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Tissue specific effects of ommochrome pathway mutations in *Drosophila melanogaster*

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Summary

The tissue-specific effects of 17 mutations affecting the synthesis of brown eye pigment (xanthommatin) have been investigated by combining them with *chocolate* and *red cells*, two mutations causing ectopic pigmentation of the Malpighian tubules and larval fat body (which normally only synthesize pigment precursors). The majority of mutations block the pigmentation of four organs: the normally pigmented eyes and ocelli, and ectopically pigmented tubules and fat body. They represent genes that would appear to be required for the normal operation of the pathway *per se* and are likely to encode structural proteins. Mutations at 5 loci affect pigmentation of a subset of organs: *cd* and *po* affect only the eyes and ocelli; *kar* affects the eyes, ocelli and fat body; *car* causes excretion of pigment from tubules; and *z* affects pigmentation of the eyes alone. Of these loci, only *z* has been shown to encode a regulatory protein and the role of the remaining four gene products is not clear. Two mutations affecting the red eye pigments (drosopterins), *bw* and *mal*, do not substantially perturb brown pigment synthesis in any of the four organs.

1. Introduction

The compound eye of *Drosophila melanogaster* contains two types of light-screening pigments, the brown ommochrome (xanthommatin) and a series of red pteridines (drosopterins). Xanthommatin is derived from tryptophan via the ommochrome biosynthetic pathway, which consists of four steps and has been well characterized biochemically (Fig. 1). Enzymes catalysing each step of the pathway have been partially purified and characterised (Sullivan, Kitos and Sullivan, 1976; Sullivan and Kitos 1976; Moore and Sullivan 1978; Wiley and Forrest 1981) with the genes encoding tryptophan oxygenase (*vermilion*) and kynurenine-3-hydroxylase (*cinnabar*) identified and cloned (Searles and Voelker, 1986; Walker *et al.* 1986; W. Warren and A. J. Howells pers. comm.). The other two steps in the pathway are catalysed by more than one form of the enzyme (Moore & Sullivan, 1978; Wiley & Forrest, 1981) and have not been uncovered mutationally. [Because kynurenine formamidase is found in the same tissues as tryptophan oxygenase and in vast excess to that enzyme (Moore & Sullivan, 1978) the conversion of tryptophan to kynurenine is usually treated as a single step.] In addition to *v* and *cn*, lesions in about 25 other genes have a major effect on the pathway, as judged by altered eye or ocellus pigmentation (Lindsley & Grell, 1968; Phillips & Forrest, 1980). Most of these mutations affect the formation of both xanthommatin and the drosopterins.

The pathway operates in four organs and at two stages in the organism's life history (Fig. 2). During larval life dietary tryptophan is taken up by the Malpighian tubules and the larval fat body. The tubules convert excess tryptophan (over and above that required for protein synthesis) to 3-hydroxykynurenine and the fat body converts tryptophan to kynurenine (Beadle, 1937*a*, *b*; Nissani, 1975). These intermediates are stored in the tissues which synthesize them.

Around the time of pupariation the larval stores of pigment precursors are released into the haemolymph, and along with tryptophan from the degradation of larval proteins, are taken up by the developing eyes (Beadle & Law, 1938). Pigment deposition begins about 48 hours after pupariation. The capacity of the developing eyes to synthesize pigment from tryptophan alone (Nissani, 1975) indicates that all the

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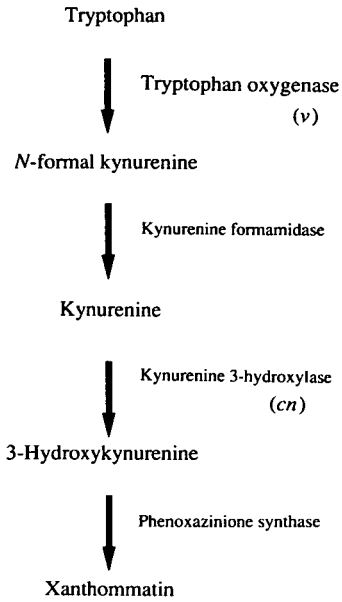

Fig. 1. The ommochrome biosynthetic pathway. The genes encoding enzymes which control two steps in the pathway have been identified: *vermilion* (*v*) which encodes tryptophan oxygenase and *cinnabar* (*cn*) which encodes kynurenine-3-hydroxylase.

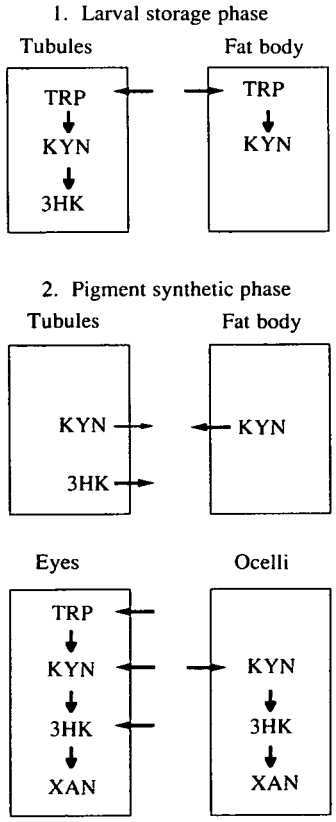

Fig. 2. Temporal and tissue specific expression of the ommochrome biosynthetic pathway in *D. melanogaster*. Pigment precursors are stored in the Malpighian tubules and larval fat body during the larval phase. During the pupal phase the stored precursors and tryptophan are taken up by the eyes and ocelli. The substrates and products of these steps in the pathway are tryptophan (TRP), kynurenine (KYN), 3-hydroxykynurenine (3HK) and xanthommatin (XAN).

