Genetica **98**: 249–262, 1996.

Molecular characterization of the *cinnabar* region of *Drosophila melanogaster*: Identification of the *cinnabar* transcription unit

William D. Warren*, Stephanie Palmer & Antony J. Howells
Division of Biochemistry and Molecular Biology, Faculty of Science, The Australian National University, Canberra, ACT, 2601, Australia
**Present address: Peter MacCallum Cancer Institute, St Andrews Place, East Melbourne, Vic. 3002, Australia*

Received 28 February 1996 Accepted 24 May 1996

Key words: cinnabar, eye pigmentation, FAD binding, kynurenine 3-monooxygenase, mitochondrial membrane targetting sequence

Abstract

Early studies of eye pigmentation in *Drosophila melanogaster* provided compelling evidence that the *cinnabar* (*cn*) gene encodes the enzyme kynurenine 3-monooxygenase (EC 1.14.13.9). Here we report the cloning of approximately 60 kb of DNA encompassing the *cn* gene by chromosome walking in the 43E6-F1 region of chromosome 2. An indication of the position of *cn* within the cloned region was obtained by molecular analysis of mutants: 9 spontaneous *cn* mutants were found to have either DNA insertions or deletions within a 5 kb region. In addition, a 7.8 kb restriction fragment encompassing the region altered in the mutants was observed to induce transient *cn* function when microinjected into cn^- embryos. The *cn* transcription unit was identified by Northern blotting and sequence analysis of cDNA and genomic clones from this region. The predicted *cn* protein contains several sequence motifs common to aromatic monooxygenases and is consistent with the assignment of *cn* as encoding the structural gene for kynurenine 3-monooxygenase.

Introduction

The brick red colour of the compound eye of *Drosophila melanogaster* is determined by the presence of the brown (ommochrome) and red (drosopterin) screening pigments that optically isolate the individual ommatidial units. These pigments are located mainly in specialized pigment cells that form a sleeve around each ommatidium. The biosynthetic pathways for the production of the two classes of pigments, which are biochemically distinct, are under strict developmental control. Ommochrome deposition in the developing eye first begins around 47 h after pupariation and continues until two days after eclosion (Ryall & Howells, 1974), while the drosopterins first appear about 70 h after pupariation and are deposited until two to three days after adult emergence (Fan et al., 1976). These features of tissue-specific and temporal control, in combination with the identification of over 50 genes that have a primary effect on pigment production (Lindsley & Zimm, 1992), make eye pigment synthesis in *Drosophila melanogaster* an attractive system for studying coordinated gene regulation.

Xanthommatin, the main ommochrome screening pigment found in dipteran eyes, is biosynthetically derived from tryptophan via a series of oxidation reactions that involve N-formylkynurenine, kynurenine, and 3-hydroxykynurenine as intermediates (see reviews by Linzen, 1974; Phillips & Forrest, 1980; Summers, Howells & Pyliotis, 1982; Sullivan, 1984). Four eye colour genes of *D. melanogaster* – *scarlet*(*st*), *white*(*w*), *vermilion*(*v*), and *cinnabar* (*cn*)– encode products that are absolutely required for xanthommatin synthesis because null mutations at these loci abolish brown pigment production entirely. Two of these genes (*st* and *w*) code for products that are involved in the uptake and storage of xanthommatin precursors (Sullivan & Sullivan, 1975; Howells & Ryall, 1975). Both *st* and *w* have been cloned and an examination of the amino acid sequences of their

putative protein products (Mount, 1987; Tearle et al., 1989; Pepling & Mount, 1990; Ewart et al., 1994) has shown that they both belong to the Traffic ATPase (ABC) super-family of membrane transporters (Ames, Mimura & Shyamala, 1990; Hyde et al., 1990). The other two genes code for enzymes required for xanthommatin biosynthesis. The *v* gene encodes the pathway enzyme tryptophan oxygenase (Baglioni, 1960; Baillie & Chovnick, 1971; Walker, Howells & Tearle 1986). This gene has been cloned (Searles & Voelker, 1986; Walker, Howells & Tearle, 1986) and its molecular structure fully characterized (Searles et al., 1990). Compelling biochemical data indicates that the *cn* gene encodes the third enzyme of the pathway, kynurenine 3-monooxygenase (EC 1.14.13.9);[1] no activity of this enzyme was detected in adults or pupae of strains homozygous for three different mutant alleles of *cn* (cn^1, cn^3, and cn^{35K}) (Ghosh & Forrest, 1967; Sullivan, Kitos & Sullivan, 1973). In pupae that have one, two, or three copies of the cn^+ allele, enzyme activity is proportional to the cn^+ dose (Sullivan, Kitos & Sullivan, 1973).

