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Mutational Analysis of the Traffic ATPase (ABC) Transporters Involved in Uptake of Eye Pigment Precursors in *Drosophila melanogaster*

IMPLICATIONS FOR STRUCTURE-FUNCTION RELATIONSHIPS*

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The *white*, *brown*, and *scarlet* genes of *Drosophila melanogaster* encode three proteins that belong to the Traffic ATPase superfamily of transmembrane permeases and are involved in the transport of guanine and tryptophan (precursors of the red and brown eye pigments). We have determined the nucleotide sequences of two mutant *white* alleles (w^{co2} and w^{Bwx}) that cause reduced red pigmentation but have no effect on brown pigmentation. In w^{co2} the effect is only observed when interacting with the bw^6 allele or a newly isolated allele (bw^{T50}). These alleles of the *brown* gene were cloned and sequenced. In w^{co2} the codon for glycine 588 is changed to encode serine; in w^{Bwx} the triplet ATC encoding isoleucine 581 is deleted; asparagine 638 is changed to threonine in bw^6, and glycine 578 is changed to aspartate in bw^{T50}. No other relevant changes to the gene structures were detected. P-element-mediated germline transduction was used to construct a fly strain containing a *white* gene with a mutation of the nucleotide binding domain. Such flies had white eyes, indicating that the mutated *white* gene was unable to support either guanine or tryptophan transport. The implications of these mutations are discussed in terms of a model of the *Drosophila* pigment precursor transport system.

The red-brown eye color of wild-type *Drosophila melanogaster* is due to the biosynthesis and deposition, in the pigment cells of the eyes, of two pigment types; drosopterins, which are red colored and are synthesized from GTP, and ommochromes, which are brown and are synthesized from tryptophan (Summers *et al.*, 1982). According to a current model (discussed below), the precursors for these pathways, guanine and tryptophan, are transported into the pigment cells by separate membrane permeases that are members of the Traffic ATPase (terminology of Ames *et al.* (1990)) or ABC (terminology of Hyde *et al.* (1990)) family of membrane transporters.

Early work on the physiological characterization of *Drosophila* eye color mutant strains by Sullivan's group (Sullivan *et al.*, 1979, 1980; Sullivan and Sullivan, 1975) identified three genes, *white* (w^+), *scarlet* (st^+), and *brown* (bw^+), as being involved in the uptake of the pigment precursors by cells in developing eyes. Explanted tissues from mutants with null alleles at either *scarlet* (st^0) or *white* (w^0) had reduced capability to take up tryptophan, and tissue from mutants with null alleles at *brown* (bw^0) or *white* (w^0) had reduced capability to transport guanine. When subsequent cloning and sequencing of the cDNAs revealed that the w^+, bw^+, and st^+ genes encode three related proteins belonging to the Traffic ATPase family with hydropathies characteristic of membrane proteins, it was proposed that the guanine transporter contains heterodimers of subunits encoded by the *white* and *brown* genes, and the tryptophan transporter contains heterodimers of subunits encoded by the *white* and *scarlet* genes (O'Hare *et al.*, 1984; Pepling and Mount, 1990; Dreesen *et al.*, 1988; Tearle *et al.*, 1989).

Traffic ATPase transporters in general are involved in a wide variety of membrane transport processes in nature and include periplasmic permeases of bacteria (Ames, 1986; Cox *et al.*, 1988; Dassa and Hofnung, 1985); the yeast STE 6 protein, which transports the a-factor mating pheromone (Berkower and Michealis, 1991); as well as human proteins of medical significance; the cystic fibrosis transmembrane conductance regulator (CFTR)[1] (Collins, 1992); the P-glycoprotein responsible for development of multidrug resistance in tumor cells (Higgins and Gottesman, 1992; Chen *et al.*, 1986; Gros *et al.*, 1986); and the proteins involved in delivering antigenic oligopeptides to the major histocompatability complexes in the endoplasmic reticulum (Powis *et al.*, 1992). The guanine and tryptophan transporters of *Drosophila* are unusual among the eukaryotic Traffic ATPases in that they transport their substrates into the cell rather than pumping molecules out of the cell (see Higgins, 1992).

Comparison among Traffic ATPase proteins reveals that, although the conservation of amino acid sequence may be low between any two members of the family, the overall predicted structural arrangement is highly conserved (Higgins, 1992). Consequently, the general model of these transporters indicates that they contain four domains; two nucleotide binding folds located in a hydrophilic portion of the complex and two membrane spanning regions where the polypeptide chain is predicted to form five or six membrane spanning α-helices (Hyde *et al.*, 1990). An additional domain on a separate subunit, called the periplasmic substrate binding protein, is present in the bacterial periplasmic permease complexes, but involvement of such a subunit has not been established for any eukaryotic members of the family. The nucleotide binding folds are conserved in many proteins that bind ATP or GTP and they are

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[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s).

characterized by a stretch of around 200 amino acids containing two highly conserved smaller sequence elements corresponding to the Walker A and B motifs (Walker *et al.*, 1982). The role of the nucleotide binding folds in the Traffic ATPase transporters is presumably to provide the energy, by hydrolysis of ATP, to drive conformational changes in the proteins that are necessary for the transport mechanism. The requirement for ATP hydrolysis has been convincingly demonstrated for the histidine permease of *S. typhimurium* (Ames and Joshi, 1990) and nonhydrolyzable analogues of ATP have been shown to be incapable of supporting chloride ion transport by CFTR (Anderson *et al.*, 1991a; Anderson and Welsh, 1992).

Although the four domains are always present, they can be located on from one to four polypeptides, depending on the individual transporter. In the case of the *Drosophila* tryptophan and guanine transporters, each of the putative subunits encoded by the *white*, *brown*, and *scarlet* genes contains one nucleotide binding fold and one transmembrane domain. The apparent requirement for at least two copies of each domain in the native Traffic ATPase transporters is consistent with the proposed heterodimeric composition of the *Drosophila* transporters discussed above (see also Dreesen *et al.*, 1988).

In this paper we describe the identification of nucleotide changes in two alleles each of *white* and *brown* that affect function of the *Drosophila* guanine transporter, but not the tryptophan transporter. The corresponding changes to the amino acid sequences are all located in the C-terminal hydrophobic portion of the two proteins. In addition, we report site-directed alterations of highly conserved amino acids in the nucleotide binding domain of the *white*-encoded subunit that cause loss of function of both the guanine and tryptophan transporters.