enzymes of the pathway are present in the eyes. The ocelli, which appear to become pigmented somewhat later than the eyes, are dependent on exogenous sources of kynurenine (Beadle, 1937*b*; Nissani, 1975) and only the steps in the pathway from kynurenine to xanthommatin definitely occur in the ocelli.

As shown in Fig. 2, not all steps in the pathway operate in all organs, implying tissue-specific regulation of genes encoding structural pathway components. Because of the organ autonomous nature of most of the pathway mutants (Beadle & Ephrussi, 1936), the requirement for a particular gene product in the eyes and ocelli can be gauged from the effect a mutation has on pigmentation of these organs. Analysis of the effect of mutations on the pathway in the tubules and fat body, however, presents a major difficulty as they do not normally deposit pigment. While it is possible to use biochemical assays to determine the levels of kynurenine and 3-hydroxykynurenine, isolation of the tissues is difficult and tedious. This problem can be overcome by utilizing mutants that cause these tissues to deposit pigment.

Lesions in two genes, *chocolate* (*cho*) and *red Malpighian tubules* (*red*) cause the ectopic synthesis of ommochrome in the Malpighian tubules from early in larval life (Lindsley & Grell, 1968). They also alter eye colour to a dark brown and darken the colour of the ocelli. Mutations at two other loci, *red cells* (*rc*) and *lysine* (*lys*), can cause the ectopic deposition of ommochrome in the remnants of the disintegrating larval fat body at 48 h after pupariation – the same time as it is occurring in the eyes (Jones & Lewis, 1957; Grell, 1961). The eyes and ocelli are normally pigmented.

The aim of the work presented here is to begin to investigate the tissue-specific regulation of pathway genes by analysing the organ-specific effects of mutant genes affecting ommochrome synthesis. This paper details the effects of 17 such eye colour mutations on the tubule phenotype of *cho*, and fat body phenotype of rc^2, and combines these data with eye and ocellus phenotypes to delineate the organ-specific effects of the genes. The possible modes of action of several genes are also discussed.

2. Materials and methods

Mutant stocks of *D. melanogaster* were obtained from a number of different sources, the majority from the Bowling Green and Caltech stock centres. The alleles used and their nomenclature are given in Table 1. Material to be examined was maintained at 25 °C except for stocks involving rc^2, which were maintained at 18 °C to enhance pigmentation of the larval fat body.

To ensure that the eye, ocellus, tubule and fat body phenotypes could all be attributed to the ommochrome mutation, and were not due to an unrelated lesion on the same chromosome, two measures were taken.

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Table 1. *Alleles of eye colour loci tested in combination with* cho *and* rc². *Alleles marked with an asterisk were backcrossed to the wild-type strain Canton S for 25–30 generations to standardize the genetic background*

Locus	Allelic combinations tested with *cho*	with *rc²*
brown (bw)	$*bw^1$	$*bw^1$
cardinal (cd)	$*cd^1$, cd^{KP2}, $*cd^{wo}$, $*cd^1/cd^{KP2}$	$*cd^1$
carmine (cm)	$*cm^1$	$*cm^1$
carnation (car)	$*car^1$	$*car^1$
cinnabar (cn)	$*cn^1$, cn^{35k}, $*cn^1/cn^{35k}$	$*cn^1$
claret (ca)	$*ca^1$, ca^3, $*ca^1/ca^3$	$*ca^1$
garnet (g)	$*g^1$, $*g^{53d}$, $*g^1 1*g^{53d}$	$*g^{53d}$
karmoison (kar)	$*kar^1$, *Df(3R)kar-SZ11/Df(3R)kar-SZ12*	$*kar^1$
light (lt)	lt^1	lt^1
lightoid (ltd)	$*ltd^1$, $*ltd^1/Df(2R)Cy^L bw^{VDelR}$	$*ltd^1$
maroonlike (mal)	mal^{bz}	mal^{bz}
orange (or)	$*or^1$, or^{48a}, $*or^1/or^{48a}$	$*or^1$
pale ocelli (po)	po^1	po^1
pink (p)	$*p^p$, $*p^{snb}$, $*p^p/*p^{snb}$	$*p^p$
ruby (rb)	$*rb^1$, rb^H	$*rb^1$
scarlet (st)	$*st^1$, st^1/st^{83c}	$*st^1$
vermilion (v)	$*v^1$, v^{36f}, $*v^1/v^{36f}$	$*v^1$
white (w)	$*w^1$, w^{1118}, $*w^1/w^{1118}$	$*w^1$
zeste (z)	$*z^1$, z^a	$*z^1$

Firstly 17 mutant stocks were backcrossed to Canton-S for 25–30 generations to remove extraneous mutations and to standardize the genetic backgrounds. Where more than one allele was available for a locus or a deficiency existed, transheterozygotes were tested with *cho*. For crosses to rc^2, only outcrossed mutant stocks were used.