The activity of kynurenine 3-monooxygenase in *D. melanogaster* has been shown to vary in a developmentally specific manner, with peaks in both larval and pupal life (Sullivan, Kitos & Sullivan, 1973). Grillo (1983) determined that the larval activity can be attributed almost entirely to expression in the Malpighian tubules and that pupal kynurenine 3-monooxygenase activity can be detected only in developing eye tissue. Thus the *cn* gene is of interest because of its distinct tissue and temporal specificity, being expressed only in the larval Malpighian tubules and in the developing eyes and ocelli of the pharate adult. The enzyme is also of interest because of its subcellular localization. In *Saccharomyces* (Bandlow, 1972), *Neurospora* (Cassady & Wagner, 1971), rat liver (Okamoto et al., 1967) and in the Mediterranean flour moth *Ephestia* (Stratakis, 1981), kynurenine 3-monooxygenase has been shown to be associated with the outer mitochondrial membrane. It seems likely that this is also the case in *D. melanogaster* because it has been localized to the mitochondria (Sullivan, Grillo & Kitos, 1974) and shown to be hydrophobic (Grillo, 1983).

Three of the four genes known to be essential for the production of xanthommatin have been cloned. This paper describes the cloning of the fourth, *cn*. As described below, a short chromosome walk was undertaken in the 43E-F region of chromosome 2, the chromosomal region to which *cn* has been localized by genetic and cytogenetic analysis (Lindsley & Zimm, 1992). By analysing the genomic DNA from a series of *cn* mutants using Southern blotting, a 5 kb region was identified as being the likely location of the *cn* gene. This and adjacent regions were sequenced and shown to contain a transcription unit with the potential to encode a polypeptide with several amino acid sequence motifs characteristic of monooxygenase enzymes.

Materials and methods

Drosophila *stocks*

Wild-type and mutant strains of *Drosophila melanogaster* were obtained from a number of sources, listed in Table 1. All stocks were routinely reared at either 18 ° or 25 °C on standard cornmeal-treacle medium (Roberts, 1986).

Library screening and Southern blotting

For each step of the chromosome walk, three genome equivalents of an amplified Canton S genomic DNA library, constructed in EMBL3A (Walker, Howells & Tearle, 1986), were screened using standard procedures (Maniatis, Fritsch & Sambrook, 1982; Bender, Spierer & Hogness, 1983). cDNA clones were isolated from libraries constructed from seven- to nine-day-old pupal RNA (Poole et al., 1985) and from size fractionated cDNA derived from newly emerged adult heads (Dreesen, Johnson & Henikoff, 1988). For both genomic and cDNA libraries, replica nitrocellulose filter lifts were hybridized with nick translated probe DNA at 60 °C as described by Walker, Howells, and Tearle (1986).

Genomic DNA was isolated from adult flies as described by Bender, Spierer, and Hogness (1983). Bacteriophage lambda and plasmid DNA were prepared as described by Sambrook, Fritsch, and Maniatis (1989). Restriction digestions and Southern blotting were performed as described by Walker, Howells, and Tearle (1986) except that genomic Southern blots were hybridized at 42 °C in 50% formamide, 5 × SSPE, 1 × Denhardt's solution, 10% dextran sulphate, 0.1% SDS and 50 μg/ml herring-sperm DNA and washed at a final stringency of 0.5 × SSC/0.1% SDS at 65 °C.

[1] Formerly known as kynurenine 3-hydroxylase (EC 1.14.1.2 pre-1978, and EC 1.99.1.5 pre-1964)