EXPERIMENTAL PROCEDURES

Fly Strains—The w^{co2} *v*,bw^{6} fly strain was obtained from Dr. James Farmer (Brigham Young University, Provo, UT). The strain containing w^{Bwx} was obtained from the *Drosophila* Stock Center, California Institute of Technology, Pasadena, CA. The strain T50, which contained the novel bw^{T50} allele, came from the wild-type stock collection of Prof. J. Gibson (The Australian National University, Canberra, Australia).

Amplification of white and brown Gene Fragments from Genomic DNA—Genomic DNA was isolated from approximately 100 flies according to the method of Lifton as modified from Bender *et al.* (1983) and was quantitated by measurement of A_{260}. Oligonucleotides to be used as primers for PCR and sequencing experiments (see Table I) were designed by reference to the *white* gene sequence of O'Hare *et al.* (1984) or the *brown* cDNA sequence of Dreesen *et al.* (1988). PCR amplifications were performed on a Perkin-Elmer Cetus DNA thermocycler in 50-μl reaction volumes containing template genomic DNA (250 ng), primers (20 pmol of each), dNTPs (200 μM each), and thermostable DNA polymerase from *Pyrococcus furiosis* (native *Pfu* polymerase; 1.25 units; from Stratagene) in the recommended buffer supplied with the enzyme. The temperature profiles programmed to the thermocycler for amplification experiments were as follows: for *white* gene fragments, 30 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 3 min; for *brown* gene fragments, 35 cycles of 94 °C for 45 s, 63 °C for 1 min, 72 °C for 3 min.

Recombinant DNA Technology Using Escherichia coli—The w^{co2}, w^{Bwx}, bw^{6}, and bw^{T50} gene fragments produced by PCR were excised from agarose gels and purified using the Geneclean II kit (BIO 101, Inc.). They were then treated with suitable restriction enzymes to cleave the sites incorporated into 5′ ends of the oligonucleotide primers (see Table I) and ligated into the pBluescript SK⁺ (Stratagene) vector which had been prepared by cleavage with the same enzymes. Plasmid DNA for double-stranded sequencing was prepared from three to five independent XL1-Blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F′*proAB, lacI*^{q}ZΔ*M15,* Tn*10*(tet^{r})]) transformant colonies by the alkaline lysis method of Ish-Horowicz (Sambrook *et al.*, 1989).

DNA Sequencing—The Sequenase version 2 kit (U. S. Biochemicals Corp.) was used to perform DNA sequencing of alkali-denatured plasmid DNA using the dideoxy chain-terminating method of Sanger *et al.* (1977). Routine sequence reactions were performed with [α-^{35}S]dATP, and the 7-deaza-dGTP labeling and termination mixes were used to minimize the number of regions containing compressions. Any remaining compressions were resolved with the dITP-containing mixtures. To prepare the template for each sequencing reaction, the double-stranded DNA (~4 μg) was incubated for 5 min at room temperature in the presence of 200 mM NaOH, 0.2 mM EDTA in a final volume of 20 μl. Then ammonium acetate was added (2 μl of a 2 M solution at pH 4.6) to neutralize the reaction, and the DNA was precipitated with 3 volumes of ethanol. Prior to sequencing, the denatured plasmid was further purified using the Geneclean II kit, omitting the agarose gel electrophoresis step. This final step was found to improve the quality and consistency of the sequence data. The sequencing strategies for the *white* and *brown* alleles are depicted in Figs. 1 and 2, respectively.

Although the thermostable *Pfu* DNA polymerase is known to be a high fidelity polymerase due to the presence of a proofreading function (Lundberg *et al.*, 1991), all the mutations observed were confirmed in at least three independent clones of the relevant PCR fragment to protect against the possibility that they were changes introduced by the enzyme during PCR.

Site-directed Mutagenesis of the white Gene and P-element-mediated Transformation of Drosophila—The *E. coli* plasmid pRK3 was obtained from Dr. P. Schedl (Princeton University, Princeton, NJ). It harbors a P-element vector containing the *white* minigene and regulatory sequences flanked by the boundary domains scs and scs′ that insulate the *white* gene against chromosomal position effects (Kellem and Schedl, 1991). To construct a P-element transformation vector with a selectable marker independent of the *white* gene, the bacterial neomycin resistance gene under control of the *Drosophila* hsp70 promoter was isolated from the plasmid pUChsneo (Steller and Pirotta, 1985) as a 1.6-kb *AseI*/*Hin*dIII fragment and ligated into the unique *Xho*I site of pRK3 between the scs element and the *white* regulatory sequences. After treatment with Klenow to create blunt termini the vector and insert DNA fragments were ligated together, the resultant vector is called pRK3hsneo (Fig. 3).

Site-directed mutagenesis of the ATP binding site of the *white* gene was performed using Amersham's *in vitro* mutagenesis kit (version 2.1). A 1.8-kb *Xba*I/*Sac*I fragment of pRK3hsneo containing the 5′ region of the *white* gene was subcloned into the phagemid pBluescript SK⁺, and single-stranded DNA was isolated in the strain XL1-Blue after infection with M13 KO7 helper phage (Vieira and Messing, 1987). The mutagenic oligonucleotide 91–122 (Table I) was annealed and all reactions were performed as described in the kit's instruction manual, with the exception that *Pvu*I was used for the nicking step prior to T5 exonuclease treatment. An *Xba*I/*Sac*I fragment containing the desired mutation was then subcloned back into pRK3hsneo, replacing the corresponding wild-type sequence and producing the plasmid pRK3hsneo.mut1.

P-element-mediated transformation of *D. melanogaster* was performed essentially as described by Spradling (1986). Freshly laid eggs were obtained from a *w*; P[*ry*⁺(Δ2–3)] (99B) strain and injected with either pRK3hsneo (P[w^{+}; *neo*^{r}]) or pRK3hsneo.mut1 (P[w^{mut1}; neo^{r}]). Surviving G0 flies were crossed to a w^{1118} strain, and germline transformants were selected on the basis of eye color and/or G418 (0.2 mg/ml) resistance, as described under "Results." This level of the drug was initially chosen for the selection of transformants, on the basis of preliminary experiments to determine the minimum concentration of G418 that neither of the w^{0} background strains were able to survive. It was subsequently endorsed by the result that when the G1 progeny of P[w^{+}, *neo*^{r}] recipient flies that had wild-type eye color were crossed to the w^{1118} strain and the G2 progeny grown on 0.2 mg/ml G418, only flies with colored eyes survived. To confirm the presence of the *white* P-element construct in transformants, genomic DNA was isolated from individual flies (Jowett, 1986), and PCR was performed using a Corbett capillary thermocycler as detailed in the legend of Fig. 4.