Most mutations affect the level of both ommochrome and drosopterin pigments of the eyes. To score the effect of mutations on just ommochrome, mutations were combined with either w^{Bwx} or *bw* and then compared to w^{Bwx} or *bw* alone. (w^{Bwx} is an unusual allele of *w* that virtually eliminates drosopterins from the eyes without greatly affecting xanthommatin.) *bw* also eliminates drosopterins and reduces the level of ommochromes somewhat (Ferré *et al.* 1986). In cases where combinations with both w^{Bwx} and *bw* scored (e.g. w^{Bwx}; *st* against w^{Bwx} and *bw; st* against *bw*) no differences in phenotype were detected. The amount of pigment in the ocelli of the various mutants was estimated visually and scored on a scale from 0 (no pigment) to 3 (same as wild type). The size of these organs and their proximity to the eye precludes biochemical estimations.

The effect of mutations on the eyes and ocelli of rc^2 was noted by comparing newly eclosed adults carrying either the mutation of interest, or the mutation in combination with rc^2 (e.g. *ltd* against rc^2 *ltd*). Pigmentation of the disintegrating larval fat body was noted by comparing, e.g. rc^2 and rc^2 *ltd* newly eclosed adults. By eclosion this tissue is scattered throughout the fly but large clumps tend to accumulate just under the cuticle, along the midline of the thorax, and this region was routinely scored for pigmentation.

cho affects both ommochrome and drosopterin pigment formation. The alteration to ommochromes is complex, probably involving the synthesis of forms other than xanthommatin (Linzen, 1974; Ferré *et al.* 1986) while the levels of drosopterins are severely reduced. This complexity precludes simple biochemical analysis. To clarify the effect of *cho* on ommochrome pigmentation of the eye, *bw* or w^{Bwx} were again used. For example the eyes and ocelli of newly eclosed *cho; ltd bw* were compared to those of *ltd bw*. To estimate tubule pigmentation, mutants were combined with *cho* and then compared to *cho* alone. The tubules of 3rd instar larvae and newly eclosed adults were routinely dissected out and scanned under a high power binocular microscope for pigment granules.

In all cases, several control and mutant background animals of the same age from the same set of cultures were scored together and the experiments was repeated at least twice. Such scoring is by necessity qualitative, but differences were consistent, repeatable and verifiable.

3. Results and discussion

Mutations at 17 loci were scored for eye, ocellus, tubule and fat body phenotype in the appropriate genetic backgrounds (Table 2). The loci have been grouped into several categories on the basis of these phenotypes. Most genes are required for normal ommochrome synthesis in all four organs (Group 1), but mutations in five affect only some of the organs: the eyes and ocelli (Group 2), eyes, ocelli and fat body (Group 3), eyes, ocelli and tubules (Group 4) and the

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Table 2. *Phenotypes of the eyes, ocelli, tubules and fat body of eye colour mutations. The probable null phenotype is given where possible*

Locus	Eyes[a] (%)	Eyes[b] with *cho*	Ocelli[c]	Ocelli[d] with *cho*	Tubules[e]	Fat body[f]
Wild type	100	drk	3	drk	+	+
Group 1*a*						
v	3	no △	0	no △	−	−
cn	4	no △	0	no △	−	−
Group 1*b*						
w	0	no △	0	no △	−	−
st	2	no △	0	no △	−	−
ltd	8	no △	0	no		
ca	30	no △	0	no △	−	−
p	16	no △	0	no △	−	−
dor	15	no △	0	no △	−	−
g	nd	no △	1	no △	−	−
lt	10	no △	0	no △	−	−
or	29	no △	2	no △	−	−
cm	43	no △	1	no △	−	−
rb	38	no △	2	no △	−	−
Group 2						
cd	12	drk	0	drk	+	+
po	69	drk	1	drk	+	+
Group 3						
kar	28 ·	drk	0	drk	+	−
Group 4						
car	60	no △	2	no △	+[g]	+
Group 5						
z	nd	no △	3	drk	+	+
Group 6						
bw	71	drk	3	drk	+	+
mal	97	drk	3	drk	+	+

[a] Xanthommatin levels taken from Ferré *et al.* (1986). nd, not determined.
[b] Effect on eye pigmentation when *cho* is added to the mutant in a *bw* or w^{Bwx} background. drk, darkened eye; no △, no change.
[c] Ocellus pigmentation scored on an arbitrary scale of 0–3 (0, no pigment; 3, wild-type level of pigmentation).
[d] Effect on ocellus pigmentation when *cho* is added to the mutant. drk, darkened ocellus.
[e] Pigmentation of the tubules scored in a *cho* background. +, pigment; −, no pigment.
[f] Pigmentation of the fat body scored in a rc^2 background. +, pigment; −, no pigment.
[g] Excretes pigment from tubules.

eyes alone (Group 5). Each group will be dealt with separately here.

(i) *Group 1: Mutants affecting all tissues*

These mutants have been divided into two subgroups: genes which encode (*a*) pathway enzymes or (*b*) other functions.

