 85 % (v/v) 50 mM ammonium acetate in 15 % (v/v) methanol at a flow rate of 1 ml/min. PDA was monitored by the absorbance at 383 nm.

Assay of GST (glutathione S-transferase)

GST activity with CDNB (1-chloro-2,4-dinitrobenzene) as substrate was measured as described by Habig et al. [18]. The standard reaction mixture contained 1 mM GSH, 0.1 M potassium phosphate buffer (pH 6.5) (mixture of 38.1 ml of 1 M K_2HPO_4 and 61.9 ml of 1 M KH_2PO_4, which was further diluted to 1000 ml with distilled water) and 100 μg of purified enzyme. The reaction was initiated by addition of 1 mM CDNB at 25 °C, and activity was measured as the increase in absorbance at 340 nm. Thiol transferase activity was measured by the method of Axelsson et al. [19] with HED (β-hydroxyethylene disulfide) as the substrate. The standard reaction mixture contained 0.2 mM NADPH, 1 mM GSH, 50 mM phosphate buffer (pH 7.6), 2 units of glutathione reductase and 30 μg of purified enzyme. The reaction was initiated by the addition of 2 mM HED at 30 °C, and activity was measured as the decrease in NADPH absorbance at 340 nm. Glutathione-dependent DHA (dehydroascorbate) reductase activity was determined by following DHA reduction spectrophotometrically at 265 nm. The standard reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 1 mM GSH and 5 μg of purified enzyme, and the reaction was initiated by adding 1 mM DHA after a 1 min pre-incubation at 30 °C [20]. All initial velocities with a range of substrates were measured within the range of 15–20 % consumption of each substrate.

Genomic sequencing

To perform PCR analyses, genomic DNA was isolated from adult flies. Thirty flies were homogenized in a glass homogenizer containing 400 μl of Buffer A (100 mM Tris/HCl, pH 7.6, 100 mM EDTA, 100 mM NaCl and 0.5 % SDS) and incubated for 30 min at 65 °C. After adding 800 μl of Buffer B (1.5 M potassium acetate and 4.5 M LiCl) to the homogenate, the material was thoroughly mixed, chilled in ice-water and centrifuged for 10 min at 20 000 **g**. The supernatant was retained and the DNA was precipitated with 0.6 vol. of propan-2-ol. CG6781 was amplified with the primer set 5′-CTATCACCACTTGCATCTCTGGACC-3′ and 5′-GGAACCGGTTATGGACTGCATTTAT-3′. PCR was carried out under the following conditions: one cycle of 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 56 °C for 45 s, 72 °C for 1 min 10 s; and 1 cycle of 72 °C for 7 min. The product was gel-purified and cloned into pGEM®-T Easy Vector (Promega). Two or three clones were sequenced using T7 and SP6 primers.

Construction of plasmids for transgenic rescue

A cDNA fragment of the ORF (open reading frame) of CG6781 was generated by RT–PCR. Total RNA was extracted with TRIzol® reagent from 30 pupae of *Oregon-R*; 2 μg of the total RNA was used as the template for first strand cDNA synthesis with RevertAid M-MuLV RT (MBI Fermentas) according to the manufacturer's instructions, and PCR was carried out with the following pair of primers: Bgl2-5′CG6781(5′-GG<u>AGATCT</u>ATGA-GTAACGGCAGGCAT-3′) and Kpn1-3′CG678(5′-<u>GGTACCA</u>-TCAGGCATCCTTGACCA-3′) (the underlining indicates the recognition sequences for restriction endonucleases mentioned). The PCR product was cloned into pGEM®-T-Easy vector and sequenced. A plasmid that had the correct sequence was selected.

It was digested with BglII and KpnI, and the resulting fragment was cloned into the BglII/KpnI site of pUAST vector to construct pUAST-CG6781.

P-element-mediated germline transformation and transgenic rescue

Transgenic flies were generated by microinjecting pUAST-CG6781 into w^{1118} embryos. The microinjection was carried out at Genexel (Daejeon, Korea). Multiple transgenic lines were generated and those with transgenes mapped to the second chromosome were used in the transgenic rescue experiments. For transgenic rescue of *sepia* phenotypes, UAS [upstream activation sequence (CGGAGTACTGTCCTCC), to which GAL4 binds in order to activate gene transcription] transgenic lines and a GMR-gal4 ('glass multiple reporter' promoter-dependent gal4 construct/transgene) driver line were crossed into the se^1 background by standard *Drosophila* genetic techniques using appropriate fly strains. For dosage–effect analysis, *y;GMR-gal4 UAS-CG6781/CyO;se* was generated by recombining the UAS transgene into the chromosome bearing GMR-gal4. Photographs of *Drosophila* eyes were taken with an Axiocam (Carl Zeiss) mounted on a Stemi 2000-C (Carl Zeiss).

Quantification of content of PDA and drosopterins

Boiled head extracts (20 heads) prepared in 0.05 ml of 100 mM Pipes buffer (pH 7.5) containing 5 mM GSH, 70 mM 2-mercaptoethanol and 10 mM $MgCl_2$ were analysed for their PDA content by HPLC. Portions (0.03 ml) were injected on to a reversed-phase C_{18} column (Inertsil ODS-3: 4.6 mm × 250 mm; GL Sciences) connected to the HPLC apparatus described above. UV absorbance was monitored at 383 nm and PDA content was determined by integrating the area of the PDA peak. Drosopterin content was assayed spectrophotometrically as described in [21]. Ten fly heads (five from male and five from female 2-day-old adults) split longitudinally into halves were placed in 1 ml of AEA [30 % (v/v) ethanol acidified with HCl to pH 2.0] for 30 h with agitation. After centrifugation followed by filtration through glass wool, the absorbance of the supernatant was measured at 482 nm.