Materials—All enzymes and chemicals used were of the highest quality available. Oligonucleotides were synthesized by the Biomolecular Resource Facility, The Australian National University, Canberra, Australia. [α-^{35}S]dATP was obtained from Amersham (Australia) Pty. Ltd. All standard molecular biological methods not otherwise described were performed essentially as described in Sambrook *et al.* (1989).

RESULTS

Amino Acid Substitutions in the Interacting Alleles w^{co2} and bw^{6}—The specific interaction between the eye pigmentation alleles w^{co2} and bw^{6} was first described by Farmer and Fairbanks (Farmer, 1977; Farmer and Fairbanks, 1986). Characterization of the mutations in these alleles is of particular interest because both are phenotypically silent except in w^{co2},bw^{6}

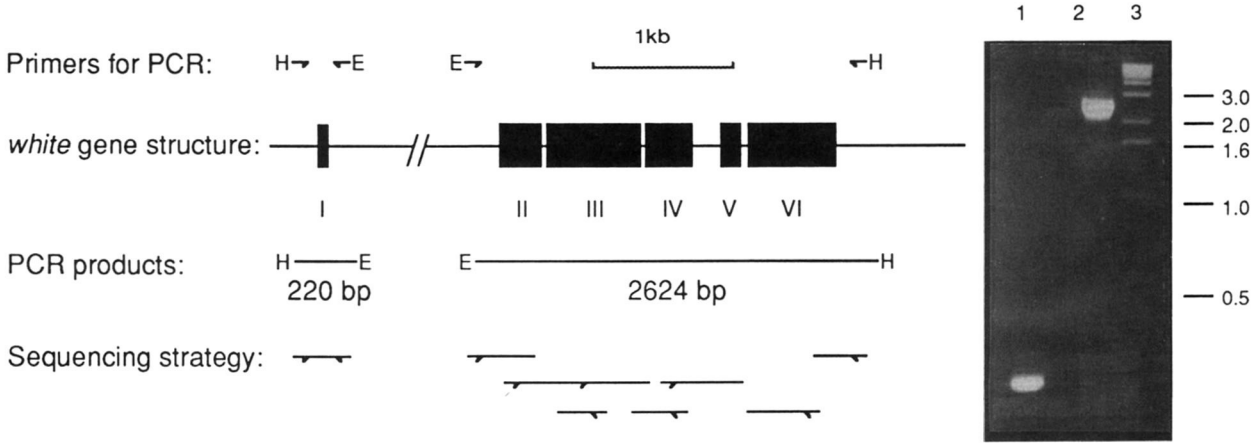

FIG. 1. **Strategy for amplification, cloning, and sequencing of *white* gene fragments from genomic DNA.** Genomic DNA prepared from mutant fly strains was used as template for PCR reactions, as described under "Experimental Procedures," to amplify the coding regions of the *white* gene as two fragments: exon 1 (220-bp fragment; primers 91–134 and 91–135) and exons 2–6 (2624-bp fragment; primers 91–136 and 91–73). Each of the four primers used had the recognition sequence for restriction by either *Eco*RI (*E*) or *Hin*dIII (*H*) incorporated at their 5′ ends to aid in the subsequent cloning of the PCR-generated fragments to pBluescript SK⁺ for sequencing. The additional primers used and the indicated sequencing strategy allowed the complete sequence of all *white* exons to be determined. The *inset gel photograph* shows the PCR products generated from DNA from flies containing the w^{co2} allele: *lane 1*, the exon 1 fragment; *lane 2*, the exons 2–6 fragment; *lane 3*, molecular weight markers with sizes (kb) shown to the *right*. Similar results (not shown) were obtained for amplifications from the w^{Bwx} allele.

double mutant flies that contain no other functional alleles of *white* or *brown*. In that case, flies with brown eyes are observed due to the almost complete absence of the red pteridine pigments. Flies homozygous or hemizygous (*white* is an X-linked gene) for w^{co2} have apparently wild-type eyes in the presence of just one copy of bw^+, and similarly, flies homozygous for bw^6 appear wild-type if w^+ is present. In terms of the heterodimer model of the *D. melanogaster* eye pigment precursor permeases (O'Hare *et al.*, 1984; Pepling and Mount, 1990; Dreesen *et al.*, 1988; Tearle *et al.*, 1989), these observations clearly indicate that both the w^{co2} and bw^6 alleles must produce proteins that are capable of folding and assembling into the membrane in a near wild-type conformation. Apparently, a guanine transporter containing one or the other of the mutant w^{co2} and bw^6-encoded subunits is functional. Only when both subunits of the transporter are mutant is activity severely compromised. Furthermore, the observation that ommochrome levels are close to wild-type in w^{co2},bw^6 homozygous flies is evidence that the w^{co2}-encoded protein is able to dimerize normally with the *scarlet*-encoded subunit to form an active transporter for tryptophan.

The strategy for determining the nucleotide sequence of the protein coding regions of the w^{co2} allele is depicted in Fig. 1. The w^{co2} gene was amplified by PCR from genomic DNA as two fragments: one fragment of 220 bp containing exon 1 flanked by short stretches of 5′-untranslated and intron 1 DNA and another fragment of 2624 bp containing exons 2–6 and corresponding untranslated DNA. The PCR products were cloned for sequencing as described under "Experimental Procedures," and it was consequently confirmed that they contained the expected regions of the w^{co2} gene.

By comparison with the wild-type genomic (O'Hare *et al.*, 1984) and cDNA (Pepling and Mount, 1990) sequences, the w^{co2} sequence was identical with the exception of a single A to G transition at position 5311 in exon 6 in the genomic sequence. This change results in the amino acid substitution G588S in the encoded protein.