Table 1. Sources of *Drosophila* stocks

Strain	Background	Origin	Source	Reference
Canton S	standard wild-type	wild-type	ANU	Lindsley and Grell, 1968
cn^{1}	outcrossed to Canton S	Spontaneous in wild-type	ANU	Tearle, 1987
cn^{2}	*SM5*	Spontaneous in *In(2R)Cy*	I. Alexandrov	Lindsley and Zimm, 1992
cn^{3}	*T(Y;2)C*	Unknown¶	BG	Lindsley and Zimm, 1992
	T(Y;2)C	Unknown	ID	Lindsley and Zimm, 1992
cn^{4}	$In(2LR)ds^{33k}ab^{2}cn^{4}bw^{V1}$	Unknown¶	BG	Lindsley and Zimm, 1992
cn^{35k}	wild-type	Spontaneous in *B bb*	BG	Lindsley and Zimm, 1992
	wild-type	Spontaneous in *B bb*	ID	Lindsley and Zimm, 1992
cn^{38j}	$net^{38j}b^{38j}cn^{38j}bw^{38j}$	Unknown¶	BG	Lindsley and Zimm, 1992
cn^{83c}	wild-type	Spontaneous	ANU	Tearle, 1987
cn^{84h}	wild-type	Spontaneous	ANU	Tearle, 1987
cn^{84g}	wild-type	Spontaneous in $y^{2}su(w^{a})$	ANU	Tearle, 1987
cn^{86i}	wild-type	Spontaneous	ANU	A. Howells unpublished
cn^{rbr}	wild-type	Spontaneous in *vg*	A. Fontevilla	Valadé del Rio, 1982
cn^{88d}	wild-type	MR induced	M. Green	M. Green unpublished
cn^{MR1}	wild-type	MR induced	M. Yamomoto	M. Yamomoto unpublished
$l(2)cn^{84h80}$	$SM5/l(2)cn^{84h80}$	radiation induced	I. Alexandrov	Alexandrov, 1984
$Df(2R)cn^{79b9}$	$SM5/Df(2R)cn^{79b9}$	radiation induced	I. Alexandrov	Alexandrov, 1984
Df(2R)CA53	*CyO/Df(2R)CA53*	radiation induced	TM	Lindsley and Zimm, 1992
Df(2R)cn-h3	*CyO/Df(2R)cn-h3*	radiation induced	TM	Lindsley and Zimm, 1992

Abbreviations: ANU, The Australian National University; BG, Bowling Green State University, Bowling Green Ohio, USA; ID, Indiana University, Bloomington Indiana, USA; TM, Max-Plank-Intitut für Entwicklungsbiologie, Tübingen Germany.
¶The origin of these alleles is not reported in the literature.

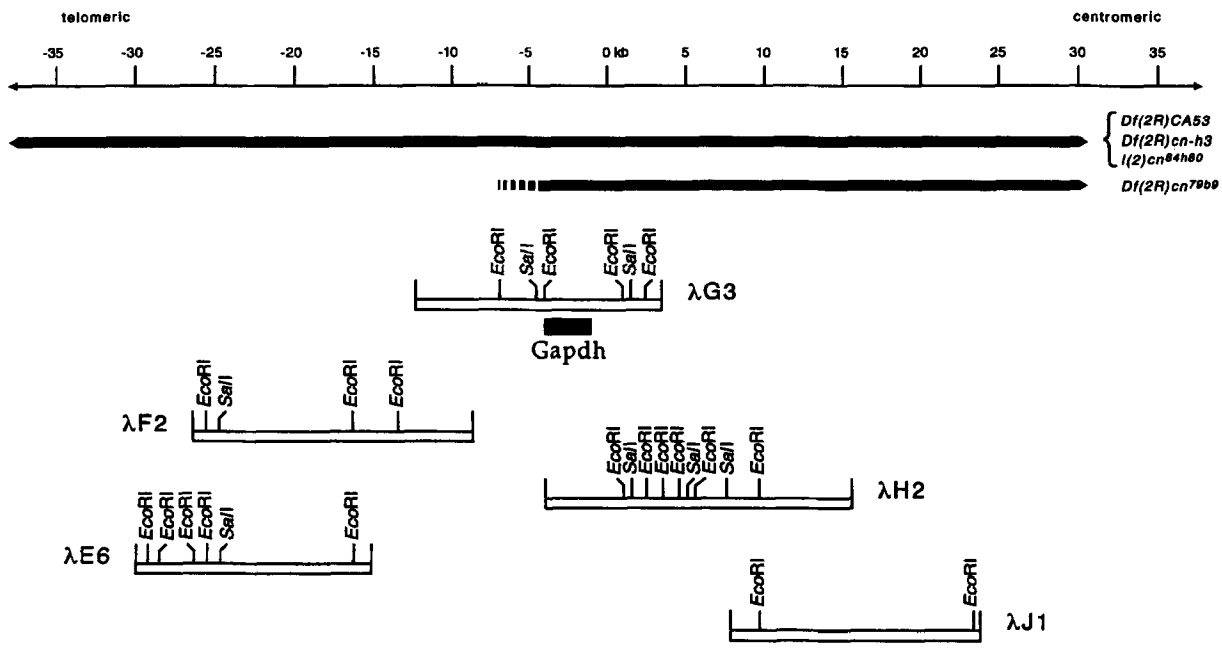

Figure 1. Basic restriction maps of the recombinant clones isolated from the 43E polytene band region. The orientation of the walk in relation to the centromere and telomere and the location of the *Gapdh* gene are shown. Solid black bars indicate the regions deleted in *Df(2R)CA53*, *Df(2R)cn-h3*, $Df(2R)cn^{79b9}$ and $l(2)cn^{84h80}$. Coordinates originate at the unique *Xho*I site in λH2 (see Figure 5).