Northern-blot analysis

Total RNA was extracted from wild-type *D. melanogaster* (*Oregon-R* strain) at various developmental stages using TRIzol® reagent. Poly(A)$^+$ (polyadenylated) mRNA was then purified using an Oligotex® mRNA mini kit (Qiagen). Aliquots (2.5 μg) of mRNA or 90 μg aliquots of total RNA were separated on agarose gels containing 2.2 M formaldehyde, and blotted on to Nytran® N membranes (Schleicher and Schuell) by the conventional capillary transfer method with a TurboBlotter system (Schleicher and Schuell). Blotted membranes were probed with a ^{32}P-labelled cDNA probe covering the full-length ORF of CG6781 using QuikHyb® hybridization solution (Stratagene), and autoradiographed. A probe recognizing the RNA coding for ribosomal protein 49 (*rp49*) was used as control to ensure that comparable amounts of RNA were loaded in all lanes.

Miscellaneous methods

The molecular mass of native enzyme was estimated with a Sephacryl HR S 200 column (1.5 cm × 85 cm) calibrated with standard proteins and Blue Dextran (2 000 000 Da). The column was developed with 0.1 M potassium phosphate buffer (pH 7.0) at a flow rate of 10 ml/h, and elution of the marker proteins was monitored with a Diode-Array spectrophotometer (Hewlett–Packard,
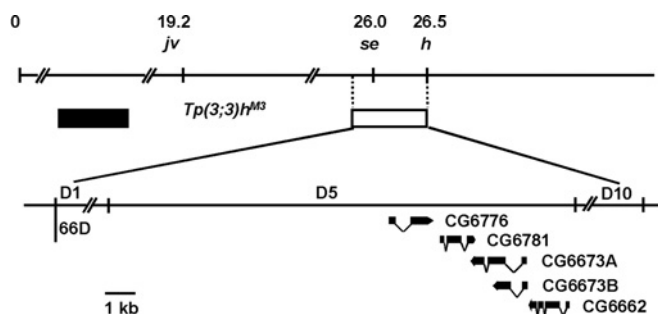
Figure 1   Genetic and cytological map of chromosome *3L* showing *sepia* and its environs

The fact that *sepia* is included in the deletion in *Tp(3;3)h^M3* predicts that the *sepia* gene is located in the interval 66D1 to 66D10 on the cytological map. The upper and lower solid lines indicate the genetic and cytological locations of the five candidate CGs of PDA synthase found in the 66D5 region. The open box represents the deletion that includes the *sepia* gene, and the filled box the material transposed in *Tp(3;3)h^M3*. The dotted vertical lines indicate the limits of the chromosomal deletion, while the solid oblique lines indicate their limits on the cytogenetic map. *jv* (*javelin*), *se* (*sepia*) and *h* (*hairy*) have been mapped by recombination to 3–19.2, 3–26.0 and 3–26.5 respectively. The breakpoints of *Tp(3;3)h^M3* include 61E, 66D1 − 3 and 66D10 on the cytological map, and its 66D10 break is located at − 10.8 to − 9.35 kb on the DNA map of *h*.

U.S.A.). Protein concentration was determined by the Bradford method [22], with BSA as the standard. SDS/PAGE was performed with SDS/4–20 % acrylamide gradient gels. After electrophoresis, proteins on the gel were visualized with Coomassie Brilliant Blue R.

RESULTS

Search for candidate PDA synthase genes at the *sepia* locus

Previous work [13] has shown that three eye colour mutants, i.e. *purple*, *sepia* and *clot*, are deficient in PDA as well as in the 'drosopterins'. *Purple* has a mutation in the structural gene for 6-PTP synthase [7,8] and the structural gene for *clot* is CG11024, which maps to 25E5 and encodes a protein related to the thioredoxin-like enzyme superfamily [17].

The *sepia* gene has been mapped by recombination to 3-26.0, very close to the 66D region on the cytological map [23]. In 1985, Ingham et al. [24] generated a new mutant allele, *Tp(3;3)h^M3*, in the course of a genetic analysis of the *hairy* locus. *Tp(3;3)h^M3* is associated with transposition to a more distal location on chromosome *3L* of material extending distally from the *hairy* locus and including a region whose breakpoints are at 61E, 66D1− 3 and 66D10 on the cytological map (Figure 1). The 66D10 break is located at − 10.8 to − 9.35 kb on the DNA map of *h*, which means that the *sepia* gene is in the 66D1 to 66D10 region [25]. It is also known that PDA synthase requires the presence of GSH, which is thought to supply electrons for the conversion of 6-PTP into PDA [13], and that it appears to be composed of two identical polypeptide chains of 24 kDa [15].

Based on this information, we began to search for the gene encoding PDA synthase. First, we analysed the biochemical functions of all the uncharacterized CGs (computed genes) in the putative *sepia* locus from 66D1 to 66D10, using the InterPro program (http://www.ebi.ac.uk/InterProScan/). Two main criteria were used: (i) high probability of interaction with glutathione and (ii) calculated molecular mass around 24 kDa. As a result, we identified five candidates, the products of CG6776, CG6781, CG6673A, CG6673B and CG6662 (Figure 1). All five products possess the functional domains of GSTs and are

highly similar to the Omega class of GSTs, such as human GSTO1 (Omega class GST 1) [Entrez Protein database (National Center for Biotechnology Information, U.S. National Library of Medicine) accession no. NP_004823], human GSTO2 (Entrez Protein database accession no. NP_899062), mouse GSTO1 (Entrez Protein database accession no. NP_034492) and rat GSTO1 (Entrez Protein database accession no. NP_001007603) (Figure 2). Members of this class have a unique N-terminal extension, and a cysteine residue at the active site, rather than the tyrosine and serine residues found in the active sites of other eukaryotic GSTs [26,27]. In addition to sequence homology, the calculated molecular masses of the products of these candidate CGs, approx. 28 kDa, were similar to the expected molecular size of PDA synthase.

Identification and characteristics of the gene encoding PDA synthase

We cloned the ORFs of the five candidate CGs into the pET 15b vector, expressed the proteins in *Escherichia coli* and purified them by Ni$^+$-affinity column. The conserved active site cysteine residue of Omega class GSTs is able to form a disulfide bond with glutathione and these enzymes have glutathione-dependent thiol transferase and DHA reductase activity, reminiscent of thioredoxin and glutaredoxin enzymes [26,27]. Although there were some differences among the five recombinant proteins, all had relatively high thiol transferase and DHA reductase activities and low activity towards CDNB, a general GST substrate (Table 2). These properties are characteristic of Omega class GSTs.