Because the *brown* genomic sequence has not been published and hence the arrangement of exons and introns was not known to us, we designed PCR primers for the amplification of bw^6 sequences entirely with reference to the wild-type (Oregon-R strain) cDNA sequence (Dreesen *et al.*, 1988). We opted to attempt amplification of this allele in two overlapping fragments (see Fig. 2) and hence designed two pairs of primers for the purpose; the pair 92–57/92–64 for the 5′ end of the gene, and the pair 92–63/92–58 for the 3′ end (see Table I for details of the primers). The PCR experiments with these pairs produced DNA fragments of ~1.1 and ~1.6 kb, respectively, both of which were a little larger (due to the presence of introns) than the sizes of the regions flanked by the oligonucleotides in the cDNA sequence (989 and 1358 bp, respectively). In preparation for cloning of these DNA fragments into pBluescript, they were digested with *Eco*RI and *Hin*dIII which revealed the presence of an internal *Hin*dIII site in the ~1.1-kb PCR product for the 5′ end of the gene. Consequently this part of the gene had to be cloned as two fragments: one of ~550 bp with *Eco*RI and *Hin*dIII termini and another of ~500 bp with two *Hin*dIII ends (see Fig. 2). The PCR product encompassing the 3′ end of the *brown* gene was cloned as a single fragment of ~1600 bp. To obtain the entire sequence of this fragment, it was necessary to synthesize an additional oligonucleotide (92-67; Table I) and to construct another plasmid in which the 3′ portion of bw^6 was deleted up to a unique *Cla*I site (known to be present from the cDNA sequence), so that additional sequence could be obtained using the universal priming site in the pBluescript multiple cloning site.

The presence of sequences corresponding to the *brown* cDNA was confirmed in all three cloned PCR fragments. Analysis of the complete sequence revealed that the protein-encoding region of the *brown* gene is split into eight exons interrupted by seven small introns (Fig. 2), the largest being intron 1 of 89 bp, and the smallest being intron 6 of 52 bp.

Comparison with the published wild-type *brown* cDNA sequence revealed five single base differences in the coding region of the bw^6 allele (Table II). One of these results in the substitution of a threonine for asparagine at position 638 of the putative amino acid sequence. The other four observed differences are silent with respect to the amino acid sequence and probably represent polymorphisms due to the different background strains being compared.

Isolation and Characterization of a Second w^{co2}-interacting Brown Allele—From a survey of wild-type and laboratory *D. melanogaster* stocks in crosses to the w^{co2} strain, Farmer (1977) observed a surprisingly high incidence of strains carrying an allele that behaved like bw^6 (failing to suppress w^{co2}). Accordingly, in an attempt to identify other bw^6-like alleles, we screened our laboratory strains with apparently normal eye

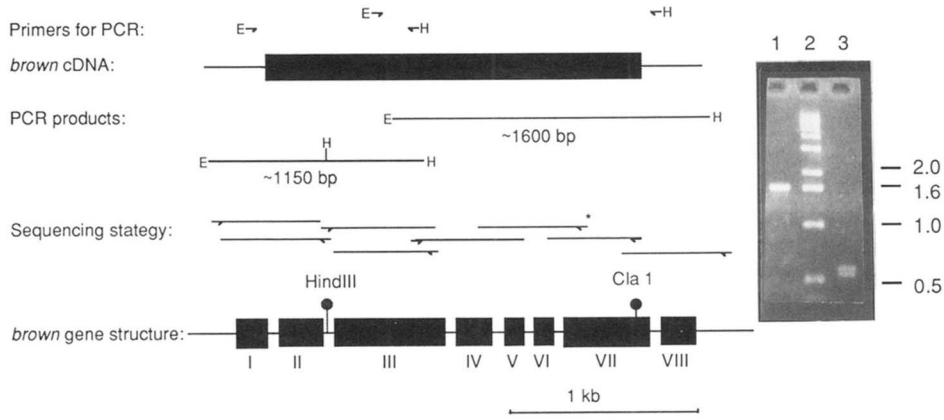

FIG. 2. **Strategy for amplification, cloning, and sequencing of *brown* gene fragments from genomic DNA.** The relative locations of binding in the cDNA are shown for the two pairs of primers used for amplification (92–57 and 92–64 for the 5′ end and 92–63 and 92–58 for the 3′ end) of bw^6 and bw^{T50} allele sequences. The *Eco*RI (*E*) or *Hin*dIII (*H*) restriction enzyme recognition sequences incorporated in their 5′ ends are also indicated. In the diagram of cDNA structure, the *thick line* indicates the protein coding sequences. The bw^6 PCR products, after digestion with *Eco*RI and *Hin*dIII, are shown in the *inset gel photograph*: *lane 1*, the 3′-terminal fragment; *lane 2*, size markers; *lane 3*, the 5′-terminal fragments (an additional site for *Hin*dIII in the ~1150 bp product was seen). The three fragments were cloned to pBluescript SK$^+$ and sequenced as discussed in the text. The region sequenced from the primer 92–67 is indicated as starting with the *asterisk*. All other sequence data was obtained using either the PCR primers or the universal or reverse sequencing primers that have binding sites in the pBluescript vector. The deduced arrangement of exons and introns in the *brown* gene is illustrated in the *lower portion* of the diagram.

TABLE I

Primers used in PCR, mutagenesis, and sequencing reactions

Primer	Sequence (5′ to 3′)[a]	Site of 3′ base binding[b]	Used for...	Strand[c]
white gene				
91–134	TTGAAGCTTGAGTGATTGGGGTG	−51	Exon 1 PCR	+
91–135	GCAGAGAATTCGATGTTGCAATCGC	123	Exon 1 PCR	−
91–136	AACCGAATTCGTAGGATACTTCG	3132	Exon 2–6 PCR	+
91–73	GATGAAGCTTATCTTGTTTTTATTGGCAC	5714	Exon 2–6 PCR	−
91–TW5	CGGCAGCTGGTCAACCGGACA	3409	Sequencing	+
91–68	CCACGACATCTGACCTATCG	3824	Sequencing	+
91–69	ACACCTACAAGGCCACCTGG	4886	Sequencing	+
91–70	GATCGTGTGCTGACATTTGC	3872	Sequencing/PCR	−
91–71	CTTTTACGAGGAGTGGTTCC	4537	Sequencing	−
91–72	GATGTGCAGCTAATTTCGCC	5431	Sequencing	−
91–122	GTTCCGGTGCCCTGCAGACGACCCTG	3615	Mutagenesis	+
brown gene				
92–57	GGCGAGAATTCGGCACATCACATAGC	−60	5′ end PCR	+
92–64	TGAAAAGCTTGAAGATGTCCGACGTCG	878	5′ end PCR	−
92–63	GCAGGAATTCCATCGAGATGGAGGTCG	759	3′ end PCR	+
92–58	AAATTAAGCTTAAGCAAGTTCTGTAAC	2066	3′ end PCR	−
92–67	TAGTAGGCGGACAGGCTGTAGGTG	1464	Sequencing	−

[a] Recognition sequences for restriction by *Eco*RI, *Hin*dIII and *Pst*I endonucleases are underlined.