(*a*) *Group 1a: Pathway enzyme genes* [vermilion (v) *and* cinnabar (cn)]. Lesions in these genes block pigmentation in all four organs. The eyes of $w^{Bwx}\ v^{36f}$ are pure white and those of $w^{Bwx}\ v^{1}$ and $w^{Bwx}\ v^{1}/w^{Bwx}\ v^{36f}$ slightly off-white. The addition of *cho* has no effect on v^{36f} (i.,e. w^{Bwx} *cho* v^{36f} eyes remain white) whereas w^{Bwx} *cho* v^{1} have slightly more pigmented eyes than $w^{Bwx}\ v^{1}$. The eyes of the transheterozygote (w^{Bwx} *cho* v^{36f}/w^{Bwx} *cho* v^{1}) lie between the two homozygotes, and in all three crosses involving *cho* the tubules remain unpigmented. The ocelli of v^{1}, v^{36f} and v^{1}/v^{36f} are colourless. v^{1} also blocks pigmentation of the fat body of rc^2 flies and the eyes of v^{1}; rc^2 are indistinguishable from v^{1} alone.

The eyes of cn^{1} *bw*, cn^{35k} *bw* and cn^{1} *bw*/cn^{35k} *bw* are pure white. When *cho* is added to these mutant combinations no pigmentation results. The ocelli remain unpigmented in all of these combinations. The tubules of *cho*; cn^{1}/cn^{35k} and the fat body of rc^2 cn^{1} do not deposit pigment.

Although mutations in *v* and *cn* block pigmentation of all four organs, their nonautonomous behaviour complicates the conclusions that can be drawn about

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their patterns of expression. v^+ must be expressed in the eyes and *cho* tubules because these organs can pigment when their only source of kynurenine is endogenous to them. Conversely pigmentation of the ocelli is dependent on exogenous kynurenine (Fig. 2) so v^+ is not necessarily expressed in this organ. v^+ is expressed in wild-type fat body because kynurenine is found there at a time when there are no exogenous sources (Rizki & Rizki, 1968), and presumably in rc^2 fat body as well. v^+ is therefore expressed in the eyes, *cho* tubules and rc^2 fat body, with expression in the ocelli remaining an open question.

For *cn* the situation is also complex. The eyes and ocelli have previously been shown to synthesize pigment when reliant on their own sources of 3-hydroxykynurenine (Beadle & Ephrussi, 1937). Similarly *cho* tubules can synthesize pigment during larval life when their only source of 3-hydroxykynurenine is endogenous. rc^2 fat body begins to pigment after pupariation, when exogenous 3-hydroxykynurenine is usually available. However in the *cho*; rc^2 double mutant discussed below the fat body can still pigment, even though all of the tubule 3-hydroxykynurenine is locked up as pigment, so rc^2 fat body must also express cn^+. cn^+ is therefore expressed in the eyes, ocelli, *cho* tubules and rc^2 fat body. Both *v* (Walker *et al.* 1986; Searles & Voelker, 1986) and *cn* (W. Warren and A. J. Howells, pers. comm.) have been cloned so that definitive analysis of v^+ and cn^+ expression patterns is now possible.

(*b*) *Group 1b: Other genes* [white (w), lightoid (ltd), scarlet (st), pink (p), claret (ca), deep orange (dor), ruby (rb), carmine (cm), garnet (g), light (lt), orange (or)]. Lesions in these genes, which reduce or eliminate pigmentation of the eyes and ocelli, also block pigmentation of the tubules and fat body. The level of eye pigmentation varies considerably, from *w*, *bw*; *st* and *ltd bw* having white eyes, to *cm*; *bw*, which is only a little paler than *bw*. Pigmentation of the ocelli also varies, but not necessarily in concert with eye pigmentation. Some mutants which deposit considerable amounts of eye pigment have colourless ocelli (e.g. *ca*) whereas other mutants which have more lightly pigmented eyes do in fact deposit some pigment in the ocelli (e.g. g^{53d}). Although there is some difficulty in accurately determining the level of pigmentation in the ocelli, the differences detected above are so obvious that this lack of correlation must reflect real differences in the effects of these eye colour mutants on pigmentation of the two organs.

When the Group 1b mutations are combined with *cho* (in a *bw* or w^{Bwx} background), eye pigmentation does not change. *cho* also fails to alter ocellus pigmentation. The tubules and fat bodies of mutants for these loci, in combination with *cho* and rc^2 respectively, do not deposit pigment.

Three of these loci are absolutely required for the synthesis of ommochrome in any tissue: *w*, *st* and *ltd*. The remaining loci (*p*, *ca*, *dor*, *rb*, *cm*, *g*, *lt* and *or*) are also required for normal pigmentation of all four organs, but do allow some pigmentation of at least the eyes.

Unlike *v* and *cn*, Group 1b mutants (and all of the following mutants) are autonomous (Beadle & Ephrussi, 1936), so their patterns of expression can be inferred from the tissues they affect. All 11 are required for normal pigmentation of the eyes, ocelli *cho* tubules and rc^2 fat body and thus are expressed in all four tissues.