We assayed each recombinant protein for PDA-synthesizing activity as described in the Experimental section. Interestingly, only the product of CG6781 had this activity (Table 2 and Figure 3C), despite the high level of homology between the primary sequences of the five enzymes. The reaction product had a UV absorption spectrum identical with that of authentic PDA [14]. The molecular mass of the CG6781 recombinant protein was estimated to be 28 kDa by SDS/PAGE analysis (Figure 3A). Size-exclusion chromatography on a calibrated Sephacryl HR S 200 column gave its mass as 56 kDa (Figure 3B), indicating that the protein forms homodimer under native conditions.

se^1 has a frameshift mutation in the ORF of CG6781

Having identified CG6781 as the PDA synthase gene, we tested whether it is the locus of *sepia*. As a first step, we investigated the nature of the mutation in *se^1* by comparing the sequence of CG6781 in wild-type and *se^1* flies. We generated primers targeted to the untranslated regions at the 5′- and 3′-ends of CG6781 (Figure 4A) based on sequence information from the *Drosophila* Genome Annotation (release 4.1), and amplified and sequenced the genomic region of CG6781 as described in the Experimental section. This revealed the presence in the *se^1* mutant of a change of nt 190–194 (numbered from the ATG translation initiation codon), from AAGAA to GTG, leading to a frameshift. This frameshift creates a stop codon (TGA) at nt 273–275 in the second exon (Figure 4B). We also sequenced approx. 1 kb of the upstream region of CG6781, but detected no other mutations.

Transgenic expression of CG6781 in the eye rescues the phenotype of *se^1*

We next enquired whether expression of CG6781 *in vivo* is sufficient to rescue the mutant phenotype of *se^1*, utilizing the Gal4-UAS binary expression system [28]. The full ORF of CG6781 used in the *in vitro* enzyme assays was subcloned into pUAST, and transgenic *se^1* flies harbouring this construct, *UAS-CG6781*,

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CG6781     1  --MSNGRHLAKGSPMPDVPEDGILRLYSMRFCPFAQRVHLVLDAKQIPYHSIYINLTDKP
CG6776     1  --MSSGKHLAKGSPKPVLPDDGVLRLYSMRFCPYAQRAHLVLNAKNVPYHSVYINLTEKP
CG6662     1  --MSNTQHLTIGSPKPVFPDDGPILKLYSMRFCPYAHRVHLVLDAKQIPYHAIYINLRDKP
CG6673A    1  -MALPQKHFKRGSTKPELPEDGVPRFFSMAFCPFSHRVRLMLAAKHIEHHKIYVDLIEKP
CG6673B    1  -MALPQKHFKRGSPKPEIPEDGVLRYYSMRFCPYSQRAGLVLAAKKIPHHTVYIDLSEKP
mGSTO1     1  MSGESSRSLGKGSAPPGPVPEGQIRVYSMRFCPFAQRTLMVLKAKGIRHEVININLKNKP
rGSTO1     1  MSGASARSLGKGSAPPGPVPEGQIRVYSMRFCPFAQRTLMVLKAKGIRHEIININLKNKP
hGSTO1     1  MSGESARSLGKGSAPPGPVPEGSIRIYSMRFCPFAERTRLVLKAKGIRHEVININLKNKP
hGSTO2     1  MSGDATRTLGKGSQPPGPVPEGLIRIYSMRFCPYSHRTRLVLKAKDIRHEVVNINLRNKP
                                          ▲

CG6781    59  EWLLEKNPQGKVPALEIVREPGPPVLTESLLICEYLDEQYP-LRPLYPRDPLKKVQDKLL
CG6776    59  EWLVEVSPLLKVPALQLVAEKGEPSLIESLIIAEYLDDKYP-ENPLLPKDPLKRAQDKIL
CG6662    59  EWFSLVSSSTKVPALELVKEQGNPVLIESLIICDYLDEKYP-EVPLYPKDLLKKAQEKIL
CG6673A   60  EWYKDFSPLGKVPALQLTGVKDQPTLVESLIIAEYLDQQYP-QTRLFPTDPLQKALDKIL
CG6673B   60  EWYIDYSPLGKVPAIQLPNLPGQPALVESLVIAEYLDEQYPEGSLFPKDPLQKALDRIL
mGSTO1    61  EWFFEKNPLGLVPVLENSQGH---LVTESVITCEYLDEAYP-EKKLFPDDPYKKARQKMT
rGSTO1    61  EWFFEKNPFGLVPVLENTQGH---LITESVITCEYLDEAYP-EKKLFPDDPYEKACQKMT
hGSTO1    61  EWFFKKNPFGLVPVLENSQGQ---LIYESAITCEYLDEAYP-GKKLLPDDPYEKACQKMI
hGSTO2    61  EWYYTKHPFGHIPVLETSQCQ---LIYESVIACEYLDDAYP-GRKLFPYDPYERARQKML

CG6781   118  IERFR-AVLGAFFKASDGGD-------LEPFWSGLDIYERELARRGTEFFGGEQTGILD
CG6776   118  LERFS-SITSAFINILVQGTG------LEDYWTALDIFEEELTKRGTPYFGGNKPGFVD
CG6662   118  IERFG-QFINAFYYLLLHDNP---EQLVDTDHYAGLVVYEEELKRRCTKFFGGDSPGMLD
CG6673A  119  IERFAPVVSAIYPVLTCNPNA---PKDAINFETALDVFEVELGKRGTPYFAGGHIGIVD
CG6673B  120  IERLSPAVSAIYPVLFTK-NP---PADAIKNFETALDVFEQEITKRGTPYFGGNKIGIAD
mGSTO1   117  LESFS-KVPPLIASFVRSKRK-EDSPNLREALENEFKKLEEGMDN-YKSFLGGDSPSMVD
rGSTO1   117  FELFS-KVPSLVTSFIRAKRK-EDHPGIKEELKKEFSKLEEAMAKKRTAFFGGNSLSMID
hGSTO1   117  LELFS-KVPSLVGSFIRSQNK-EDYAGLKEEFRKEFTKLEEVLTNKKTTFFGGNSISMID
hGSTO2   117  LELFC-KVPHLTKECLVALRCGRECTNLKAALRQEFSNLEEILEYQNTTFFGGTCISMID

