

Mutations in the *white* gene of *Drosophila melanogaster* affecting ABC transporters that determine eye colouration

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Abstract

The *white*, *brown* and *scarlet* genes of *Drosophila melanogaster* encode proteins which transport guanine or tryptophan (precursors of the red and brown eye colour pigments) and belong to the ABC transporter superfamily. Current models envisage that the *white* and *brown* gene products interact to form a guanine specific transporter, while *white* and *scarlet* gene products interact to form a tryptophan transporter. In this study, we report the nucleotide sequence of the coding regions of five *white* alleles isolated from flies with partially pigmented eyes. In all cases, single amino acid changes were identified, highlighting residues with roles in structure and/or function of the transporters. Mutations in *w^{cf}* (G589E) and *w^{sat}* (F590G) occur at the extracellular end of predicted transmembrane helix 5 and correlate with a major decrease in red pigments in the eyes, while brown pigments are near wild-type levels. Therefore, those residues have a more significant role in the guanine transporter than the tryptophan transporter. Mutations identified in *w^{err}* (H298N) and *w¹⁰¹* (G243S) affect amino acids which are highly conserved among the ABC transporter superfamily within the nucleotide binding domain. Both cause substantial and similar decreases of red and brown pigments indicating that both tryptophan and guanine transport are impaired. The mutation identified in *w^{E187}* alters an amino acid within an intracellular loop between transmembrane helices 2 and 3 of the predicted structure. Red and brown pigments are reduced to very low levels by this mutation indicating this loop region is important for the function of both guanine and tryptophan transporters. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Pigmentation of the eye of *Drosophila melanogaster* is due to the synthesis and deposition in the pigment cells of red pigments (drosopterins), which are synthesised from guanine, and brown pigments (ommochromes) which are synthesised from tryptophan [1]. It has been proposed that the pigment

Abbreviations: ABC, ATP binding cassette; CFTR, cystic fibrosis transmembrane regulator; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); TM, transmembrane

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precursors are transported into pigment cells by membrane transporters belonging to the ABC transporter superfamily and encoded by the *white* (*w*), *scarlet* (*st*) and *brown* (*bw*) genes of *D. melanogaster* [2]. Genetic and biochemical evidence suggests that the gene products of *white* and *scarlet* together form a tryptophan transporter; and the *white* and *brown* gene products together form a guanine transporter [3–6].

The ABC transporter (or traffic ATPase) superfamily is a large and growing group of active membrane transporters found in procaryotes and eucaryotes [7–9]. Members of this superfamily share a similar overall predicted topology and usually transport substrates against a concentration gradient at the expense of ATP hydrolysis. A large variety of substrates is known, with each transporter specific for a particular substrate or group of related substrates. A typical ABC transporter complex is predicted to be composed of two membrane spanning domains, each composed commonly of six α -helices; and two cytoplasmic domains which harbour the ATP binding motifs A and B [10] in addition to the ABC transporter ‘signature sequence’ – also known as the linker peptide [11]. These four domains may be found either in four different polypeptides (as in the phosphate transporter of *Escherichia coli* [12]), in two polypeptides, each of which has one transmembrane domain and one cytoplasmic domain (as appears to be the case with the *D. melanogaster* pigment precursor transporters) or on a single polypeptide, as with the most extensively studied eucaryotic ABC transporters such as the cystic fibrosis transmembrane regulator (CFTR) and the multiple drug resistance proteins (MDR) in humans and mice. Within the cytoplasmic domain, the ATP binding and signature motifs are located within a region encompassing approximately 200 residues which forms the ATP binding cassette and is highly conserved among the superfamily. The Walker motifs A and B are found in a large number of ATP and GTP binding proteins, and their role in ATP and GTP binding has been well characterised [13–16]. The ‘signature sequence’, however, is exclusive to ABC transporters and is predicted to have an important functional role in the mechanism of transport [11,17–22].

Mutagenesis studies of a number of ABC transporters have revealed that helices 5 and 6 in the transmembrane domains (this includes helices 11 and 12 in the transporters with both transmembrane domains on a single polypeptide chain) have an important structural and/or functional role in the transport mechanism. In the *D. melanogaster* White/Brown heterodimeric guanine transporter, for example, mutations were identified within predicted transmembrane helices 5 and 6 of the White and Brown subunits which led to the conclusion that transmembrane helix 5 of the *white* encoded subunit and helices 5 and 6 of the *brown* encoded subunit must interact in the functional guanine transporter [23].

Particular intracellular loops linking the transmembrane helices have also been shown to be important for function in ABC transporters. In procaryotic transporters, a conserved protein motif known as the EAA motif which is around 30 residues long, between transmembrane helices 4 and 5 [24,25] has been identified. Certain of the residues in the motif have been shown to be important for function and it has been suggested [24] that the EAA motif interacts with the nucleotide binding domain. Shani et al. [26] report that some eucaryotic ABC transporters (e.g. adrenoleukodystrophy protein (ALDp)) have a 15-amino acid motif resembling the procaryotic EAA motif. It has been suggested [17] that the ABC ‘signature sequence’ interacts with the transmembrane domain through this EAA-like motif in eucaryotes.

In this paper, we characterise a further five *white* alleles from partially pigmented eye colour mutant strains of *D. melanogaster*, namely w^{cf} , w^{crr} , w^{sat} , w^{101} and w^{Et87} . These *white* alleles were chosen because: (a) the restriction maps in the case of w^{101} , w^{cf} , w^{crr} , and w^{sat} are indistinguishable from the derivative wild-type w^+ allele [27,28] and therefore do not contain any gross genetic lesions; (b) the presence of pigments, although reduced, indicates that both the White/Brown, and White/Scarlet complexes are assembled in the membrane, but that their function is impaired. We describe the nature and location of the point mutations, which identify functionally important regions of the *D. melanogaster* guanine and tryptophan ABC transporters.

2. Materials and methods

2.1. *D. melanogaster* strains

The *D. melanogaster* strain containing the w^{Et87} mutation was induced by ethyl methanesulphonate (M.M. Green and A.J. Howells, unpublished). The *D. melanogaster* strains containing the X-ray-induced w^{cf} allele [29], the two spontaneous alleles w^{err} [30], and w^{sat} [31] were obtained from the *Drosophila* Stock Center, California Institute of Technology, Pasadena, as were Canton-S strain containing the wild-type w^+ allele and the strain lacking eye pigmentation w^{1118} . Chromosomal DNA extracted from the *D. melanogaster* strain containing the N-ethyl-nitrosourea-induced w^{101} allele [28] was obtained from A. Pastink.