[b] Binding sites are numbered with +1 as the A of the translation start codon in the published *white* genomic (O'Hare *et al.*, 1984) and *brown* cDNA (Dreesen *et al.*, 1988) sequences.

[c] + indicates the coding strand; − indicates the complementary strand.

TABLE II

Sequence differences between the mutant and wild-type white and brown alleles

Allele	Phenotype	Mutation in DNA[a]	Effect on amino acid sequence
w^{co2}	Interaction with bw^6 and bw^{T50} reduces red pigments	G to A (5311)	Gly^{588} to Ser
bw^6	Interaction with w^{co2} reduces red pigments	A to C (1913)	Asn^{638} to Thr
		A to T (822)	Silent
		A to C (843)	Silent
		A to G (1335)	Silent
		A to C (1365)	Silent
bw^{T50}	Interaction with w^{co2} reduces red pigments	G to A (1733)	Gly^{578} to Asp
w^{Bwx}	Brown eye color due to reduced red pigments	Δ(5290)ATC	Ile^{581} deleted

[a] The nucleotide positions in parentheses are relative to the first base of the start codon in the published *white* genomic (O'Hare *et al.*, 1984) and *brown* cDNA (Dreesen *et al.*, 1988) sequences.

color, as follows. Male flies from our various strains (bw^*/bw^*; where * indicates an unidentified allele) were mated to female w^{co2} v;bw^6 flies and eye color of the male progeny (w^{co2} v/Y; bw^6/bw^*) was assessed. Males with reduced red pigments were carriers of a potentially novel w^{co2}-interacting allele. The vermilion (v) marker was incorporated in the screen to block ommochrome synthesis and thereby enable small changes in the level of pteridine pigments to be more easily observed.

From a total of the 36 strains that were screened in this way, one of the crosses produced male flies with yellow eyes among the progeny. These flies contained a bw^6-like allele, which we have named bw^{T50} (after the strain T50 from which it was

isolated). To rule out the possibility that this new allele was a *brown* null, flies from the T50 strain were also crossed to bw^0 (w^+ v^+) flies. All of the resulting progeny had wild-type eye color, consistent with the bw^{T50} allele requiring a specific interaction with w^{co2} to produce a detectable reduction in pteridine pigmentation. If bw^{T50} had been a null allele, that proportion of the progeny that were bw^0/bw^{T50} should have had brown eyes.

For characterization of the mutation in the bw^{T50} allele, DNA was isolated from four of the male flies with yellow eyes produced in the initial cross to w^{co2} v;bw^6 females. The *brown* gene fragments were amplified and cloned for sequencing as described for bw^6 in the previous section (results not shown).

Sequencing revealed that the bw^{T50} allele was not identical to bw^6. The new allele contains a single missense mutation with a G to A transition corresponding to position 1733 of the cDNA sequence. This results in the substitution of glycine 578 by aspartate.

Identification of a Single Codon Deletion in the w^{Bwx} Allele: Another Mutation in White That Affects the Guanine Transporter but Not the Tryptophan Transporter—Although the *white*-encoded subunit is involved in transport of both guanine and tryptophan, in w^{Bwx} there is a preferential reduction in the pteridine level, whereas the ommochrome level is virtually unaffected (Zachar and Bingham, 1982; Lindsay and Grell, 1968). As for w^{co2}, this indicates that the w^{Bwx} allele produces a protein that is capable of inserting in the membrane in such a way that it interacts with the *scarlet*-encoded subunit, to form an active tryptophan transporter in *Drosophila* eyes. However, clearly the mutant form of the w^{Bwx} protein is unable to support normal function of the guanine transporter.

To identify any mutations in the w^{Bwx}-encoded subunit, we amplified and sequenced the allele from genomic DNA as described for the w^{co2} allele (results not shown). The sequence data showed that the triplet ATC, encoding Ile^{581}, has been deleted from the w^{Bwx} gene (Table II). Otherwise, the sequence of the entire coding region and all splice donor and acceptor sites was identical to the published *white* sequences.

A Site-directed Mutation of Residues in the Nucleotide Binding Domain of the White-encoded Subunit That Results in Loss of Function of Both Transporters—The invariant amino acid doublet Gly-Lys of the Walker motif A has been proposed to be directly involved in binding of ATP in the nucleotide binding fold (Parsonage *et al.*, 1987), and mutations to these residues in various members of the Traffic ATPase family are known to eliminate or severely reduce function (Berkower and Michealis, 1991; Azzaria *et al.*, 1989; Cox *et al.*, 1989). To establish the significance of the nucleotide binding fold in the *white*-encoded subunit of *Drosophila* guanine and tryptophan transporters, we used oligonucleotide-directed mutagenesis to alter the codons for the Gly^{135}-Lys^{136} pair in the putative nucleotide binding fold to encode Leu-Gln. The mutated *white* minigene replaced the wild-type gene in pRK3hsneo (P[w^+, neo^r]) (Fig. 3), to create the plasmid pRK3hsneo.mut1 (P[w^{mut1}, neo^r]). Plasmid constructs containing either the wild-type or mutated *white* genes were subsequently used in P-element-mediated germline transduction experiments as described under "Experimental Procedures." The eye color of the G1 progeny was assessed and then G2 flies were selected for resistance to G418 at 0.2 mg/ml. Two of the G0 flies from embryos injected with the P[w^+, neo^r] construct produced G1 progeny with the expected wild-type eye color. In contrast, none of the G1 progeny from P[w^{mut1}, neo^r] recipient G0 flies made any detectable pigments in their eyes. However, when the P[w^{mut1}, neo^r] G1 flies were allowed to interbreed and lay eggs in medium containing 0.2 mg/ml G418, fertile G2 adult flies were recovered that had escaped the drug selection.