The sites of lesion of many of these mutants have been investigated previously, mainly through biochemical analysis and tissue transplantation (reviewed in Linzen, 1974; Phillips & Forrest, 1980; Summers, Howells & Pyliotis, 1982). In all Group 1b mutants that have been tested, the four enzymes of the pathway function normally (Glassman, 1956; Sullivan *et al.* 1973; Sullivan & Kitos, 1976; Wiley & Forrest, 1981), so no genes coding for products that directly affect enzyme function are represented here. These genes must encode products that are involved in other biochemical functions pertinent to pathway function, e.g. uptake, intracellular movement and storage of precursors, access of precursors to enzymes, removal of waste products, etc.

The *w* and *st* genes have been cloned and sequenced (O'Hare *et al.* 1984; Tearle *et al.* 1989; D. B, Boyle, R. G. Tearle, A. Elizur and A. J. Howells, unpublished data). They show substantial amino-acid homology to each other and to components of bacterial permeases (Mount, 1987; Tearle *et al.* 1989 and unpublished data), supporting the hypothesis that the *w* and *st* protein products are involved in the transport of pigment precursors (Sullivan & Sullivan, 1975). However these lesions (and those of *p*, *ltd* and *ca*) cause similar complex biochemical phenotypes: appreciable amounts of tryptophan enter cells and some 3-hydroxykynurenine is synthesized (Howells, Summers & Ryall, 1977), but little or no pigment is formed.

These observations cannot be explained simply by a failure to transport precursors into the cell, and I propose that the site of action of the putative permease should be extended to include: transport of any precursor across the plasma membrane and into the cell; transport of kynurenine across the outer mitochondrial membrane to interact with kynurenine-3-hydroxylase which is probably located on the outer surface of the inner mitochondrial membrane (Okamoto *et al.* 1967; Stratakis, 1981), and transport of 3-hydroxykynurenine across a membrane associated with pigment granule genesis to form xanthommatin. This proposal is outlined in Fig. 3.

Some bacterial permeases consist of multiple protein components (Ames, 1986). Hybridization between fragments carrying the *p* and *w* genes (A. J. Howells, pers. comm.) suggests that the *p*-encoded protein may be another component of the ommochrome precursor

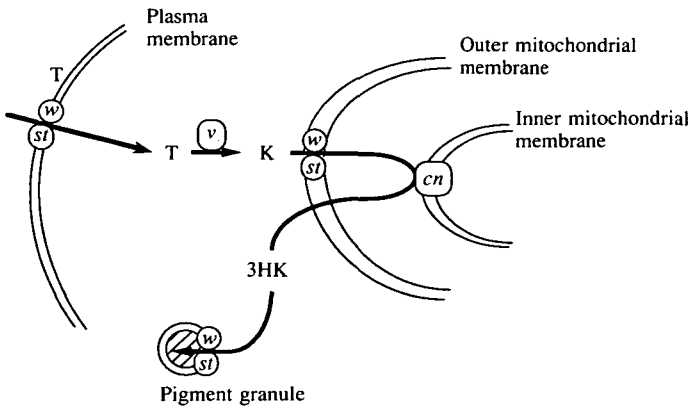

Fig. 3. Model of proposed sites of action of the *white* and *scarlet* gene products. These gene products are required for the transport of tryptophan (T) across the plasma membrane, kynurenine (K) across the outer mitochondrial membrane and 3-hydroxykynurenine (3HK) across the pigment granule membrane. *v*, *vermilion*; *cn*, *cinnabar*; *st*, *scarlet* and *w*, *white* gene products.

transport system. The biochemical phenotypes of *ca* and *ltd* also make them candidates.

If the *w*, *st*, *p*, *ca* and *ltd* proteins do complex to form a permease, why is the permease not required for tryptophan uptake and kynurenine storage by the fat body (Beadle & Ephrussi, 1937)? The fat body is an organ of intermediary metabolism and there may well be other more generalized mechanisms for transporting tryptophan across the plasma membrane for protein synthesis, with only the later steps of the pathway which involve the traversing of other membranes showing a critical requirement for a permease.

Two Group 1b loci, *dor* and *lt*, are lethal when amorphic (Lindsley & Grell, 1968; Lindsley & Zimm, 1985, 1990). The viable hypomorphic alleles analysed here affect both ommochrome and pteridine synthesis. Several genes which encode pteridine biosynthetic pathway enzymes (e.g. *Punch*) can also perturb ommochrome biosynthesis (Ferré *et al.* 1986) and the lethality associated with lack of function *dor* and *lt* is probably due to perturbance of pteridine biosynthesis alone.

The remaining Group 1b loci (*cm*, *g*, *or* and *rb*) remain relatively uncharacterized biochemically and so will not be discussed further here.

(ii) *Group 2: Mutations affecting the eyes and ocelli* [cardinal (cd) *and* pale ocelli (po)]

The eyes of $w^{Bwx}; cd^{1}$, $w^{Bwx}; cd^{KP2}$ and $w^{Bwx}; cd^{1}/cd^{KP2}$, are pale brown. The similarity of the eyes of the two homozygotes, the transheterozygote and other EMS induced *cd* alleles (A. J. Howells, pers. comm.) suggests that this is probably the phenotype of null mutations at this locus. The addition of *cho* to both $w^{Bwx}; cd^{1}$ and $w^{Bwx}; cd^{1}/cd^{KP2}$ considerably darkens the eye colour. However the ocelli remain colourless in all of the above mutant combinations.