2.2. Amplification of white gene fragments from genomic DNA

Genomic DNA was extracted from approximately 50 flies as previously described [32] and was quantitated by measurement of A_{260} . Single stranded DNA primers for PCR and sequencing were designed using the published *white* gene sequence [3] and are listed in Table 1. DNA fragments containing the *white* gene sequences were amplified by PCR as described previously [23] using the nucleotide primers complementary to the 5'- and 3'-regions flanking Exon 1. Exons 2–6 were amplified in a separate PCR reaction using nucleotide primers flanking this region of the gene. The thermostable *Pfu* DNA polymerase was

used in order to minimise introduction of errors by PCR, since this enzyme has a proofreading function [33]. To ensure that mutations detected after DNA sequencing were not introduced by random polymerase errors, amplified fragments were cloned and sequenced from at least two separate PCR reactions.

2.3. Recombinant DNA technology using *Escherichia coli*

The gene fragments of each mutant *white* allele produced by PCR were purified using the Bresaclean Kit (Bresatech). The fragments were digested with the appropriate restriction enzymes to cleave the sites incorporated into 5'-ends of the oligonucleotide primers (see Table 1) and ligated into the pBluescript SK⁺ (Stratagene) plasmid vector which had been digested with the same restriction enzymes. The ligation reaction was then used to transform CaCl₂ competent *E. coli* strain XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, [F'*proAB*, *lacI^q*Δ*M15*, Tn10(*tet^r*)]). Small scale plasmid purifications were performed on overnight cultures grown from transformant colonies using a kit supplied by Qiagen.

2.4. DNA sequencing

The ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase, FS (Perkin-Elmer) was used, following the instructions of the supplier. The extension products

Table 1
Oligonucleotide primers used in sequencing and PCR reactions

Primer	Sequence (5' to 3') ^a	Site of 3'-base binding ^b	Use	Strand ^c
91-134	TTGAAGCTT <u>GAGT</u> GATTGGGGTG	-81	Exon 1 PCR	+
91-135	GCAGAGAATTCGATGTTGCAATCGC	123	Exon 1 PCR	-
91-136	AACCGAATTCGTAGGATACTTCG	3102	Exon 2-6 PCR	+
91-73	GATGAAGCTTATCTTGTGTTTTATTGGCAC	5714	Exon 2-6 PCR	-
91-68	CCACGACATCTGACCTATCG	3824	Sequencing	+
91-69	ACACCTACAAGGCCACCTGG	4467	Sequencing	+
91-70	GATCGTGTGCTGACATTTGC	3872	Sequencing	-
91-71	CTTTTACGAGGAGTGGTTCC	4537	Sequencing	-
91-72	GATGTGCAGCTAATTTTCGCC	5431	Sequencing	-

^aUnderlined sequences are recognition sites for restriction by *Bam*HI, *Eco*RI and *Hind*III.

^bBinding sites are numbered with +1 as the A of the translation start codon in the published *white* genomic [3] sequences.

^c+ indicates the coding strand; - indicates the complementary strand.

were separated by polyacrylamide gel electrophoresis by the Biomolecular Resource Facility of the Australian National University. Some of the sequencing was performed using the dideoxy chain-termination method [34], as described previously [23]. Each mutation was confirmed in at least two independent clones carrying DNA fragments from separate PCR experiments to be sure the nucleotide changes were not a result of an error by the DNA polymerase.

2.5. Extraction of eye colour pigments from *D. melanogaster*

Modified small-scale methods described previously [35,36] were used to extract xanthommatin and drosopterin pigments, respectively, from adult, 10-day-old *D. melanogaster*. The ages of flies used for extraction of pigments were standardised to 10 days old since eye colour changes as the fly matures. For extraction of xanthommatin pigments, 20 adult heads were homogenised in 0.3 ml of 2 M HCl using a small homogeniser. The homogenate was transferred to an Eppendorf tube and vortexed intermittently with 0.4 ml *n*-butanol and 1 mg sodium meta bisulphite for 10 min. The tube was then centrifuged in an Eppendorf bench top centrifuge for 2 min and 200 µl of the upper (butanol) layer was removed and the absorbance measured at 492 nm. *n*-Butanol was used as a blank. For extraction of drosopterin pigments, 10 adult heads were homogenised in a 1:1 mixture (0.4 ml each) of 0.1% aqueous ammonia and chloroform using a small homogeniser. The homogenate was transferred to an Eppendorf tube and centrifuged for 2 min. A 200-µl portion of the upper (aqueous) phase was removed and the absorbance

measured at 485 nm. Aqueous ammonia (0.1%) was used as a blank. Duplicate xanthommatin and drosopterin extractions were performed. Duplicate readings were averaged and results were expressed as a percentage of the of absorbance readings obtained from extractions from heads of the *D. melanogaster* wild-type strain Canton-S. The heads of the *D. melanogaster* strain *w¹¹¹⁸* which lacks pigmentation were used as a negative control.

2.6. Protein sequence alignments

Sequence comparisons were performed using ClustalW alignment program [37]. The settings used were as follows. Pairwise alignment mode: slow. Pairwise alignment parameters: open gap penalty 10; delay divergent 40%; extend gap penalty 0.1; gap distance 2; similarity matrix used was blosum. Multiple alignment parameters: open gap penalty, 10; extend gap penalty, 0.1; delay divergent, 40%; gap distance, 2; similarity matrix, blosum. Protein sequences for HisP [38], Snq2 [39], Pdr5 [40], Bfr1 [41], human CFTR [42] and human MDR1 [43,44], were obtained from the SwissProt data base. The predicted transmembrane domains were obtained from the SwissProt data base and checked for validity by hydrophathy analysis [45]. For the Snq2 protein, a further three potential transmembrane α -helices were predicted, encompassing the following amino acids: 523–541; 556–572 and 628–647, on the basis of hydrophathy analysis and homology between Snq2, Bfr1 and Pdr5. Positions of potential transmembrane helices for White, and Brown were taken from previously reported topological model of these proteins [23].

Table 2

Point mutations identified in the white gene of *D. melanogaster* eye colour mutants

White allele	Mutation in DNA ^a	Amino acid change	Predicted location in protein
<i>w¹⁰¹</i>	T to G (3270) G to A (3925)	Leu-49 to Arg Gly-243 to Ser	Nucleotide binding domain
<i>w^{crr}</i>	C to A (4090)	His-298 to Asn	Nucleotide binding domain
<i>w^{cf}</i>	GC to AA (5315) T to G (3270)	Gly-589 to Glu Leu-49 to Arg	Transmembrane helix 5
<i>w^{sat}</i>	TT to GG (5317)	Phe-590 to Gly	Transmembrane helix 5
<i>w^{Et87}</i>	G to A (5075)	Gly-509 to Asp	Intracellular loop between transmembrane helices 2 and 3

^aMutation sites are numbered with +1 as the A of the translation start codon in the published *white* genomic DNA sequence [3].