To confirm that the P[w^{mut1}, neo^r] G2 survivors of G418 selection were authentic transformants, PCR was used to amplify the 5′ end of the *white* minigene from genomic DNA isolated from individual G418 survivors. One primer was designed to bind to the 5′-nontranslated region, and the other was designed to bind in exon 3, downstream of the mutation site. The production of a 1.2-kb fragment after PCR confirmed that the *white* minigene (containing a shortened intron 1) was present in the genome of the flies, and digestion of the PCR product by *Pst*I into two fragments of approximately 940 and 300 base pairs was proof that the gene contained the site-directed mutation (see Fig. 4).

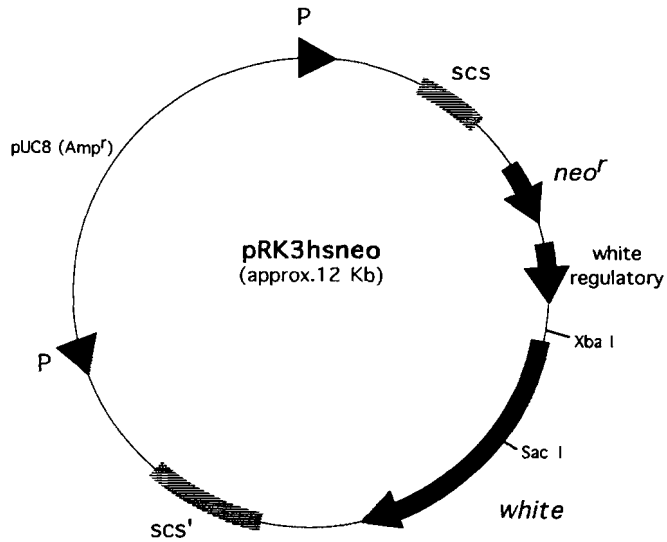

FIG. 3. **The plasmid pRK3hsneo used in P-element-mediated transformation of *D. melanogaster*.** The arrangement and approximate relative sizes is shown for genes and DNA motifs referred to in the text that are flanked by the P-element inverted repeat sequences. The unique *Xba*I and *Sac*I restriction endonuclease sites, used for cloning the equivalent fragment of the *white* minigene containing the site-directed mutation in pRK3hsneo.mut1 (see "Experimental Procedures"), are also shown. The *E. coli* origin of replication derived from pUC8 and the β-lactamase gene (conferring ampicillin resistance) are located in the segment external to the P-element termini.

Therefore, we conclude that a *white* gene with a Gly^{135}-Lys^{136}/Leu-Gln mutation in the putative nucleotide binding fold is unable to complement eye color in recipient flies with a defective *white* gene. This result also demonstrates that a functional nucleotide binding fold on the *white*-encoded subunit is necessary for activity of both the guanine and tryptophan transporters of *D. melanogaster*. The nucleotide binding folds of neither the *scarlet* nor *brown* gene products are able to support transport on their own.

DISCUSSION

The mutations detected in the two *white* (w^{co2}, w^{Bwx}) and two *brown* (bw^6, bw^{T50}) alleles described in the present work result in changes to amino acids located in the hydrophobic C-terminal regions of the two encoded proteins. The hydropathy plots of the amino acid sequences of the two proteins (Dreesen *et al.*, 1988), together with a comparison with other members of the ABC transporter family, suggest that there are six membrane-spanning regions for each of the subunits. Models of the orientation of these putative α-helices and the amino acids predicted to be buried in the membrane are depicted in Fig. 5, and the residues altered in the w^{co2}, w^{Bwx}, bw^6, and bw^{T50} alleles are indicated.

In the model of the *white*-encoded subunit the G588S mutation in w^{co2} is located in the C-terminal end of the fifth putative transmembrane helix. Since this mutation does not affect eye color in bw^+ flies, it can be concluded that the change does not