CG6781   169  YMIWPWCERLELLKLQRGEDYNYDQSRFPQLTLWLERMKRDPAVMAFYMEAEVQAEFLRT
CG6776   170  YMIWPWFERLSVIELKLQKEYNFNESRFPKITKWIALLKADSVVQSFYATPEQHNEFWRT
CG6662   174  YMMWPWCERFDSLKYTFEQKFELSPERFPTLIKWRDLMIQDRAVKCFYLDGQTHAKYMNS
CG6673A  176  YMIWPWFERFPSMKINTEQKYELDTKRFEKLLKWRDLMTQDEVVQKTALDVQLHAEFQKS
CG6673B  176  YMIWPWFERFPALKYTLDEPYELDKTRYQNLLKWRDLVAQDEAVKATALDARIHAKFMKT
mGSTO1   174  YLTWPWFQRLEALELK------ECLAHTPKLKLWMAAMQQDPVASSHKIDAKTYREYLNL
rGSTO1   175  YLIWPWFQRLEALELN------ECIDHTPKLKLWMATMQEDPVASSHFIDAKTYRDYLSL
hGSTO1   175  YLIWPWFERLEAMKLN------ECVDHTPKLKLWMAAMKEDPTVSALLTSEKDWQGFLEL
hGSTO2   176  YLLWPWFERLDVYGIL------DCVSHTPALRLWISAMKWDPTVCALLMDKSIFQGFLNL

CG6781   229  RSLGRPNYNLLVKDA------
CG6776   230  RKAGNANYDLLA---------
CG6662   234  RRSGQADYNMLYNEAKRVKLG
CG6673A  236  KTLGNPQYDIAFKGTP-----
CG6673B  236  RHENKPDYDVAFQPL------
mGSTO1   228  YLQDSPEACDYGL--------
rGSTO1   229  YLQDSPEACDYGL--------
hGSTO1   229  YLQNSPEACDYGL--------
hGSTO2   230  YFQNNPNAFDFGLC-------
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	CG6776	CG6662	CG6673A	CG6673B	mGSTO1	rGSTO1	hGSTO1	hGSTO2
					%identity/ %similarity			
CG6781	56/76	52/70	43/66	45/70	37/56	38/57	39/56	36/55
CG6776		49/72	47/70	50/74	36/54	34/55	35/57	33/51
CG6662			44/67	46/70	33/50	31/51	32/50	32/52
CG6673A				65/82	29/51	29/53	31/50	30/49
CG6673B					29/53	31/55	32/50	30/50

Figure 2   Multiple sequence alignment

The five candidates for PDA synthase were predicted to be Omega class GSTs using the InterPro program. Multiple sequence alignment of the candidates at 66D5 on the cytological map was performed with human GSTO1 (hGSTO1), human GSTO2 (hGSTO2), mouse GSTO1 (mGSTO1) and rat GSTO1 (rGSTO1) using the ClustalW program. Identical and semi-conserved amino acids are highlighted in black and grey respectively. The arrowhead indicates the putative binding site for the substrate GSH which is characteristic of Omega class GSTs. A % identity/% similarity matrix is shown at the bottom.

were generated. In order to drive overexpression in the eyes of these flies, we introduced the eye-specific GMR-gal4 driver into one such transgenic line by recombination and obtained *y;GMR-gal4 UAS-CG6781/+;se* flies. The photographs in Figure 5(A) demonstrate that *in vivo* expression of CG6781 rescues the *sepia* eye colour phenotype. We also measured the amount of PDA, PDA synthase activity and red pigments in these flies. The results in Figures 5(B) and 5(C) reveal that the levels of PDA, PDA synthase

activity and drosopterin pigments increased to 30, 49 and 77 % respectively of wild-type levels. To determine whether this partial rescue was due to an insufficient level of transgene expression, the dosage of the transgene was increased to two copies by an additional cross yielding *y;GMR-gal4 UAS-CG6781/UAS-CG6781;se* flies, as described in the Experimental section. This resulted in 43, 75 and 95 % rescue of PDA, PDA synthase activity and drosopterins levels respectively (Figures 5B and 5C).

Table 2   Enzyme activity of recombinant candidate CG products with various substrates

The activity for each substrate was determined under standard conditions as described in the Experimental section. All values are the means for three determinations. ND, not detectable.

	Specific activity (units/mg)				
	CG6781	CG6673A	CG6673B	CG6776	CG6662
PDA synthase*	124	ND	ND	ND	ND
GST activity for CDNB†	ND	0.01	0.08	ND	ND
Thiol transferase‡	5.62	2.83	54.26	1.06	0.55
DHA reductase§	3.00	74.34	133.29	0.71	0.26

* One unit of enzymatic activity is defined as the amount of enzyme needed for the formation of 1 nmol of PDA from H_2NTP in 2 h at 30 °C in the presence of excess PTP synthase.

† One unit of enzymatic activity is defined as the amount of enzyme catalysing the displacement of 1 μmol of chloride per min at 25 °C.

‡ One unit of enzymatic activity is defined as the amount of enzyme catalysing the formation of 1 μmol of GSSG per min at 30 °C.

§ One unit of enzymatic activity is defined as the amount of enzyme catalysing the formation of 1 μmol of DHA per min at 30 °C.

Figure 3   Molecular mass and PDA synthase activity of the CG6781 product

(**A**) Recombinant CG6781, purified with His · Bind resin and subjected to electrophoresis on an SDS/4–20 % acrylamide gradient gel, has a molecular mass of approx. 28 kDa. Molecular masses of standards are indicated. 1, standard markers; 2, crude extract before induction; 3, crude extract after induction; 4, purified enzyme. (**B**) The native molecular mass of the enzyme (○) was estimated to be 56 kDa by size-exclusion chromatography on a calibrated Sephacryl HR S 200 column (1.5 cm × 85 cm), as described in the Experimental section. Standard proteins (▲) used for calibration were: alcohol dehydrogenase (150 000 Da), BSA (67 000 Da), chymotrypsinogen A (25 000 Da) and cytochrome c (12 500 Da), and a calibration curve of log molecular masses of protein markers versus partition coefficients, K_{av}, was constructed. (**C**) PDA synthase activity of CG6781 was assayed as described in the Experimental section. The UV–visible spectrum of the reaction product eluted between 21 and 22 min was identical with that of PDA. The characteristic UV–visible spectrum of PDA determined with photodiode array detector (Waters 996) is shown.