3. Results

3.1. Amino acid substitutions and eye colour pigment levels in the *white* alleles w^{cf} , and w^{sat} : mutations affecting transmembrane spanning helix 5 of the *white* protein

The coding region for the *white* gene was amplified from genomic DNA isolated from the fly strains w^{cf} , and w^{sat} . The strategy for PCR and sequencing is illustrated in Fig. 1. The gene was amplified as two PCR fragments to avoid the large non-coding region of 3.1 kilobases between Exons I and II. The PCR products produced from the two PCR reactions were analysed on a 0.9% agarose gel and shown to have the predicted sizes (Fig. 1). The PCR products were cloned and sequenced as described in Section 2. The sequences obtained from the cloned PCR products were compared to the published wild-type *white* genomic [3] and cDNA [4] sequences.

Two nucleotide changes affecting the amino acid sequence were identified in w^{cf} : G589E, and L49R (Table 2). The G589E substitution is predicted to occur at the C-terminal end of putative transmembrane spanning α -helix 5 of the protein structure modelled previously [23] and illustrated diagrammatically in Fig. 2. L49R occurs near the amino terminus in a region which is not conserved in amino

acid sequence among ABC transporters, nor homologs of *white* in other species [46].

The pigment levels of flies carrying the w^{cf} allele were assayed and the drospterin levels (red pigments) were found to be 29% of wild-type levels, while the xanthommatin (brown pigments) were found to be 64% of wild-type levels (Fig. 3). These pigment levels result in the eye colour phenotype depicted in Fig. 4. This result indicates that the mutation identified in w^{cf} reduces the function of the guanine transporter, while having a lesser effect on the tryptophan transporter.

In the w^{sat} allele, only one nucleotide change was identified which altered the amino acid sequence and this resulted in the substitution F590G. This residue is adjacent to G589 which has been substituted in the w^{cf} allele (see above). F590 is located on the extracellular, C-terminal end of transmembrane helix 5 in the White protein model [23] (see Fig. 2). The drospterin pigments in flies carrying the w^{sat} allele were 4% of wild-type, while the xanthommatin level was 79% (Fig. 3). These pigment levels result in the eye colour phenotype depicted in Fig. 4. This result suggests that the F590G substitution has caused a significant loss of function of the guanine transporter, while the tryptophan transporter function is at near wild-type levels.

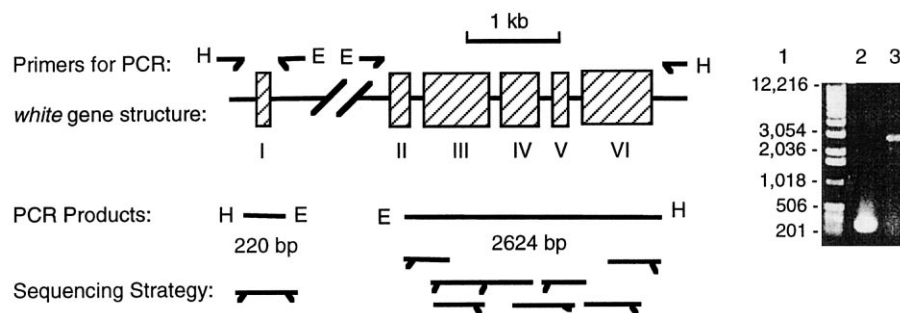


Fig. 1. Strategy for amplification, cloning and sequencing of *white* gene fragments from genomic DNA. Coding regions of the *white* gene were amplified by PCR as two DNA fragments using genomic DNA prepared from mutant fly strains as template DNA as described in Section 2. Exon 1, a 220-bp fragment was amplified using primers 91–134 and 91–135. Exons 2–6, a 2624-bp fragment was amplified using primers 91–136 and 91–73. Each primer had a recognition sequence for restriction by either *Eco*RI(E) or *Hind*III(H) incorporated at their 5'-ends to allow subsequent cloning of the PCR-generated fragments into 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^{sat} allele: lane 1, molecular weight markers with sizes (kb) shown to the left; lane 2, exon 1 fragment; lane 3, the exons 2–6 fragment. Similar results (not shown) were obtained for amplifications of w^{101} , w^{err} , w^{E187} and w^{cf} .

3.2. Amino acid substitutions and eye pigment levels of white alleles w^{crr} , w^{101} and w^{Et87} : mutations predicted to be within regions of the White protein located in the cytoplasm

The coding regions of the three *white* alleles: w^{crr} , w^{101} and w^{Et87} were cloned and sequenced as described for w^{cf} and w^{sat} . The only alteration to amino acid sequence found in the w^{crr} allele was the substitution H298N. This histidine residue is within the cytoplasmic ATP binding domain and is highly conserved among ABC transporters. The pigment levels in flies carrying this allele are significantly reduced, with drosoterins 11% of wild-type and xanthommatins 19% of wild-type levels (Fig. 3). These pigment levels result in the eye colour phenotype depicted in Fig. 4.

In the w^{101} allele, two nucleotide changes were identified which altered the amino acid sequence: G243S and L49R. Both of these residues are located within the ATP binding domain. G243S alters a highly conserved residue within the ABC transporter 'signature sequence'. This motif and the position of G243, is shown in the sequence alignment in Fig. 5A

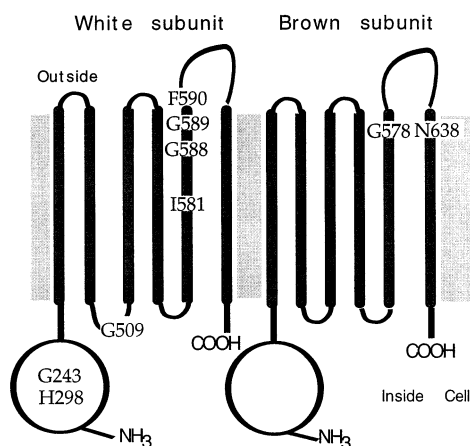


Fig. 2. Model of the topology of the protein products encoded by the *white* and *brown* genes of *D. melanogaster*. This figure is a simplified representation of the published model [23] and illustrates the relative positions of the amino acids which are altered due to mutations in the *white* gene affecting eye colour described in this paper. In addition, the previously reported mutations in the *brown* gene [23] (referred to in the text), bw^{TS0} (G578D) and bw^6 (N638T) are also shown. The grey-filled vertical rods represent putative membrane spanning α -helices; circles represent the ATP binding domains; lines connecting the transmembrane helices represent intra- or extra-cellular loop regions.

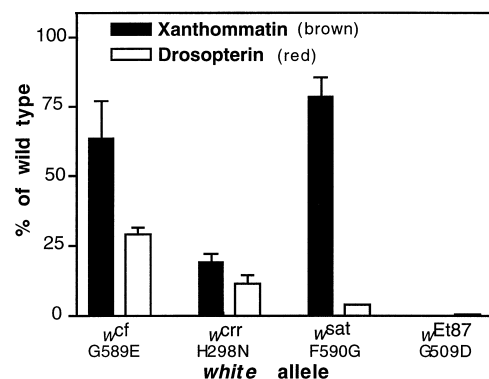


Fig. 3. Comparison of levels of eye colour pigments xanthommatins (brown pigments) and drosoterins (red pigments) extracted from the compound eyes of flies carrying mutant *white* alleles. Eye colour screening pigments were extracted from adult 10-day-old flies as described in Section 2. Pigment levels are expressed as a percentage of the optical densities obtained for pigment extractions from the wild-type *D. Melanogaster* strain Canton-S. Duplicate values from duplicate extractions were obtained and the mean used in the histogram. The error bars represent the standard error of the mean.