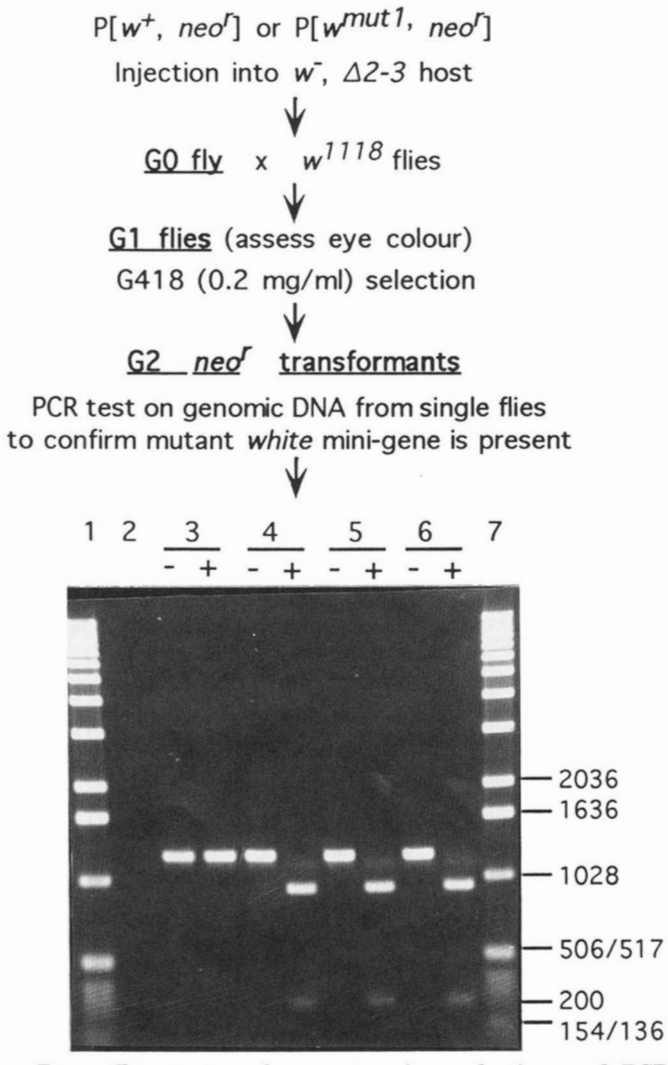

FIG. 4. **The strategy for construction, selection, and PCR screening of P[w^{mut1}; neo^r] transformant flies.** For the PCR test, genomic DNA was isolated from single flies and approximately 100 ng was used in PCR reactions with oligonucleotides 91–134 and 91–70 to amplify the 5′ portion of the white minigene (across the shortened intron 1). A product of 1.2 kb indicates that the flies contained the P-element-borne *white* gene. The PCR products were separated from unreacted primers and "primer dimers" by the Geneclean™ method and were then tested for the presence of an internal *Pst*I site that would have been introduced by successful mutagenesis with oligonucleotide 91–122. *Lanes 1* and 7, DNA markers with sizes (bp) indicated on the *far right-hand side. Lanes 2–6*, purified PCR products, before (–) and after (+) digestion with *Pst*I, amplified from the following flies: *lane 2*, a noninjected *w*, *Δ2–3* fly; *lane 3*, a G2 fly (with wild-type eye color) derived from an egg injected with pRK3hsneo. *Lanes 4–6*, three G2 flies (white-eyed) derived from eggs injected with pRK3hsneo.mut1.

appreciably affect the ability of the w^{co2}-encoded subunit to function in active guanine and tryptophan transporters. Therefore, the global structure of the mutant subunit must be nearly identical to wild-type, and any structural alterations due to the introduction of a hydroxyl moiety or the increased size of the serine residue compared with glycine must be relatively localized and insignificant with respect to function of the heterodimers. Similarly, it can be argued that the amino acid substitutions in either bw^6 (N638T) or bw^{T50} (G578D) do not cause disruption of subunit structure or function in heterodimers with w^+-encoded subunits. As for w^{co2}, the mutant residues in the bw^6 and bw^{T50}-encoded subunits are predicted to be near the external surface of the membrane. In bw^{T50} the G578D mutation is in a similar position in transmembrane helix 5 as the change in w^{co2}, and in bw^6 the N638T change is located near the membrane surface in transmembrane helix 6. Notably, the three mutations all give rise to residues capable of H-bonding, and it would seem likely that H-bonding between serine at position 588 in the *white*-encoded subunit and either aspartate 578 or threonine 638 in the *brown*-encoded subunit is responsible for the observed phenotype although other explanations are possible. These interactions would imply that helix 5 of the *white* protein is close to helices 5 and 6 of the *brown* protein (see Fig. 5). As such interactions cause loss of function, yet individually have no effect, it is possible that some dynamic aspect of transporter function has been affected. Furthermore, with reference to the folding models, the predicted location of all three mutations near the external surface of the membrane makes it tempting to speculate that function associated with the mouth of the pore through which the substrate passes may be affected.

The w^{Bwx} allele, containing a mutation that results in the deletion of Ile^{581}, causes almost no pteridine pigment to be synthesized while leaving the ommochrome levels unaffected (Zachar and Bingham, 1982; Lindsay and Grell, 1968). This is an identical phenotype to that produced by the w^{co2} bw^6 interaction, and again it indicates that the mutant *white*-encoded subunit is capable of interacting with the *scarlet*-encoded subunit to form an active tryptophan transporter and is therefore assembled and is sufficiently structurally similar to the wild-type subunit to form this functional interaction. We propose, however, that the deletion of Ile^{581} from transmembrane helix 5 in the w^{Bwx}-encoded subunit causes loss of a functional interaction with the *brown* protein. Deletion of an amino acid from an α-helix changes the faces of the helix due to the rotation in position, by 1 amino acid residue, of the helical surface on the C-terminal side of the deletion relative to the surface on the N-terminal side. Obviously, this could have significant effects on the interactions of that helix with the other transmembrane helices of the complex. Furthermore, if a face of the helix lined the channel through which the substrate is transported, then the deletion may alter the chemical properties of the channel surface by changing the positions of residue side chains which might be critical to the transport mechanism. Therefore, we propose that transmembrane helix 5 of the *white*-encoded subunit is involved in specific interactions with the *brown*-encoded subunit in formation of the guanine transporter. This conclusion is consistent with the interaction between helices 5 and 6 of these subunits proposed on the basis of the changes observed in the w^{co2}, bw^6, and bw^{T50} alleles, and it may be of relevance that the amino acid deleted is just two turns of an α-helix away from the interacting residue affected in w^{co2}. A corollary to this deduction is that a similar interaction between transmembrane helix 5 of the *white*-encoded subunit and the *scarlet*-encoded subunit does not occur in the tryptophan transporter, since ommochrome levels are wild-type in both w^{Bwx} and w^{co2},bw^6 flies.

Although the existence of additional mutations in the noncoding DNA of the w^{Bwx} allele has not been ruled out in this work, it seems unlikely that the phenotype could be caused by effects on the regulation of gene expression. It should be noted that previous molecular investigation of the w^{Bwx} allele using Southern blotting has shown it to have a gross gene structure identical to the wild-type gene with the exception of a 150-bp deletion between positions –18 and –19.5 on the gene map of *white* (Zachar and Bingham, 1982). However, a causative effect of this deletion on the expression of the gene is excluded by the results of Hazelrigg *et al.* (1984) who showed that flies transformed with a *white* gene construct which does not contain DNA that far downstream of the coding region regulate expression of *white* normally.