RNA extraction, blotting, and RT-PCR procedures

RNA was extracted from frozen animals by an adaptation of the method described by Bingham and Zachar (1985). 0.2 mg of frozen material was homogenized in 2.5 ml of 10 mM Tris-HCl (pH8)/350 mM NaCl/2% SDS/7M urea/10 mM EDTA and immediately vortexed in 1 ml of buffered phenol followed by 1 ml of chloroform. The aqueous phase was then collected, reextracted as above, and ethanol precipitated. Poly A^+ RNA was then isolated from this crude preparation by oligo dT cellulose chromatography (Nakazato & Edmonds, 1974).

Northern blot analysis was performed by electrophoresing equal quantities (approx. 10 μg) of glyoxylated poly A^+ RNA through 1% agarose, blotting onto nylon membrane (Hybond N+, Amersham), and hybridizing to a randomly primed ^{32}P labelled DNA probe in 50% deionized formamide/2 × SSPE/1% SDS/1% BSA/0.5 mg/ml sheared herring testis DNA/10% dextran sulphate at 50 °C. Blots were washed at a final stringency of 0.1 × SSC, 1% SDS at 60 °C prior to autoradiography.

cDNA was made from DNase treated poly A^+ RNA using a commercial cDNA synthesis kit (Pharmacia P-L Biochemicals) utilizing the oligonucleotide 5′-AACTGGAAGAATTCGCGGCCGCAGGAA$(T)_{18}$-3′ to prime the reverse transcription (RT) reaction. Two rounds of hemi-nested PCR were performed on 1 μg of cDNA in 100 μl reactions. Each amplification contained 2.5 U Taq DNA polymerase/0.2 mM dNTPs/2 mM $MgCl_2$/50 mM KCl/10 mM Tris pH 8.3/100 pmol of each primer. Primers 5′-CAGAATCAAAACGATCTCCTG-3′ and 5′-CATTTGCGACCTGGCCATGTAC-3′, which are specific to *cn* exon 2, were used in combination with primer 5′-AAGAATTCGCGGCCGCAGGAAT-3′. Thermal cycling conditions for both primer combinations were as follows: 96 °C (5 min), 35 cycles of 96 °C (10 s), 65 °C (15 s), 72 °C (1.5 min) followed by a final extension of 72 °C (5 min).

Plasmid cloning and sequencing

Restriction fragments derived from recombinant phage were cloned using standard procedures (Sambrook, Fritsch & Maniatis, 1989) into the pBluescribe (pBS+ and pBS−, Stratagene) phagemid vectors. PCR amplified cDNA sequences were cloned into pBluescript (Stratagene) prepared as described by Marchuk et al. (1991). All phagemids were propagated in *E. coli* strain JPA101 (a Tet^R derivative of JM101 constructed by J. Adelman).