In combination with *cho* both cd^{1} and cd^{1}/cd^{KP2} have pigmented tubules. $rc^{2}; cd^{1}$ fat body also deposits pigment. The *cd* lesion therefore reduces pigmentation of the eyes and eliminates that of the ocelli, but does not block pigmentation of the tubules nor fat body.

po^{1} has almost wild type eyes (distinguishable in a wild type but not in a *bw* or w^{Bwx} background) and partly pigmented ocelli. The eyes of *cho* versus *cho*; po^{1} and of w^{Bwx} *cho* versus w^{Bwx} *cho*; po^{1} are indistinguishable, suggesting that *po* does not have much effect on pigmentation of the eyes. *cho* does not alter pigmentation of po^{1} ocelli. The tubules of *cho*; po^{1} and fat body of rc^{2} po^{1} are pigmented. *po* therefore affects the ocelli and to a lesser extent the eyes.

Thus the genes cd^{+} and po^{+} are required for normal pathway function in the eyes and ocelli only. The phenotype of cd^{1} has been investigated at the cellular level (Stark, Srygley & Greenberg, 1981). This lesion blocks pigmentation of one of the two types of eye pigment cells, the secondary pigment cells, and causes excessive pigmentation of the other, the primary pigment cells. Unlike most mutations analysed here, the addition of *cho* to $w^{Bwx}; cd$ darkens the eyes, probably because the primary pigment cells are capable of further pigmentation. The ocelli remain unpigmented under all conditions, so *cd* is required in two of the three adult pigment cell types.

The product of the cd^{+} locus in unlikely to play a role in the differentiation of the eyes and ocelli since the temperature sensitive period of a cd^{ts} allele coincides with the onset of eye pigmentation (A. J. Howells, pers. comm.), after cell commitment and morphogenesis has occurred (Tomlinson & Ready, 1987; Cagan & Ready, 1989). The accumulation of 3-hydroxykynurenine instead of ommochrome at this time (Howells *et al.* 1977) suggests a role in the terminal step of the pathway. However *cd* is unlikely to encode the terminal enzyme, phenoxazinone synthase, because there are two forms of this enzyme, and *cd* is not deficient in either form (Wiley & Forrest, 1981). It can be concluded only that *cd* affects the terminal step in the pathway in two types of cells in some unspecified manner.

There is little genetic and biochemical information concerning *po*. Only two alleles have been reported, po^{1} used in this study and po^{2}, a hypomorphic allele which has been shown to accumulate normal levels of 3-hydroxykynurenine during larval life but slightly reduced levels during pupal life (Howells *et al.* 1977). The relationship of this slightly altered pattern in precursor flux to reduction in the level of ocellar pigmentation is far from clear.

(iii) *Group 3: Mutations affecting the eyes, ocelli and fat body* [karmoison (kar)]

Mutant alleles at the *kar* locus resemble those of

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cd. bw; kar^{1} adults have very pale brown eyes and the addition of *cho* darkens the eye colour (although less than for *cd*). The ocelli remain unpigmented regardless of genetic background. The tubules of *kar*^{1}, *kar*^{1}/*Df(3R)kar-SZ11* and two overlapping deficiencies for *kar*(*Df(3R)kar-SZ11/DF(3R)kar-SZ12*) all deposit pigment in combination with *cho*. However the fat body of *rc*^{2}; *kar*^{1} does not deposit pigment. Lesions at this locus therefore affect pigmentation of three organs – the eyes, ocelli and fat body. The *kar*$^{+}$ gene must be expressed in these organs.

The possibility that either *cd* or *kar* (or both) may have encoded or regulated phenoxazinone synthase prompted an analysis of combinations of lesions at the two loci. The eyes of *w*^{Bwx}; *kar*^{1} *cd*^{1} adults are less pigmented than for either mutant alone. With *cho* the eyes deposit somewhat more pigment, but again less than for either mutant alone. The ocelli are colourless in all cases. The tubules of *cho; kar*^{1} *cd*^{1} appear to deposit as much pigment as *cho; kar*^{1} and *cho; cd*^{1}. Thus in the absence of both *kar*$^{+}$ and *cd*$^{+}$ gene products the eyes and tubules can still synthesize and deposit pigment. These results argue quite strongly that neither encode a phenoxazinone synthase.

Like *cd*, *kar* continues to accumulate 3-hydroxykynurenine after pigment synthesis normally begins in the eye, but the mutant also accumulates excess 3-hydroxykynurenine during larval life (Howells *et al.* 1977). There is no simple model involving perturbation of pigment precursor synthesis that explains all aspects of the *kar* phenotype.