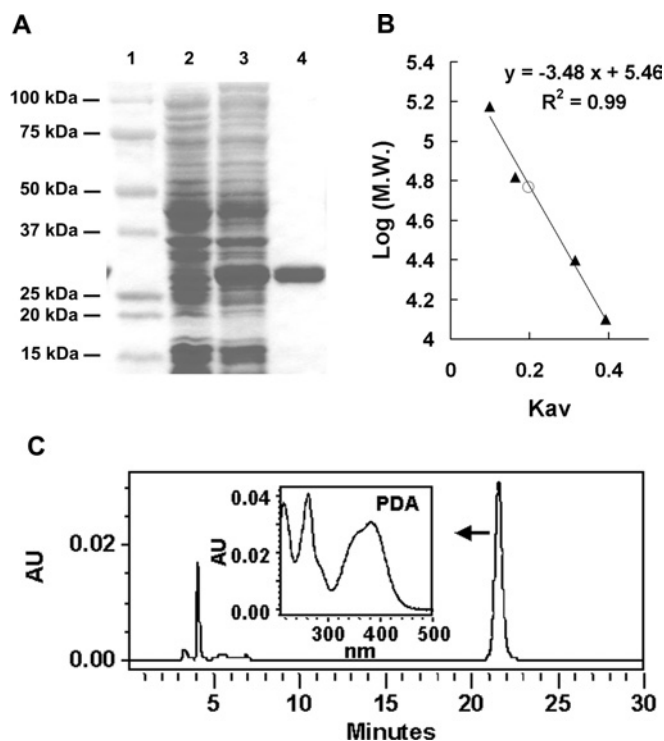## Expression of *CG6781* during development

We carried out a Northern-blot analysis to examine the expression profile of CG6781 during development and in adult flies. Blots were probed with a sequence covering the full-length *CG6781* ORF. A single species of mRNA (0.83 kb) was detected (Figure 6). This mRNA only appeared at the late pupal stage when it was present at a high level; it was also evident in the early adult (0–2 days old) at a substantially lower level (Figure 6A). Northern blots prepared separately from adult heads and bodies showed that CG6781 is expressed exclusively in the adult head (Figure 6B). It should be noted that GTP cyclohydrolase I [29] and 6-PTP synthase [30], which are involved in the biosynthesis of drosopterins, generally appear at their highest level in the late pupa and the newly emerged adult. Moreover, the developmental profile and head-specific expression of CG6781 agree well with the fact that the formation of red eye pigments starts during the late pupal stage and continues over several days in the eclosed fly [31].

DISCUSSION

Drosopterins are one of the major groups of pigments present in the eyes of *Drosophila*. They consist of a pentacyclic ring system in which two pteridine structures are joined by a pyrrole ring [2–4,32] and are produced non-enzymatically by one-to-one condensation of 7,8-dihydrolumazine or 7,8-dihydropterin with PDA [4,33]. PDA was first discovered as the 'quench spot' in fly heads because, unlike other pteridines, it quenches UV light at room temperature (25 °C), but fluoresces an intense bright green at very low temperature [34].

Wiederrecht and Brown [15], using partially purified fly head extracts, identified the enzyme activity responsible for conversion of 6-PTP into PDA, and demonstrated a requirement for glutathione for this reaction. They also suggested that the eye colour mutation *sepia* was in the structural gene for PDA synthase based on comparison of PDA synthase activity in relevant eye colour mutants. In the present study, we have for the first time identified and characterized the gene encoding PDA synthase, and provided strong biochemical and *in vivo* evidence that this gene, CG6781, is the structural gene mutated in *sepia*.

Based on previously reported information that PDA synthase requires glutathione and is composed of two identical subunits of 24 kDa [13,15], five candidate genes of PDA synthase were selected in the presumed *sepia* locus. These five candidates had already been classified as encoding Omega class GSTs based on amino acid sequence comparisons and phylogenetic analysis of GSTs [35]. We showed that all five products had thiol transferase and DHA reductase activity, reminiscent of thioredoxins and glutaredoxins and characteristic of Omega class GSTs [26,27]. Currently, insect cytosolic GSTs are divided into six classes, Delta (class I), Sigma (class II), Epsilon (class III), Zeta, Theta, Omega and unclassified [36]. Enzymes of the Omega, Zeta and Theta classes are relatively poorly characterized compared with the Delta, Epsilon [37,38] and Sigma [39] types. Our work establishes a new catalytic activity for the Omega class GSTs that CG6781 of *Drosophila* is involved in pteridine metabolism, in particular in the biosynthesis of red eye pigments. It is likely that the role of CG6781 is restricted to drosopterin biosynthesis, since (i) it is expressed only in the head in adults, (ii) it is expressed in the late pupa and newly emerged adult, and (iii) *sepia* has no other detectable phenotype than dark-brown eye colour. It is interesting to note that CG6673B has remarkably high DHA reductase activity (Table 2), and its active site motif (CPYS) is identical with that of human GSTO2 (Figure 2), whose DHA reductase activity is also 70–100-fold higher than that of