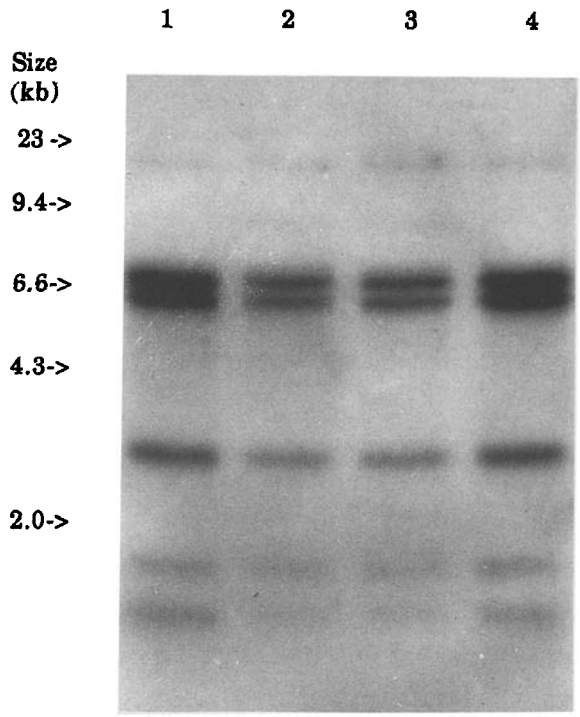

Figure 2. Localization of the λG3 clone to the region absent in both the *Df(2R)CA53* and *Df(2R)cn-h3* deficencies. Equal quantities of *Eco*RI-cut genomic DNA were probed with radiolabelled λG3 DNA. DNA samples include Canton S (lanes 1 and 4), *Df(2R)CA53/CyO* (lane 2), and *Df(2R)cn-h3/CyO* (lane 3).

Nucleotide sequences were determined from single-stranded DNA by a modification of the standard Sequenase method (Tsang & Bentley, 1988) using ^{35}S-dATP (Amersham, >1000 Ci/mmol) and ^7C-deaza-dGTP (Boehringer) in place of dGTP (Mizusawa, Nishimura & Seela, 1986). Nested deletions required for sequence analysis were generated by the method of Henikoff (1987). Sequence analyses were performed with the MacVector (IBI) or GCG (Devereux, Haeberli & Smithies, 1984) suites of sequence manipulation programs. Protein and nucleic acid sequence database searches were performed using the BLAST algorithm (Altschul et al., 1990) via the electronic-mail search facility provided by the National Center for Biotechnology Information (NCBI).

Embryo injections for transient expression assays

Embryos (less than 40 min old) that had been manually dechorionated and desiccated were microinjected with cesium chloride purified plasmid DNA (600 mg/ml) essentially as described by Rubin and

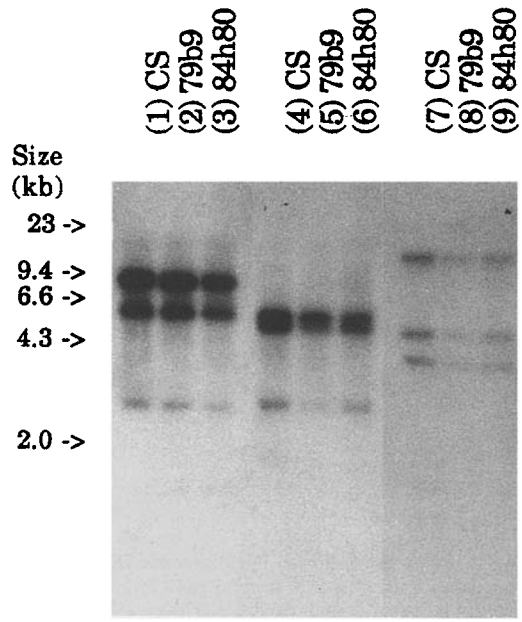

Figure 3. Localization of the λF2, λG3, and λH2 clones relative to the lesions in *Df(2R)*cn^{79b9} and *l(2)cn*^{84h80}. Replica Southern blots containing approximately equal loadings of *Eco*RI-cut genomic DNA were probed with the λF2 (lanes 1–3), λG3 (lanes 4–6), and λH2 (lanes 7–9). DNA samples analysed were Canton S (CS), *Df(2R)cn*^{79b9}*/SM5* (79b9), and *l(2)cn*^{84h80}*/SM5* (84h80). Alexandrov and Alexandrov (1992) report *Df(2R)cn*^{79b9} to be lacking the 43E5-7 to 43E19-F1 polytene band region.

Spradling (1982). The injected animals were maintained at 20 °C until eclosion. Eye phenotype was scored within 24 h of eclosion and rechecked around 72 h later.

Results

Cloning the cinnabar *region by chromosome walking*

λG3, a genomic clone originating from within polytene band 43E and containing the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene (Sullivan et al., 1985), provided an entry point into the *cn* gene region. Using standard chromosome walking techniques, two sequential steps were taken from each end of the λG3 clone to isolate overlapping clones from a Canton S derived genomic library. Preliminary restriction mapping of DNA from the recombinant phage (denoted λE6, λF2, λH2, and λJ1; see Figure 1) showed that the genomic region covered by the five clones spans just over 60 kb.