(iv) *Group 4: Mutations affecting the eyes, ocelli and tubules* [carnation (car)]

car; bw flies have a slightly lighter eye colour than *bw*, indicating that they do synthesize a reduced, albeit substantial amount of pigment. *cho* does not alter the eye colour, as evidenced by the similarity of *car; bw* and *cho car; bw*. The ocelli of *car* flies are paler than wild type, and the addition of *cho* does not alter pigmentation. Upon eclosion, *cho car* flies have heavily pigmented meconium, the only mutant when combined with *cho* to behave in this manner. The meconium is not pigmented in *car* nor *car; rc*^{2}. The tubules of *cho car* are also pigmented upon eclosion but the tubules of old flies often become unpigmented. The addition of *st* or *ltd*, mutations which block tubule pigmentation (e.g. *cho car; st*), also blocks the formation of the meconial pigment. The fat body of *car; rc*^{2} is pigmented and remains so throughout adult life.

The presence of ommochrome in the meconium of newly emerged *cho car* adults and the tendency of tubules to become unpigmented with age suggests that this double mutant excretes ommochrome from the tubules. The addition of mutations which block pigmentation in the tubules (like *st*) also block meconial pigmentation, consistent with a tubule source of pigment and evidence against non-enzymatic formation of the pigment from excreted precursor in the meconium itself. This 'leakiness' is restricted to ommochrome deposition in the tubules, as neither *car* nor *car; rc*^{2} flies have pigmented meconium.

The relationship between the presence of ommochrome in the meconium of *cho car* and the reduced ommochrome and pteridine levels in the eyes of *car* (Ferré *et al.* 1986) is not obvious. The effect of the *car* lesion therefore is to reduce pigmentation of the eyes and ocelli and to cause excretion of pigment from the tubules. *car*$^{+}$ must therefore be expressed in these three organs.

(v) *Group 5: Mutations affecting the eyes* [zeste (z)]

Mutations at the *z* locus affect expression of the *w* gene (Jack & Judd, 1979; Lifschytz & Green, 1984). One neomorphic *z* allele, *z*^{1}, and derivatives of this allele (e.g. *z*^{op6}) repress the expression of the *w* gene, giving rise to adults with pale yellow eyes. The effect of *z*^{1} and an amorphic *z* allele (*z*^{a}) on pigmentation of all four tissues was analysed.

The pale yellow eyes of *z*^{1} *w*^{Bwx} adults are indistinguishable from those of *z*^{1} *w*^{Bwx} *cho*. The ocelli of *z*^{1} are normally pigmented and the addition of *cho* darkens them. The tubules of *z*^{1} *cho* and the fat body of *z*^{1}; *rc*^{2} are pigmented. The interaction between *z*^{1} and the *w* gene is therefore limited to the eyes.

The eyes of *z*^{a} *w*^{Bwx} adults are the same colour as those of *w*^{Bwx}. However, the eyes of *z*^{a} *w*^{Bwx} *cho* adults are noticeabley lighter than those of *w*^{Bwx} *cho*. The ocelli are similarly pigmented. The tubules and fat body of *cho* and *rc*^{2} respectively are still pigmented when combined with *z*^{a}. The effect of this amorphic *z* lesion on ommochrome pigmentation is also limited to the eyes, with the addition of *cho* and consequent alteration in eye colour necessary to make it obvious.

The *z*$^{+}$ gene encodes a transcription factor that plays a redundant role in the regulation of several genes, including *w*$^{+}$ (Bingham & Zachar, 1985). The altered eye pigmentation of *z*^{a} *cho* compared to *cho* does suggest that the level of *w*$^{+}$ gene expression during eye pigment formation is reduced and *z*$^{+}$ may therefore play a subtle, but significant, role in controlling the expression of at least some genes. The fact that only the eyes of *z*^{a} and *z*^{1} flies are affected (in the appropriate genetic background) suggests that in terms of the ommochrome biosynthetic pathway the *z*–*w* interaction is eye specific.

(vi) *Group 6: Drosopterin pathway mutants* [brown (bw) *and* maroonlike (mal)]

The mutants *bw* and *mal* have been shown to primarily affect the formation of the drosopterins (Ferré *et al.* 1986; Parisi, Carfagna & D'Amora, 1976). However, both can reduce the level of ommochrome in the eye.

They have been included in these experiments to determine whether pteridine pathway mutations can substantially affect pigmentation of the tubules and fat body.

The eye colour of w^{Bwx} mal^{bz} is lighter than for w^{Bwx}. The addition of *cho* darkens the eyes a little. The ocelli are normally pigmented in both mal^{bz} and *cho* mal^{bz}. The tubules of *cho* mal^{bz} appear to be as pigmented as those of *cho*, and the fat bodies of mal^{bz}; rc^2 and rc^2 are also similarly pigmented. The *mal* lesion thus affects pigmentation in the eye to some extent, but not the other three organs.

For *bw*, no differences were observed in the pigmentation of the ocelli of wild type versus *bw*, the tubules of *cho* versus *cho; bw* nor the fat body of rc^2 versus rc^2 *bw*. *bw* has no substantial effect on ommochrome pigmentation of the ocelli, tubules or fat body.

(vii) *Group 7:* Inter se *combination of* cho *and* rc^2

The eyes of *cho*; rc^2 are dark like those of *cho*. The ocelli of *cho*; rc^2 are less pigmented than for *cho*, and the ocelli of animals raised in crowded conditions are usually colourless. The tubules and fat body of the double mutants deposit similar amounts of pigment to the respective single mutants. The only aspect of the double mutant phenotype which differs from that of the single mutant phenotypes is the reduced level of ocellar pigmentation.