A

tatcaccacttgcatctctggacccgagcacgcacatc   −1

↓Exon1   ↓Exon2

```
 M   S   N   G   R   H   L   A   K   G   S   P   M   P   D   V   P   E    18
ATG AGT AAC GGC AGG CAT TTG GCA AAA GGC TCA CCC ATG CCG GAT GTT CCC GAA   54

 D   G   I   L   R   L   Y   S   M   R   F   C   P   F   A   Q   R   V    36
GAT GGT ATC CTT CGC CTG TAC TCG ATG CGC TTC TGC CCA TTT GCC CAA CGG GTG  108

 H   L   V   L   D   A   K   Q   I   P   Y   H   S   I   Y   I   N   L    54
CAT CTG GTC CTG GAC GCC AAG CAG ATC CCG TAT CAC AGC ATC TAC ATT AAT CTC  162

 T   D   K   P   E   W   L   L   E   K   N   P   Q   G   K   V   P   A    72
ACA GAC AAG CCG GAG TGG CTG CTG GAG AAG AAT CCA CAG GGC AAG GTG CCG GCT  216

 L   E   I   V   R   E   P   G   P   P   V   L   T   E   S   L   L   I    90
CTA GAA ATC GTG CGA GAA CCT GGA CCA CCT GTG CTC ACA GAG TCG CTA CTG ATT  270

 C   E   Y   L   D   E   Q   Y   P   L   R   P   L   Y   P   R   D   P   108
TGT GAA TAT CTG GAC GAG CAG TAT CCA TTG CGA CCA CTC TAT CCA CGT GAT CCG  324

 L   K   K   V   Q   D   K   L   L   I   E   R   F   R   A   V   L   G   126
CTG AAG AAA GTG CAG GAC AAG TTA CTA ATC GAG CGA TTT AGA GCG GTG TTA GGT  378

 A   F   F   K   A   S   D   G   G   D   L   E   P   F   W   S   G   L   144
GCC TTC TTC AAG GCA TCC GAT GGC GGT GAT CTG GAG CCC TTC TGG AGC GGC CTG  432

 D   I   Y   E   R   E   L   A   R   R   G   T   E   F   F   G   G   E   162
GAC ATC TAC GAA AGG GAG CTG GCT CGA CGT GGT ACG GAA TTC TTT GGT GGC GAG  486

 Q   T   G   I   L   D   Y   M   I   W   P   W   C   E   R   L   E   L   180
CAG ACG GGC ATT CTG GAC TAT ATG ATT TGG CCC TGG TGT GAG CGC CTC GAG CTC  540

 L   K   L   Q   R   G   E   D   Y   N   Y   D   Q   S   R   F   P   Q   198
CTT AAG TTG CAG CGT GGA GAG GAT TAT AAC TAC GAT CAG AGT CGC TTC CCC CAG  594
```

↓Exon3

```
 L   T   L   W   L   E   R   M   K   R   D   P   A   V   M   A   F   Y   216
CTG ACC CTT TGG CTG GAA CGC ATG AAA CGA GAT CCG GCT GTG ATG GCC TTC TAC  648

 M   E   A   E   V   Q   A   E   F   L   R   T   R   S   L   G   R   P   234
ATG GAG GCC GAG GTT CAG GCG GAG TTC CTG CGT ACA CGG AGC CTG GGT CGA CCC  702

 N   Y   N   L   L   V   K   D   A   *                                    243
AAT TAC AAT CTG CTG GTC AAG GAT GCC TGA tgcacttggtttattgaattttttcgcatgga  763

atgaataaaacataaatgcagtccataaccg                                          794
```

B
```
      190                                                          276
        K  N   P   Q   G   K   V   P   A   L   E   I   V   R   E   P   G   P   P   V   L   T   E   S   L   L   I   C   E
OR 5' [AAGAA]TCCACAGGGCAAGGTGCCGGCTCTAGAAATCGTGCGAGAACCTGGACCACCTGTGCTCACAGAGTCGCTACTGATTTGTGAA 3'

        V   S   T   G   Q   G   A   G   S   R   N   R   A   R   T   W   T   T   C   A   H   R   V   A   T   D   L   *
se¹ 5' [GTG]TCCACAGGGCAAGGTGCCGGCTCTAGAAATCGTGCGAGAACCTGGACCACCTGTGCTCACAGAGTCGCTACTGATTTGTGA    3'
```
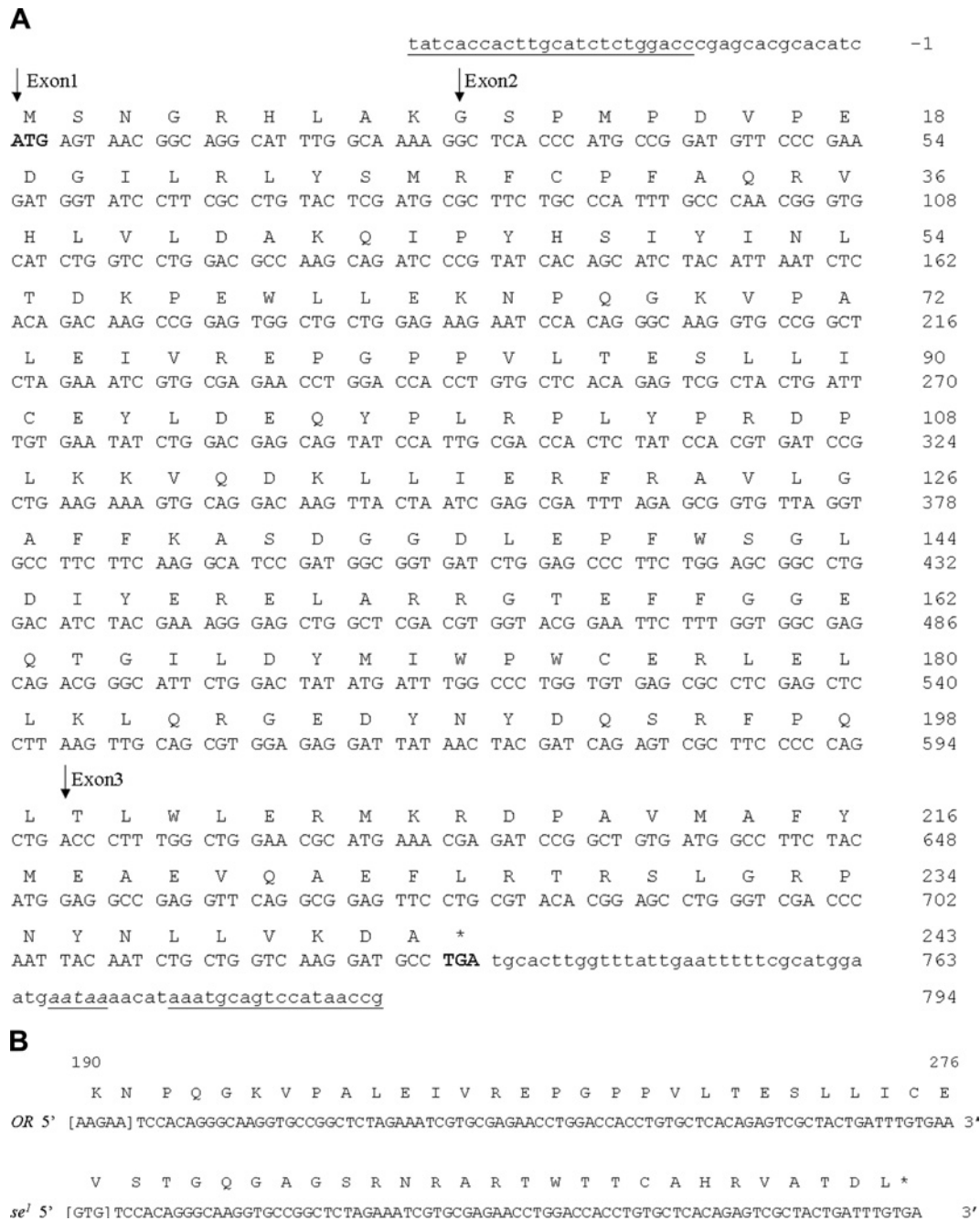
Figure 4   Nucleotide and amino acid sequence of the *se¹* allele of CG6781