To localize the cloned sequences within the 43E-F polytene band region, Southern blot analyses of genomic DNA from wild-type and from heterozygotes carrying *Df(2R)CA53* , *Df(2R)cn-h3*, *Df(2R)cn*^{79b9}, and *l(2)cn*^{84h80} were performed. All four of these chromosomes lack *cn* function as well as that of one or more flanking lethal complementation groups (Alexandrov, 1984; Alexandrov & Alexandrov, 1991; Lindsley & Zimm, 1992; Wustmann et al., 1989). Radiolabelled DNA prepared from the λG3 clone was found to hybridize to *Df(2R)CA53/CyO* and *Df(2R)cn-h3/CyO* DNA with approximately half the intensity of that observed for wild-type (Figure 2). By probing Southern blots similar to that shown in Figure 2, it was found that the sequences covered by the entire 60 kb are absent in both *Df(2R)CA53* and *Df(2R)cn-h3* (data not shown). The hybridization signal with DNA from the *l(2)cn*^{84h80} heterozygotes was reduced to about half that of wild-type for all the bands hybridizing to λF2, λG3, and λH1 (Figure 3, lanes 1, 4, and 7 compared to lanes 3, 6, and 9), as well as with λE6 and λJ1 (data not shown). This indicated that the entire genomic region covered by the walk is deleted in the *l(2)cn*^{84h80} chromosome. In contrast, DNA from the *Df(2R)cn*^{79b9} showed wild-type levels of hybridization to λF2 (Figure 3, lane 1 compared to 2), and λE6 (not shown), but reduced levels with λH2 (Figure 3, lane 7 compared to 8) and λJ1 (not shown). λG3 appears to span one of the deletion endpoints in the *Df(2R)cn*^{79b9} chromosome, as one fragment (of about 6 kb) shows normal levels of hybridization while the others show reduced levels (Figure 3, lane 4 compared to 5). Alexandrov (1984) determined that the *Df(2R)cn*^{79b9} chromosome lacks *cn* function as well as that of a juxtaposed lethal complementation group immediately centromere proximal, whereas *l(2)cn*^{84h80} lacks *cn* and the flanking lethal gene immediately distal (Alexandrov, 1984; Alexandrov, unpublished). From the orientation of the two deficiencies with respect to the chromosome it was deduced that the distal endpoint of *Df(2R)cn*^{79b9} is located within the λG3 sequences and, of the five recombinant phage, λJ1 contains sequences closest to the centromere. Because *cn* function is absent in both *Df(2R)cn*^{79b9} and *l(2)cn*^{84h80}, *cn* was concluded to lie on the centromeric side of the *Df(2R)cn*^{79b9} breakpoint, i.e., in the righthand region of λG3, in λH2, λJ1, or beyond.

Southern blot analysis of cinnabar *mutant strains*

In an attempt to locate the *cn* transcription unit within the region covered by the chromosome walk, 15 independently isolated, cytologically normal *cn* alleles (Table 1) were examined by genomic Southern analysis