(G243 is encircled). The L49R substitution resides near the amino terminus within the cytoplasmic domain of the protein and the same change was also identified in the w^{cf} allele. The pigment levels of flies carrying the w^{101} allele could not be assayed because flies were not available. However, the eye phenotype is reported to be partially pigmented [47].

In the w^{Et87} allele, the only nucleotide change identified resulting in a change in amino acid sequence was the substitution G509D. This residue is located in the transmembrane domain in a predicted intracellular loop between transmembrane helices 2 and 3 of the proposed White protein structure [23] (see Fig. 2). Both pigment levels of flies carrying this allele are dramatically reduced: drosoterins were 0.2% and xanthommatin were undetectable (Fig. 3). These pigment levels result in the eye colour phenotype depicted in Fig. 4.

3.3. Protein sequence alignments of HisP and multiple drug resistance proteins with White, Scarlet and Brown proteins of *D. melanogaster*

Protein sequence alignments were performed as described in the experimental procedures using the ClustalW program [37]. The ATP binding domain of White, Scarlet and Brown were aligned with the

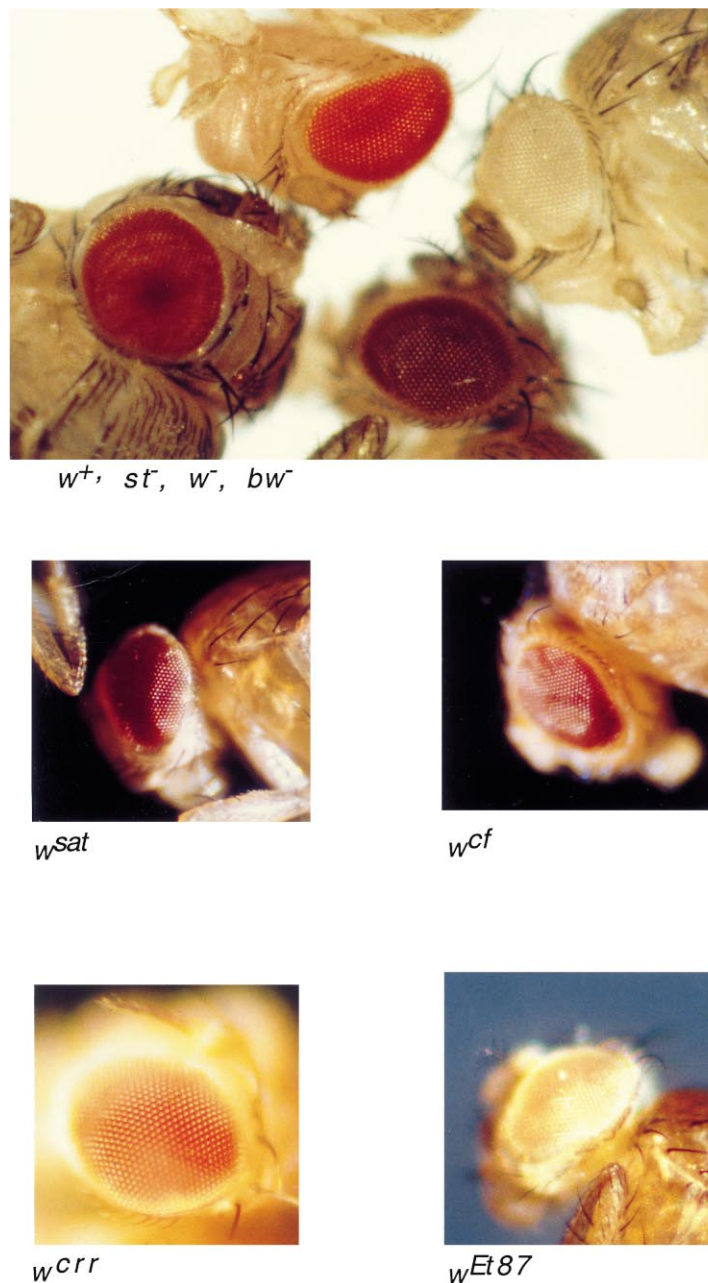


Fig. 4. Eye colour phenotypes of wild-type *D. melanogaster* and mutant strains carrying mutant *white* alleles. The top photograph represents eye colour of wild-type (left) and mutant *D. melanogaster* carrying null mutations in *scarlet* (top), *white* (right) and *brown* (bottom). The four photographs labelled w^{sat} , w^{cf} , w^{crr} and w^{cf} represent the eye colour of flies carrying the respective allele.

nucleotide binding domain of the *Salmonella typhimurium* histidine transporter, HisP [38]. The conserved sequence motifs Walker A, Walker B and ABC transporter signature motif aligned in a similar way as performed previously for other ABC transporters (data not shown) [8,42]. Fig. 5B shows the

aligned sequences including H211 of HisP which aligns with H273 of Scarlet, H291 of Brown and H298 of White, the latter corresponding to the H298N mutation identified in w^{crr} .

An alignment of yeast multiple drug resistance proteins Bfr1, Pdr5 and Snq2 from yeast and with