(**A**) Nucleotide and deduced amino acid sequence of CG6781. The sequence of PDA synthase (CG6781) is based on the *Drosophila* Genome Annotation (release 4.1). CG6781 is composed of three exons, two introns and short untranslated regions at each end. The deduced amino acid sequence is shown above the nucleotide sequence. Lower-case letters represent the untranslated region of the gene. Start and stop codons are shown in boldface, and a putative polyadenylation signal is underlined and in italics. The right-hand side of the sequence gives both nucleotide and amino acid numbering. The beginning of each exon is indicated by downward-pointing arrows. The pair of primers used for genomic sequencing is underlined. (**B**) The mutation in the coding region of CG6781 in the *se¹* allele. The cDNA sequence of the coding region of the second exon from 190 to 276 (numbered from the ATG translation initiation codon) is shown. Single-letter amino acid abbreviations are shown above the nucleotide sequence. In *se¹*, nt 190–194, AAGAA, are changed to GTG, which leads to a frameshift mutation. This frameshift mutation produces a stop codon (TGA, underlined) at nt 273–275 in the second exon.

human GSTO1 [40,41]. The biological functions of other Omega class GSTs of *Drosophila* including CG6673B will be the subject of further studies.

Interestingly, in spite of the strong homology between the five candidates, only the CG6781 protein had PDA synthase activity. Recombinant CG6781 was shown to be a homodimer of 28 kDa subunits. It is well known that all soluble GSTs form dimers and that each subunit contains two independent binding sites. The first is specific for glutathione or a closely related homologue (the G site) and is formed from a conserved group of N-terminal amino acids. The other is the site that binds the hydrophobic substrate (the H site); it is much more variable structurally and is formed from residues in the C-terminal domain [36]. Since these features are present in all five candidates, structural differences in the
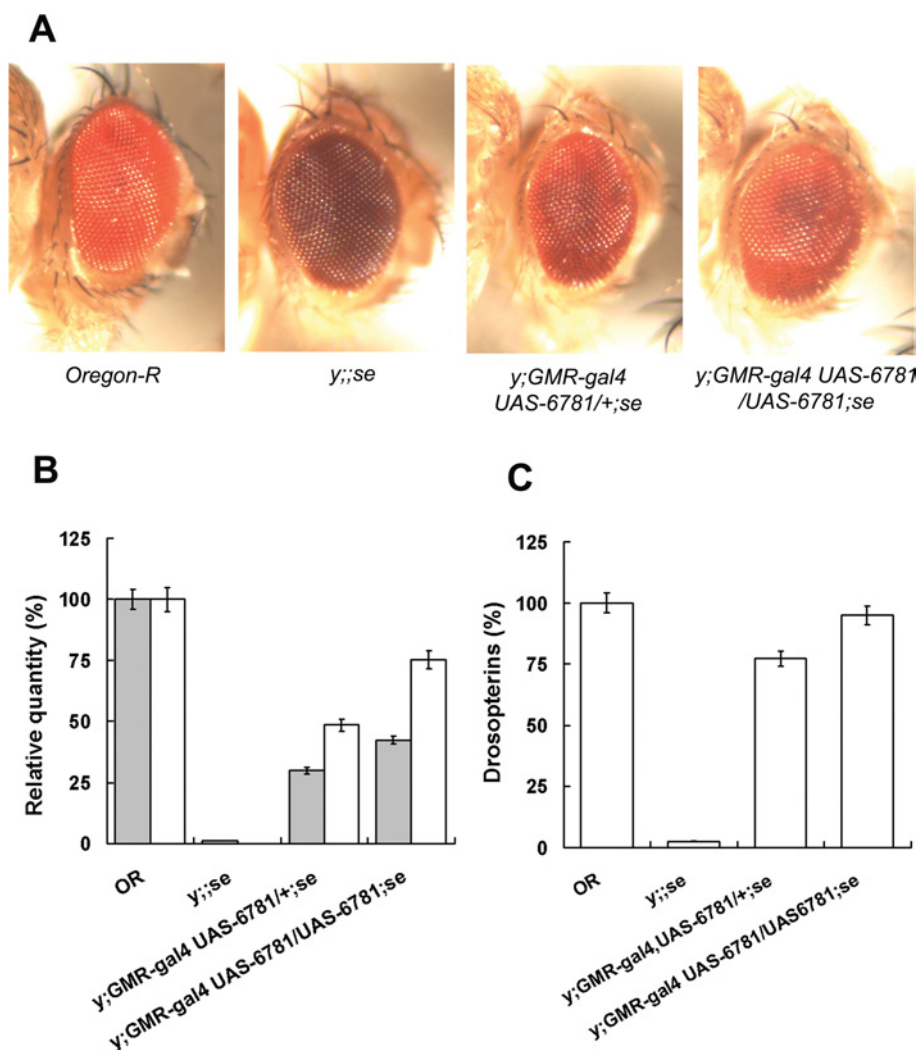
Figure 5   Phenotypic rescue of *se¹* by transgenic expression of CG6781 *in vivo*