The ocellar phenotype of *cho*; rc^2 can be understood in terms of the fate of the kynurenine that is synthesized during larval life (Fig. 2). Normally both the tubules and fat body release kynurenine into the haemolymph upon pupariation. However in *cho*; rc^2 larvae the stores of this precursor in the tubules have already been converted into pigment, and the stores from the fat body may be preferentially taken up by the eyes, which pigment at least a day earlier than the ocelli. There would then be little kynurenine available for ocellar pigmentation, an effect that would be exacerbated in crowded larval cultures.

(viii) *Mutant classes that have not been recovered*

Mutations which affect the operation of the pathway in the fat body or tubules alone have never been recovered, presumably because such lesions would not cause an eye or ocellus phenotype. Mutations which affect the pathway in both the fat body and the tubules should cause loss of ocellar pigment. These may have arisen and gone undetected because the phenotype of the ocelli is difficult to score. With the exception of *z* no mutant has been recovered which affects the eyes or ocelli alone. Some Group 2 and 3 mutants do affect the ocelli more than the eyes but whether this is due to the function of the genes, nature of the lesions or the cellular environment remains to be investigated.

(ix) *What kind of lesions are* cho *and* rc?

cho and *rc* are unusual loci in that apparent loss-of-function mutations cause tissue specific alterations to a biochemical pathway. For the thrust of the work described here it is vital to know the nature of these alterations. Ectopic pigmentation could be due to changes in some biochemical aspect of the pathway, or changes in expression of several pathway genes.

The final step in the pathway is autocatalytic, so the presence of a metabolic repressor of this reaction in wild-type animals, and its absence in *cho*, could explain both the phenotype and the recessive nature of the *cho* mutation. The little data that is available suggests this might be the case. Although the eyes of *cho* adults appear much more pigmented than wild type, the amount of xanthommatin deposited is only 66% of wild type (Ferré *et al.* 1986), so ommochrome pigments other than xanthommatin must be synthesized in *cho* eyes. Another mutation which causes tubule pigmentation, *red malphigian tubules*, interacts in a similar way to *cho* with other eye colour mutations (R. G. Tearle, unpublished data) and has also been shown to deposit novel ommochromes in the eye (Linzen, 1974). This altered spectrum of ommochromes is most consistent with the lesion affecting some biochemical aspect of the pathway, rather than causing the ectopic expression of pathway genes. Unfortunately no gene involved in the last step in the pathway has been identified, so the effect of *cho* on the expression of genes encoding proteins catalysing this step cannot be studied.

Very little information concerning *rc* is available. Grell (1961) showed that the pigmentation of larval fat cells is influenced by temperature and larval starvation, which may be taken as weak evidence for the lesion being a metabolic rather than regulatory one. If *rc* fat body pigmentation is due to altered gene expression, the primary target gene candidate would be *cn*, which controls the step in the pathway that is absent in wild-type fat body but operating in *rc* fat body. With the cloning of *cn* (W. Warren and A. J. Howells, pers. comm.) it should be possible to establish whether fat body pigmentation, in *rc*, is indeed due to the altered expression of this gene, and thus whether *rc* encodes a regulatory factor.

4. Conclusions

The interactions between the tissue specific mutants, *cho* and rc^2, and the other ommochrome biosynthetic pathway mutants provide new information about genes which are necessary for pigment production in different tissues (Table 3). They indicate that the majority of genes facilitate normal functioning of the pathway *per se*: these genes are probably involved in the final steps of the pathway and/or regulate the uptake of precursors and their movement within the cell. These interactions have also identified five genes

Table 3. *Tissue specific effects of eye colour mutants*

Gene	Organs requiring gene function[a]			
v	eyes	—	tubules	fat body
cn	eyes	ocelli	tubules	fat body
ca, ltd, p. st, w *cm, g, or, rb* *dor, lt*				
cd, po	eyes	ocelli	—	—
kar	eyes	ocelli	—	fat body
car	eyes	ocelli	tubules	—
z	eyes	—	—	—

[a] Organs: wild-type eyes and ocelli, *cho* tubules and rc^2 fat body.

that are necessary for pigmentation in some but not all tissues.

Only one of these five genes, *z*, has been shown to be a regulatory locus. At least one other, *cd*, is unlikely to regulate gene expression. There is insufficient evidence to conclude whether *kar*, *po* and *car* have their effect at a metabolic or gene regulatory level.

Although the effect of mutants on pigmentation of these four organs establishes to some extent the organs in which the genes must be expressed they do not address the question of ommochrome pathway gene expression in other tissues. The effect of *w* and other mutants on pteridine pigmentation of the testis sheath not only illustrates the intimate genetic and biochemical interrelationship between the two pigment pathways but also indicates that some of the genes designated as ommochrome pathway genes have other roles and thus must be regulated to display the appropriate patterns of tissue-specific and temporal gene expression concomitant with those roles.

The wealth of genetic and biochemical data on the ommochrome biosynthetic pathway offers great scope for determining the mechanisms involved in the coordinate regulation of gene expression and for identifying genes that carry out this regulation. With the cloning of four pathway genes (*w*, *st*, *v* and *cn*) identification of such regulatory hierarchies and their components will be made simpler.

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