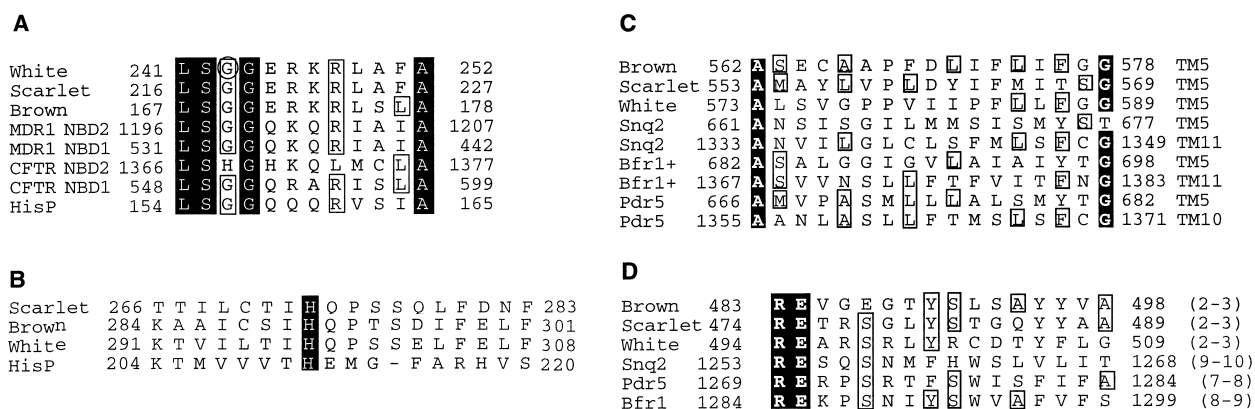


Fig. 5. Protein sequence alignments of White, Scarlet and Brown proteins with HisP and yeast multiple drug resistance proteins. The multiple alignments were performed as described in Section 2. Numbers on the left- and right-hand side of the protein sub-sequences indicate the amino acid residue number as published in the SwissProt database. (A) Alignment of the ABC transporter 'signature sequence' of White, Scarlet, Brown, human MDR1 and human CFTR discussed in the text. (B) A region of the nucleotide binding domain of White, Scarlet, Brown and HisP harbouring a highly conserved histidine residue. (C) Alignment of transmembrane helices (TM) 5 of White, Brown and Scarlet from *D. melanogaster* and transmembrane regions of yeast multiple drug resistance proteins. The TM the sub-sequence represents – where TM 1 is equivalent to the first potential TM helix from the amino terminus of the protein, is indicated on the far right hand side of the respective sequence. (D) Alignment of loop regions between TM helices 2 and 3 of White, Brown and Scarlet from *D. melanogaster* and loop regions of yeast multiple drug resistance proteins.

White, Scarlet, Brown from *D. melanogaster* revealed homology between putative transmembrane helix 5 of White, Scarlet and Brown and TM (transmembrane spanning region) 5 and 11 of Bfr1, TM 5 and 10 of Pdr5 and TM 5 and 11 of Snq2. The sequence alignment of these regions is shown in Fig. 5C. Most notable are the 100% conserved A at the N-terminal region and G at the C-terminal region of 8 of the 9 aligned sequences. The conserved A and G are separated by 15 amino acids in all cases. Other residues showing some degree of homology are also highlighted in Fig. 5C.

The sequence alignment of the above-mentioned proteins also revealed a conserved arginine–glutamate pair within a region predicted to form a loop between two TM helices. This region of homology is shown in Fig. 5D. This loop corresponds to the loop between TM 2 and 3 of White, Brown, Scarlet; TM 8 and 9 of Bfr1 which is an intracellular loop analogous to the loop of the White, Scarlet and Brown proteins; TM 9 and 10 of Snq2 which is predicated to be an extracellular loop region; and TM 7 and 8 of Pdr5 which is also extracellular. Other residues in this region which show some degree of homology between the White-related proteins and the yeast proteins are highlighted in Fig. 5D.

4. Discussion

The White protein of *D. melanogaster* is an unusual member of the ABC transporter family in that it is a subunit of two different transporters: the combination of White and Brown subunits makes a guanine transporter, while the combination of White and Scarlet subunits forms a tryptophan transporter. The fact that each of these transporters is required for deposition of red or brown pigments, respectively, in *Drosophila* eyes, makes eye colour phenotype a convenient way to monitor the function of *white* alleles [48]. The five mutant *white* alleles analysed in this work were selected from amongst many of the known *white* alleles on the criterion of partial eye pigmentation. Our expectation was that such non-knockout phenotypes would be caused by relatively small mutations in the gene that might reveal individual amino acids in the White protein with functional roles.

In the work presented here, DNA sequence analysis of the *w^{crr}*, *w^{sat}*, *w^{cf}*, *w¹⁰¹* and *w^{ET87}* alleles has identified point mutations which alter amino acids in the encoded White protein. As will be discussed below, five of these changes affect residues in regions or motifs conserved among members of the ABC trans-

porter superfamily. The mutations G243S in w^{101} and H298N, in w^{crr} , affect motifs within the nucleotide binding domain and correlate with reduced function in both of the White-containing transporters. Similarly, the mutation G509D in w^{ET87} , located in the cytoplasmic loop between helices 2 and 3, severely reduces function of both transporters. In contrast, however, the mutations G589E and F590G in w^{cf} and w^{sat} , respectively, affect function of the White/Brown guanine transporter more than the White/Scarlet tryptophan transporter.

From these and previous [23] results, we conclude: (1) that proper functioning of the nucleotide binding domain of the White subunit is essential to activity of both transporters; and (2) that the transmembrane helix 5 of the White subunit has different roles in the mechanisms of the two different transporters. These mutations are discussed further below in relation to the effects of identical or similar mutations in other ABC transporters.

4.1. Mutations in transmembrane helix 5

In addition to the mutations G589E and F590G reported herein, we have previously reported two other mutations in TM 5 of the White subunit – also identified in partially pigmented flies – which preferentially inhibit guanine transport rather than tryptophan transport. An adjacent mutation (G588S) in the *white* allele w^{CO2} [23] (see Fig. 2) was shown to affect guanine transport when in combination with either of the *brown* alleles bw^b or bw^{T50} which also have amino acid substitutions in the extracellular ends of transmembrane helix 5 or transmembrane helix 6, respectively (see Fig. 2). Functional interaction between the transmembrane helix 5/ transmembrane helix 6 regions of both the White and Brown subunits has been suggested previously [23]. It is interesting to note the gradation of effect on red pigmentation, with w^{CO2} (588) having the least affect, w^{cf} (589) having an intermediate affect while w^{sat} (590) has the greatest affect. The functional significance of transmembrane helix 5 for guanine transport was also highlighted by the previously reported (Δ Ile-581) mutation of the *white* allele w^{Bwx} [23] which completely abolished the presence of red pigments, while brown pigment synthesis was unaffected. The clustering of the mutations near the ex-

tracellular surface of the membrane in the predicted structure supports the suggestion that this region is associated with the function of the mouth of a pore specific for guanine and that helix 5 of the *white* encoded subunit interacts with helices 5 and 6 of the *brown* encoded subunit in formation of the guanine specific transporter [23]. Since these mutations in White TM 5 affecting guanine transport have little effect on tryptophan transport, it can be concluded that TM 5 of White is not intimately involved in tryptophan transport.

It has been reported in both the P-glycoprotein system and CFTR that the substrate specificity function resides in the transmembrane domain. There is biochemical evidence to suggest that transmembrane helices 5, 6, 11 and 12 are involved in drug binding and specificity in P-glycoprotein [49,50]. In CFTR, residues within and flanking transmembrane helix 6 have been proposed to line the ion conducting channel [51–53] and influence halide ion specificity [54].

It was recently reported that the White, Scarlet and Brown proteins' closest relatives based on sequence homology of the nucleotide binding domains include the yeast drug resistance proteins Snq2, Bfr1 and Pdr5 [55]. In order to investigate possible homology between the transmembrane domains of these proteins, a sequence alignment was performed and a number of conserved residues were noted. In particular, TM 5 of White, Scarlet and Brown show marked homology to Pdr5, Bfr1 and Snq2 which is highlighted in Fig. 5C. Of particular interest is the 100% conserved Gly which corresponds to the G589E mutation. This observation is suggestive of a conserved structural/functional element.