The coding region of CG6781 was cloned into pUAST, which was then used to generate *UAS-CG6781* transgenic flies by microinjection. (**A**) Eye colour phenotypes of wild-type (*Oregon-R*), *sepia* mutant (*y;;se¹*), flies with one copy each of *GMR-gal4* and *UAS-CG6781* in the *se¹* background (*y;GMR-gal4 UAS-CG6781/+;se¹*), and flies with one copy of *GMR-gal4* and two copies of *UAS-CG6781* in the *se¹* background (*y;GMR-gal4 UAS-CG6781/UAS-CG6781;se¹*). (**B**) PDA content (grey bars) and PDA synthase activity (white bars) of adult fly heads of each genotype were determined by HPLC and enzyme assays respectively. (**C**) The drosopterin content of adult fly heads of each genotype was determined spectrophotometrically as described in the Experimental section. The extent of rescue defined by the content of PDA and PDA synthase activity (**B**) or of drosopterin (**C**) is dependent on the dosage of *UAS-CG6781*. Levels are calculated relative to wild-type flies. The vertical bars represent means ± S.E.M.

C-terminal domain may be responsible for the fact that only CG6781 has PDA synthase activity.

Since the first discovery of the naturally occurring *sepia* allele (*se¹*) in 1913 [42], the nature of this mutation has remained unknown. By sequencing CG6781, we found that nt 190–194 are changed from AAGAA to GTG in *se¹* and that this leads to a frameshift mutation causing a premature stop codon (TGA) at nt 273–275 in the second exon. Although more rare than spontaneous mutations caused by one or two base changes, this type of complex mutation, involving either insertions/deletions coupled with base substitutions, or insertions coupled with deletions, has been reported in a number of spontaneous frameshift mutations [43–45]. The mechanism involved is obscure. One possibility, suggested by Ripley [46], is that it is caused by quasi-palindromic sequences that could form imperfect hairpin loop structures in which the misaligned bases provide templates for either deletions (by endonucleolytic base excision) or insertions (by gap repair).

However, there is no detectable quasi-palindromic sequence in the neighbourhood of the *sepia* mutation.

Drosopterins are produced non-enzymatically by the condensation of PDA with 7,8-dihydropterin under acidic condition [33]. Due to the lower level of PDA synthase activity in the transgenic flies, PDA would be rate-limiting for the condensation reaction. Therefore drosopterins could accumulate in ommatidial structures as they are synthesized and attain nearly wild-type levels, whereas PDA would not only be in short of supply but would be continuously consumed, so that its steady-state concentration would be less than that of drosopterin (Figures 5B and 5C). Several eye colour gene products are involved in drosopterin biosynthesis. *Punch* is in the structural gene for the first enzyme, GTP cyclohydrolase I [5,6], and the second step in the pathway is catalysed by PTP synthase encoded by the *purple* gene [7,8,10]. The mutations *sepia* and *clot* affect the production of PDA from 6-PTP, and in the present study we have provided *in vitro* as well as *in vivo*
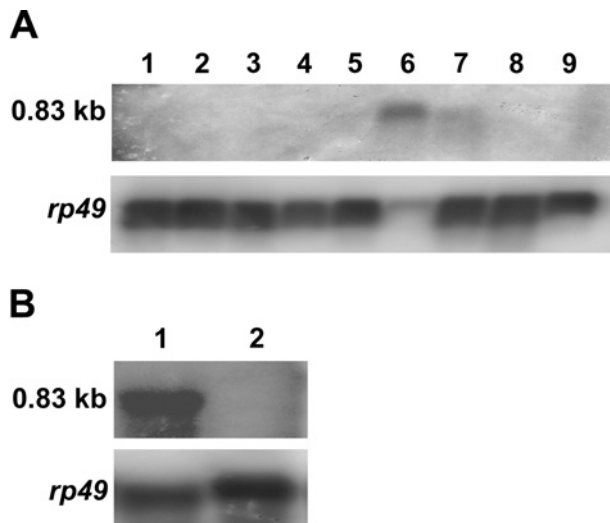
Figure 6 Expression of PDA synthase

(**A**) Poly(A)⁺ RNA (2.5 μg) extracted from wild-type *Drosophila* (*Oregon-R* strain) at different developmental stages was analysed by Northern blotting with a ³²P-labelled cDNA probe covering the full-length CG6781 ORF. Developmental stages were: 1, early embryos (0–9 h); 2, late embryos (9–18 h), 3, second instar larvae; 4, third instar larvae; 5, white pupae; 6, brown pupae; 7, 0–2-day-old adults; 8, 3–4-day old adults; 9, 5–6-day-old adults. (**B**) Total RNA (90 μg) extracted from 0–2-day-old adult fly heads (lane 1), and decapitated bodies (lane 2), was analysed by Northern blotting. The lower panel in (**A**, **B**) shows the results of hybridization of the same blot with a ³²P-labelled *rp49* cDNA probe.

evidence that the *sepia* gene is the structural gene for PDA synthase. The 'side-chain releasing enzyme' present in fly head extracts may participate in the conversion of 6-PTP into 7,8-dihydropterin, a component of drosopterin [47]. *clot* and *sepia* have similar phenotypes in that they contain neither drosopterins nor PDA, and both produce large amounts of sepiapterin [13]. It has been suggested that *clot* affects the conversion of H₂NTP into PDA indirectly because of the 'leaky' nature of its PDA synthase defect. Recently, the *clot* structural gene was shown to encode a protein related to the glutaredoxin class of the thioredoxin-like enzyme superfamily [17]. Glutaredoxin is a general GSH-disulfide oxidoreductase, catalysing thiol–disulfide exchange reactions. We have cloned the *clot* ORF and purified the recombinant protein. This did not exhibit PDA synthase activity on its own, nor did it have any effect on the production of PDA when combined with PDA synthase *in vitro* (results not shown). Whether other factors are needed for Clot to function, or whether its role is simply to balance redox potential for PDA synthesis, is not clear.

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