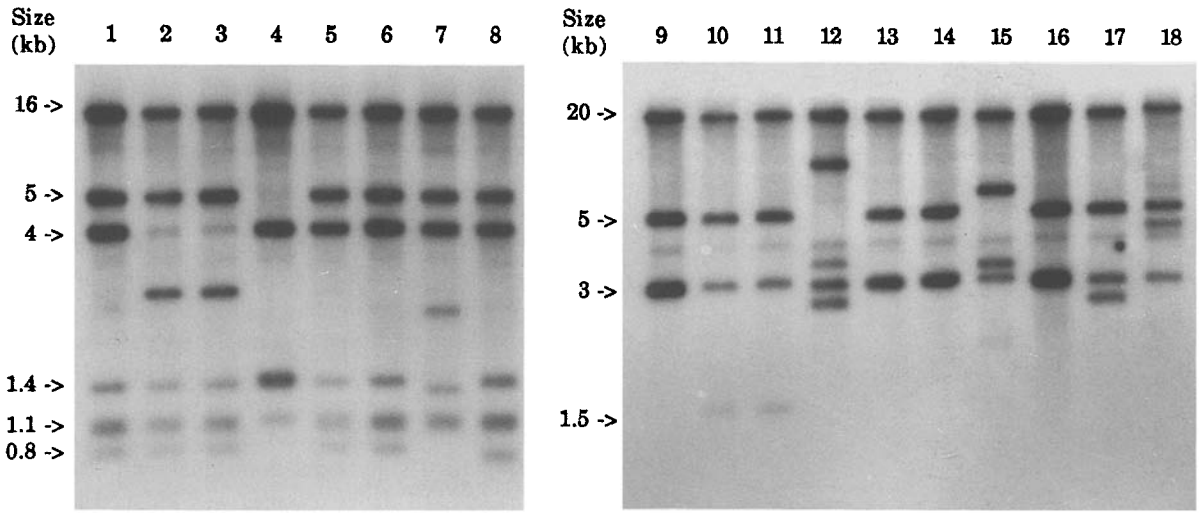

Figure 4. Hybridization of radiolabelled λH2 DNA to *Eco*RI digested (lanes 1–8) and *Sal*I digested (lanes 9–18) genomic DNA from various *cinnabar* mutants. DNA samples analysed include Canton S (lanes 1 and 9), cn^1 (lanes 2 and 10), cn^{rbr} (lanes 3 and 11), cn^3 from Bowling Green (lanes 4 and 12), cn^{83c} (lanes 5 and 13), cn^{84g} (lanes 6 and 14), cn^{84h} (lanes 7 and 15), cn^{38j} (lanes 8 and 16), cn^{35k} (lane 17), and $SM5/l(2)cn^{84h80}$ (i.e., cn^2, lane 18).

for DNA rearrangements. When λH2 was hybridized to blots carrying DNA cut with a variety of restriction enzymes, altered restriction patterns relative to wild type were found in a large number of the mutants (see Figure 4 for examples). This high level of restriction site polymorphism was not observed when the Southern filters shown in Figure 4 were stripped and hybridized with λE6, λF2, or λJ1 (data not shown).

A detailed restriction map of the sequences carried in λH2 was constructed and the nature and position of the molecular lesion in each mutant (as determined from a series of detailed genomic Southern analyses) was determined (Figure 5). DNA insertions or deletions were mapped to a region in the centre of the λH2 clone in 9 of the 15 *cn* alleles examined, suggesting that the *cn* transcription unit spans this region. Of these 9 alleles, 7 showed a single alteration while 2 alleles (cn^3 and cn^{84h}) have two. The cn^3 strain obtained from the Bowling Green Stock Center contains a 0.5 kb deletion as well as a 7.5 kb insertion located about 5 kb distally, although this insertion was not found in DNA from the cn^3 strain obtained from the Indiana Stock Center (data not shown). For cn^{84h}, two insertions were detected, one of 0.4 kb and a second of approximately 1 kb located about 4 kb distally (Figure 5). Both cn^3 and cn^{35k} show a 0.5 kb deletion that appears to be identical in both size and position. Two other alleles (cn^1, cn^{rbr}) contain identical 1.5 kb deletions (compare the relevant lanes in Figure 4 for examples) and cn^2 and cn^4 have identical 8 kb DNA insertions (data for cn^4 not shown). In addition, identical restriction patterns to that observed for cn^1 were also seen in DNA from cn^{88d} and cn^{MR1} (data not shown).

Localization of cinnabar *by transient expression*

Transient expression of microinjected DNA was used to ascertain whether the region of λH2 identified by mutant analysis could supply *cn* function *in vivo*. Transient expression of injected DNA containing the *cn* transcription unit was considered likely to induce partial xanthommatin deposition in cn^- individuals because 1) *cn* is non-cell autonomous in tissue transplantation experiments (Beadle & Ephrussi, 1936), 2) xanthommatin deposition in adult eye tissues can be partially restored by feeding cn^- larvae on a diet supplemented with 3-hydroxykynurenine (Schwabl & Linzen 1972), and 3) microinjection of $rosy^+$ and v^+ encoding DNA into $rosy^-$ and v^- embryos has previously been shown to partially restore adult eye pigmentation (Rubin & Spradling, 1982; P.W. Atkinson and W.D.W, unpublished). Plasmid DNA containing the 7.8 kb *Xho*I-*Kpn*I fragment (designated XK8, coordinates 0 to +7.8, Figure 5), which includes the region altered in the *cn* mutants, was found to induce partial xanthommatin deposition in 37% of the adults developing from microinjected $cn^1;w^{Bwx}$ embryos. Thus, the XK8 fragment was concluded to contain the *cn* gene.