4.2. Mutations in intrahelical loops

Cytoplasmic loops have been reported to be functionally important in a number of ABC transporters, including CFTR [56,57], ALDp[17] and P-glycoprotein [49,58]. The mutation G509D identified in w^{Et87} which causes almost complete knock-out of pigment levels compared to wild-type, occurs within the cytoplasmic loop between transmembrane helices 2 and 3 of the predicted structure of the *white*-encoded subunit (see Fig. 2). Sequence alignment to other ABC transporter subunits (Fig. 5D) reveals a conserved glutamate in this loop that in White precedes an

alanine residue. Thus, we proposed that this loop region may be analogous to cytoplasmic loops containing the EAA or EAA-like motifs identified in other ABC transporters, where evidence indicates that this region performs an important structural and/or functional role at the interface between the transmembrane domain and ATP binding domain [24–26,59]. In the White protein, G509 is adjacent to K510 and it may be that the G509D substitution causes neutralisation of the positive charge thereby eliminating possible functionally important electrostatic interactions between this residue and the ATP binding domain.

4.3. Mutations in the nucleotide binding domain

Three of the mutations identified in the mutant *white* alleles were located in the nucleotide binding domain of the *white* encoded subunit. In *w¹⁰¹*, two mutations were found, G243S and L49R. The *w¹⁰¹* mutation G243S (underlined in the sequence below) is located within the highly conserved ‘signature sequence’ which has the consensus sequence ‘LSGGQXXXRHyXHyA’, (Hy is any hydrophobic residue, and X is any residue) and is shown in Fig. 5A. The replacement of the single hydrogen atom of glycine with the polar hydroxyl moiety of serine with hydrogen bonding potential represents a significant alteration in residue size and chemistry. Mutations within this motif have been characterised in a number of systems and show that this sequence is important both for transport function, protein expression and stability, as well as substrate specificity [11,18–22,60,61].

Recently, the crystal structure of the ATP binding subunit of the histidine permease of *Salmonella typhimurium* has been solved [62]. Part of the signature motif in this crystal structure forms the first half of an α -helix (α 5) within a region of the protein designated arm II and which interacts with the membrane domains HisQ and HisM. According to this structure, only the residues LSGGQ (residues 154–158 in HisP) are partially exposed to the cytoplasmic side of the protein [62]. A role in the structural integrity of the folded HisP molecule was suggested [62]. It is interesting to note that the α 5 helix of the HisP crystal structure is mostly buried between the α -helices of arm II of the protein, however, it is physically con-

nected via a loop to the β -strand harbouring the highly conserved aspartic acid believed to be involved in co-ordination of Mg^{+} during ATP binding and hydrolysis [62]. Conformational changes which occur upon ATP binding and/or hydrolysis may well be propagated via this connection to the arm II proposed to interact with the membrane domains HisQ and HisM.

The second mutation identified in the *w¹⁰¹* allele, L49R, is located near the N-terminus of the protein in a region which is not conserved in primary sequence among ABC transporters. This mutation is also found in the allele *w^{cf}*. We suspect this mutation does not occur in a region of the protein important for function and may be a strain dependent polymorphism. However, it is possible that the mutation contributes to the mutant phenotype and this would need to be confirmed in transgenic flies where the effect of this mutation alone can be assessed.

The mutation identified in *w^{crr}*, H298N changes a histidine residue in the ATP binding domain which is highly conserved in ABC transporters, but not other nucleotide binding proteins. In the sequence alignment in Fig. 5A, it can be seen that H298 of White aligns with H211 of HisP which, according to the crystal structure of HisP, is involved in hydrogen bonding to γ -phosphate of ATP via a water molecular (wat-415) which is adjacent to the water molecule proposed to be the attacking water during ATP hydrolysis (wat-437) [62]. It is interesting to note that when H211 of HisP is mutated to either D, R or Y, transport function is abolished, while ATP binding is not affected. Thus, the mutation may affect ATP hydrolysis or conformational changes subsequent to ATP binding [11].

Overall, the mutations identified in the NBD of White result in an approximately equal decrease in function of both the transporters involving the White subunit. This observation is consistent with studies of P-glycoprotein indicating a strong cooperative interaction between the two nucleotide binding domains [63]. It was proposed that the two nucleotide binding sites undergo sequential or alternating cycles of ATP hydrolysis [64,65] which is consistent with the effect of the H298N mutation since if ATPase activity at the White nucleotide binding site is reduced, this would consequently reduce the ATPase activity of both the Brown and Scarlet nucleotide binding sites

to the same degree. It has been previously reported that mutation of G135 and K136 to LQ within the nucleotide binding fold motif of the *white* gene is unable to complement eye colour in recipient transgenic flies with a defective *white* gene [23]. Hence, the function of the White ATP binding domain is essential for the transport of both guanine by the White/Brown complex, and tryptophan by the White/Scarlet complex.

In summary, a cluster of mutations have been identified at the extracellular end of transmembrane helix 5 in the alleles *w^{cf}* and *w^{sat}* and the previously reported *w^{CO2}* [23] which predominantly affect guanine transport (i.e. red pigments have been reduced substantially) while the tryptophan transporter has retained function almost at wild-type levels (i.e. brown pigment levels are near wild-type levels). In contrast, mutations identified in the cytoplasmic ATP binding domain of the White protein decreased the function of both the guanine and tryptophan transporters to a similar degree. This was also the case for the *w^{Et87}* mutation which occurs in an intracellular loop of the transmembrane domain predicted to couple signals from the ATP binding domain to the transmembrane domain. The next step is to develop a model system which could be used to gain further insights into the effects of these mutations on rates of transport, substrate binding, and effects on ATP binding and hydrolysis.