Another important property of the w^{Bwx} allele which indi-

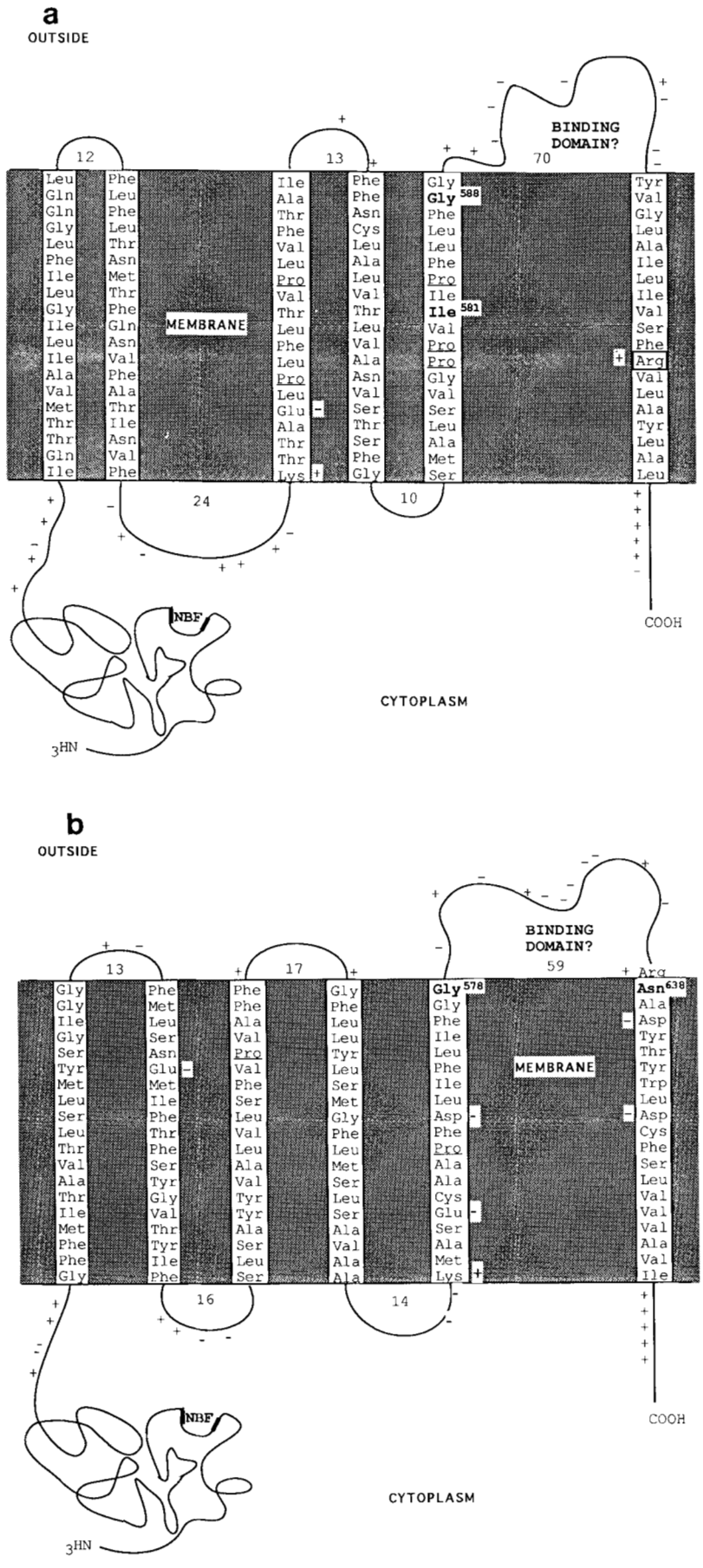

FIG. 5. *a*, model of the *white*-encoded subunit. *b*, model of the *brown*-encoded subunit. The amino acids predicted to be in the transmembrane helices in the *white*- and *brown*-encoded subunits of the guanine transporter are as indicated. The *numbers* inside the intra-helical loops indicate the number of amino acids in the loop. The relative distribution of charged amino acids is indicated by + or −. Amino acid residues that were found to be altered in the mutant alleles sequenced are in *boldface type* and have their residue position superscripted (see Table II). The putative nucleotide binding fold (*NBF*) is discussed in the text.

cates that the mutation in the protein is responsible for the phenotype is that it has a semidominant effect on pteridine production (Lindsay and Grell, 1968): w^{Bwx}/w^{+} heterozygotes have an eye color that is noticeably less red than wild-type. This is in contrast to the majority of *white* alleles in which the eye color phenotype is wild-type in heterozygotes. This suggests the interesting possibility that the native permease complex might be tetrameric (or multimeric), possessing two or more copies each of the *white*-encoded and *brown*-encoded subunits. Then it is conceivable that the presence of just one copy of the w^{Bwx}-encoded subunit in the oligomeric complex may be sufficient to disrupt the function of the transporter and that partial pigmentation results from the activity of the small proportion of complexes that form containing only the wild-type *white*-encoded subunit.

Function-altering mutations within the transmembrane helices have been identified in the MDR (Devine *et al.*, 1992; Gros *et al.*, 1991; Safa *et al.*, 1990) and CFTR (Tsui, 1992; Anderson *et al.*, 1991b) proteins. In the case of the CFTR protein, 58 missense mutations and 3 single amino acid deletions have been reported that probably cause cystic fibrosis (Tsui, 1992). Although the majority (32 mutants) of these are associated with either of the two nucleotide binding domains, other apparent hotspots for mutations are a predicted cytoplasmic loop in the C-terminal hydrophobic domain (nine mutants) and transmembrane helices 5 and 6 (six mutants). Interestingly, it has been suggested previously that these helices may have roles in substrate association and the mechanism of transport in Traffic ATPases (Devine *et al.*, 1992; Gros *et al.*, 1991; Webb *et al.*, 1992).

The effects of the site-directed mutation resulting in Gly-Lys being replaced by Leu-Gln in the Walker motif A of the *white* protein is consistent with the effects of similar mutations on other ABC transporters and on the F_0F_1-ATPase (Berkower and Michealis, 1991; Parsonage *et al.*, 1987; Azzaria *et al.*, 1989; Cox *et al.*, 1989). Structural analysis of adenylate kinase has shown that these amino acids are directly involved in binding of the phosphoryl groups of the nucleotide (Hyde *et al.*, 1990). Similar changes in the β-subunit of F_1-ATPase affect the binding and hydrolysis of ATP (Parsonage *et al.*, 1987). The results reported here are therefore consistent with the proposition that ABC transporters are energized by ATP hydrolysis. With respect to the models depicted in Fig. 5, the location of the nucleotide binding domains to the N-terminal side of the transmembrane helices in the *Drosophila* transporters is unusual among the eukaryotic ABC permeases. Similarly, the *Drosophila* transporters are also unusual among eukaryotic ABC permeases so far described, in that they transport their substrates into the cell instead of pumping molecules out of the cell (Higgins, 1992). As more and more examples of ABC permeases are characterized it will be interesting to see whether a correlation persists between the order of the hydrophilic and hydrophobic domains on the polypeptides and the direction of pumping.

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