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References

- [1] K.M. Summers, A.J. Howells, N.A. Pyliotis, Biology of eye pigmentation in insects, *Adv. Insect Physiol.* 16 (1982) 119–166.
- [2] D.T. Sullivan, S.L. Grillo, R.J. Kito, Subcellular localization of the first three enzymes of the ommochrome synthetic pathway in *Drosophila melanogaster*, *J. Exp. Zool.* 188 (1974) 225–234.
- [3] K. O'Hare, C. Murphy, R. Levis, G.M. Rubin, DNA Sequence of the *white* locus of *Drosophila melanogaster*, *J. Mol. Biol.* 180 (1984) 437–455.
- [4] M. Pepling, S.M. Mount, Sequence of a cDNA from the *Drosophila melanogaster white* gene, *Nucleic Acids Res.* 18 (1990) 1633.
- [5] T.D. Dreeson, D.H. Johnson, S. Henikoff, The Brown protein of *Drosophila melanogaster* is similar to the White protein and to components of active transport complexes, *Mol. Cell. Biol.* 8 (1988) 5206–5215.
- [6] R.G. Tearle, J.M. Belote, M. McKeown, B.S. Baker, A.J. Howells, Cloning and characterisation of the *scarlet* gene of *Drosophila melanogaster*, *Genetics* 122 (1989) 595–606.
- [7] G.F.-L. Ames, Bacterial periplasmic transport systems: structure, mechanism, and evolution, *Annu. Rev. Biochem.* 55 (1986) 397–425.
- [8] C.F. Higgins, ABC transporters: from microorganisms to man, *Annu. Rev. Cell. Biol.* 8 (1992) 67–113.
- [9] M.J. Fath, R. Kolter, ABC transporters: bacterial exporters, *Microbiol. Rev.* 57 (1993) 995–1017.
- [10] J.E. Walker, M. Saraste, M.J. Runswick, N.J. Gay, Distantly related sequences in the alpha and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold, *EMBO J.* 1 (1982) 945–951.
- [11] V. Shyamala, V. Baichwal, E. Beall, G.F.-L. Ames, Structure-functional analysis of the histidine permease and comparison with cystic fibrosis mutations, *J. Biol. Chem.* 266 (1991) 18714–18719.
- [12] B.P. Surin, H. Rosenberg, G.B. Cox, Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene–polypeptide relationships, *J. Bacteriol.* 161 (1985) 189–198.
- [13] D.C. Fry, S.A. Kuby, A.S. Mildvan, ATP-binding site of adenylate kinase: mechanistic implications of its homology with *ras*-encoded p21, *F₁-ATPase*, and other nucleotide-binding proteins, *Proc. Natl. Acad. Sci. USA* 83 (1986) 907–911.
- [14] J. Sondek, D.G. Lambright, J.P. Noel, H.E. Hamm, P.B. Sigler, GTPase mechanism of Gproteins from the 1.7 Å crystal structure of transducin alpha.GDP.AIF₄[−], *Nature* 372 (1994) 276–279.
- [15] I. Schlichting, S.C. Almo, G. Rapp, K. Wilson, K. Petratos, A. Lentfer, A. Wittinghofer, W. Kabsch, E.F. Pai, G.A. Petsko, R.S. Goody, Time-resolved X-ray crystallographic study of the conformational change in Ha-Ras p21 protein on GTP hydrolysis, *Nature* 345 (1990) 309–315.
- [16] H.R. Bourne, D.A. Sanders, F. McCormick, The GTPase superfamily: conserved structure and molecular mechanism, *Nature* 349 (1991) 117–127.
- [17] N. Shani, A. Sapag, D. Valle, Characterisation and analysis of conserved motifs in a peroxisomal ATP-binding cassette transporter, *J. Biol. Chem.* 271 (1996) 8725–8730.
- [18] B.L. Browne, V. McClendon, D.M. Bedwell, Mutations

- within the first LSGGQ motif of Ste6p cause defects in a-Factor transport and mating in *Saccharomyces cerevisiae*, *J. Bacteriol.* 178 (1996) 1712–1719.
- [19] J.L. Teem, H.A. Berger, L.S. Ostedgaard, D.P. Rich, L.-C. Tsui, M.J. Welsh, Identification of revertants for the cystic fibrosis deltaF508 mutation using STE6-CFTR chimeras in yeast, *Cell* 73 (1993) 335–346.
- [20] T. Hoof, A. Demmer, M.R. Hadam, J.R. Riordan, B. Tümmler, Cystic fibrosis-type mutational analysis in the ATP-binding cassette transporter signature of human P-glycoprotein MDR1, *J. Biol. Chem.* 269 (1994) 20575–20583.
- [21] B.S. Kerem, J. Zielenski, D. Markiewicz, D. Bozon, E. Gazit, J. Yahaf, D. Kennedy, J.R. Riordan, F.S. Collins, J.R. Rommens, L.-C. Tsui, Identification of mutations in regions corresponding to the two putative nucleotide (ATP)-binding folds of the cystic fibrosis gene, *Proc. Natl. Acad. Sci. USA* 87 (1990) 8447–8451.
- [22] G.R. Cutting, L.M. Kasch, B.J. Rosenstein, J. Zielenski, L.-C. Tsui, S.E. Antonarakis, H.H. Kazazian, A cluster of cystic fibrosis mutations in the first nucleotide-binding fold of the cystic fibrosis conductance regulator protein, *Nature* 346 (1990) 366–369.
- [23] G.D. Ewart, D. Cannell, G.B. Cox, A.J. Howells, Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in *Drosophila melanogaster*, *J. Biol. Chem.* 269 (1994) 10370–10377.
- [24] R. Kerppola, G. Ames, Topology of the hydrophobic membrane-bound components of the histidine periplasmic permease. Comparison with other members of the family, *J. Biol. Chem.* 267 (1992) 2329–2336.
- [25] W. Saurin, W. Köster, E. Dassa, Bacterial binding protein-dependent permeases: characterization of distinctive signatures for functionally related integral cytoplasmic membrane proteins, *Mol. Microbiol.* 12 (1994) 993–1004.
- [26] N. Shani, P.A. Watkins, D. Valle, *PXA1*, a possible *Saccharomyces cerevisiae* ortholog of the human adrenoleukodystrophy gene, *Proc. Natl. Acad. Sci. USA* 92 (1995) 6012–6016.
- [27] Z. Zachar, P.M. Bingham, Regulation of *white* locus expression: the structure of mutant alleles at the *white* locus of *Drosophila melanogaster*, *Cell* 30 (1982) 529–541.
- [28] A. Pastink, C. Vreeken, E.W. Vogel, The nature of N-ethyl-N-nitrosourea-induced mutations at the *white* locus of *Drosophila melanogaster*, *Mutat. Res.* 199 (1988) 47–53.
- [29] Nicoletti, *Drosophila* Information Service 34 (1960) 52–53.
- [30] Judd, *Drosophila* Information Service 39 (1964) 59–60.
- [31] Bridges, *Drosophila* Information Service 3 (1935) 18.
- [32] W. Bender, P. Spierer, D.S. Hogness, Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the bithorax complex in *Drosophila melanogaster*, *J. Mol. Biol.* 168 (1983) 17–33.
- [33] K.S. Lundberg, D.D. Shoemaker, M.W.W. Adams, J.M. Short, J.A. Sorge, E.J. Mathur, High fidelity amplification using thermostable DNA polymerase from *Pyrococcus furiosus*, *Gene* 108 (1991) 1–8.
- [34] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. USA* 74 (1977) 5463–5467.
- [35] R.L. Ryall, A.J. Howells, Ommochrome biosynthetic pathway of *Drosophila melanogaster*: Variations in the levels of enzyme activities and intermediates during adult development, *Insect Biochem.* 6 (1974) 135–142.
- [36] B.A. Evans, A.J. Howells, Control of drosoperin synthesis in *Drosophila melanogaster*: mutants showing an altered pattern of GTP cyclohydrolase activity during development, *Biochem. Genet.* 16 (1978) 13–26.
- [37] D.G. Higgins, J.D. Thompson, T.J. Gibson, Using CLUSTAL for multiple sequence alignments, *Methods Enzymol.* 266 (1996) 383–402.
- [38] C.F. Higgins, P.D. Haag, K. Nikaido, F. Ardeshir, G. Garcia, G.F.-L. Ames, Complete nucleotide sequence and identification of membrane components of the histidine transport operon of *S. typhimurium*, *Nature* 298 (1982) 723–727.
- [39] J. Servos, E. Haase, M. Brendel, Gene SNQ2 of *Saccharomyces cerevisiae*, which confers resistance to 4-nitroquinoline-N-oxide and other chemicals, encodes a 169 kDa protein homologous to ATP-dependent permeases, *Mol. Gen. Genet.* 236 (1993) 214–218.
- [40] P.H. Bissinger, K. Kuchler, Molecular cloning and expression of the *Saccharomyces cerevisiae* STS1 gene product. A yeast ABC transporter conferring mycotoxin resistance, *J. Biol. Chem.* 269 (1994) 4180–4186.
- [41] K. Nagao, Y. Taguchi, M. Arioka, H. Kadokura, A. Takatsuki, K. Yoda, M. Yamasaki, *bfr1+*, a novel gene of *Schizosaccharomyces pombe* which confers brefeldin A resistance, is structurally related to the ATP-binding cassette superfamily, *J. Bacteriol.* 177 (1995) 1536–1543.
- [42] J.R. Riordan, J.M. Rommens, B.-S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, M.L. Drumm, M.C. Iannuzzi, F.S. Collins, L.-C. Tsui, Identification of the cystic fibrosis gene: cloning and characterisation of complementary DNA, *Science* 245 (1989) 1066–1073.
- [43] C.J. Chen, J.E. Chin, K. Ueda, D.P. Clark, I. Pastan, M.M. Gottesman, I.B. Roninson, Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells, *Cell* 47 (1986) 381–389.
- [44] C.-J. Chen, D. Clark, K. Ueda, I. Pastan, M.M. Gottesman, I.B. Roninson, Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins, *J. Biol. Chem.* 265 (1990) 506–514.
- [45] J. Kyte, R.F. Doolittle, A simple method for displaying the hydrophobic character of a protein, *J. Mol. Biol.* 157 (1982) 105–132.
- [46] L.J. Zwiebel, G. Saccone, A. Zacharopoulou, N.J. Besansky, G. Favia, F.H. Collins, C. Louis, F.C. Kafatos, The *white* gene of *Ceratitis capitata*: a phenotypic marker for germline transformation, *Science* 270 (1995) 2005–2007.
- [47] A. Pastink, C. Vreeken, A. Schalet, J. Eeken, DNA sequence analysis of X-ray-induced deletions at the *white* locus of *Drosophila melanogaster*, *Mutat. Res.* 207 (1988) 23–28.

- [48] G.D. Ewart, A.J. Howells, ABC transporters involved in transport of eye pigment precursors in *Drosophila melanogaster*, *Methods Enzymol.* 292 (1998) 213–224.
- [49] T.W. Loo, D.M. Clarke, Functional consequences of glycine mutations in the predicted cytoplasmic loops of P-glycoprotein, *J. Biol. Chem.* 269 (1994) 7243–7248.
- [50] M. Hanna, M. Brault, T. Kwan, C. Kast, P. Gros, Mutagenesis of transmembrane domain 11 of P-glycoprotein by alanine scanning, *Biochemistry* 35 (1996) 3625–3635.
- [51] J.A. Tabcharani, J.M. Rommens, Y.X. How, X.B. Chang, L.C. Tsui, J.R. Riordan, J.W. Hanrahan, Multi-ion pore behaviour in the CFTR chloride channel, *Nature* 366 (1993) 79–82.
- [52] M. Cheung, M.H. Akabas, Identification of CFTR channel-lining residues in and flanking the M6 membrane-spanning segment, *Biophys. J.* 70 (1996) 2688–2695.
- [53] S. McDonough, N. Davidson, H.A. Lester, N.A. McCarty, Novel pore-lining residues in CFTR that govern permeation and open-channel block, *Neuron* 13 (1994) 623–634.
- [54] M.P. Anderson, R.J. Gregory, S. Thompson, D.W. Souza, S. Paul, R.C. Mulligan, A.E. Smith, M.J. Welsh, Demonstration that CFTR is a chloride channel by alteration of its anion selectivity, *Science* 253 (1991) 202–205.
- [55] J.M. Croop, Evolutionary relationships among ABC transporters, *Methods Enzymol.* 292 (1998) 101–116.
- [56] J. Xie, M.L. Drumm, J. Ma, P.B. Davis, Intracellular loop between transmembrane segments IV and V of cystic fibrosis transmembrane conductance regulator is involved in regulation of chloride channel conductance state, *J. Biol. Chem.* 270 (1995) 28084–28091.
- [57] J.F. Cotten, L.S. Ostedgaard, M.R. Carson, M.J. Welsh, Effect of cystic fibrosis-associated mutations in the fourth intracellular loop of cystic fibrosis transmembrane conductance regulator, *J. Biol. Chem.* 271 (1996) 21279–21284.
- [58] X. Zhang, K.I. Collins, L.M. Greenberger, Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by *MDR 1*, *J. Biol. Chem.* 270 (1995) 5441–5448.
- [59] M. Mourez, M. Hofnung, E. Dassa, Subunit interactions in ABC transporters: a conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits, *EMBO J.* 16 (1997) 3066–3077.
- [60] É. Bakos, I. Klein, E. Welker, K. Szabó, M. Müller, B. Sarkadi, A. Váradi, Characterization of the human multidrug resistance protein containing mutations in the ATP-binding cassette signature region, *Biochem. J.* 323 (1997) 777–783.
- [61] T. Dörk, U. Wulbrand, T. Richter, T. Neumann, H. Wolfes, B. Wulf, G. Maass, B. Tümmeler, Cystic fibrosis with three mutations in the cystic fibrosis transmembrane regulator gene, *Hum. Genet.* 87 (1991) 441–446.
- [62] L.-W. Hung, I.X. Wang, K. Nikaido, P.-Q. Liu, G.F.-L. Ames, S.-H. Kim, Crystal structure of the ATP-binding subunit of an ABC transporter, the histidine permease of *Salmonella typhimurium*, *Nature* 396 (1998) 703–707.
- [63] I.L. Urbatsch, B. Sankaran, J. Weber, A.E. Senior, P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site, *J. Biol. Chem.* 270 (1995) 19383–19390.
- [64] I.L. Urbatsch, B. Sankaran, S. Bhagat, A.E. Senior, Both P-glycoprotein nucleotide-binding sites are catalytically active, *J. Biol. Chem.* 270 (1995) 26956–26961.
- [65] A.E. Senior, M.K. Al-Shawi, I.L. Urbatsch, Mini-review. The catalytic cycle of P-glycoprotein, *FEBS Lett.* 377 (1995) 285–289.