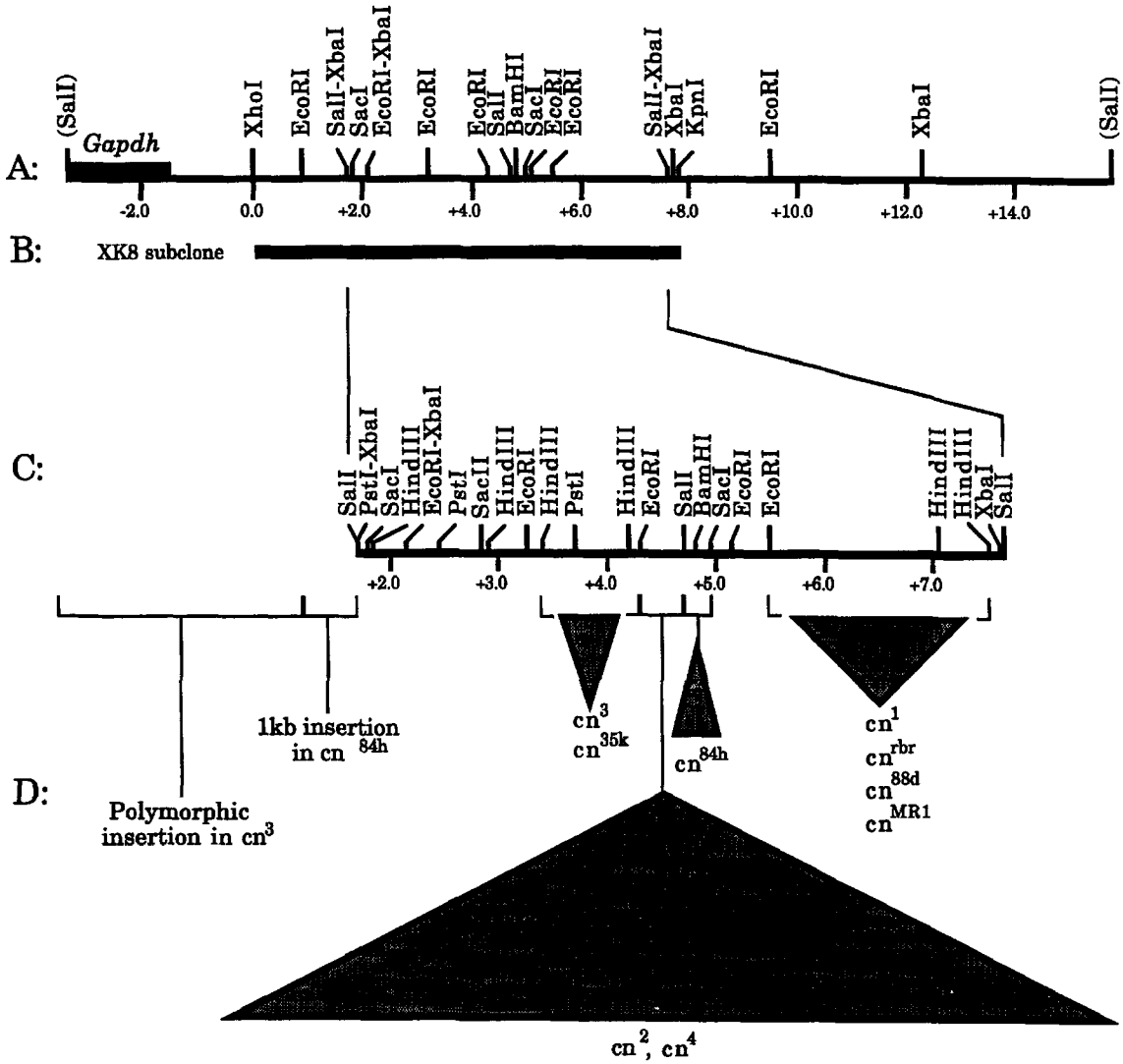

Figure 5. Summary of the molecular characterization of the *cinnabar* gene region. (A) Restriction map of the λH2 clone; (B) Location of the XK8 sequences shown to induce eye pigmentation in *cn*⁻ animals; (C) Enlarged restriction map of the central portion of λH2; (D) Molecular lesions identified by Southern blot analysis of *cn* mutants. Insertions (Δ) and deletions (∇) are indicated in proportion to their size and location. Coordinates correspond to kilobases from the unique *Xho*I site.

Sequence analysis of the cinnabar *region*

In order to establish the molecular structure of the *cn* gene region, the sequence of 12.3 kb of genomic DNA (from the *Xho*I site, coordinate 0.0, to the *Xba* I site, coordinate +12.3) was determined, as was the sequence of several partial cDNA clones and an RT-PCR product derived from this region. The portion of the genomic sequence data that includes the *cn* gene is shown in Figure 6. The *cn* gene is interupted by two introns whose splice donor and acceptor sequences compare favorably to the known *Drosophila* consensus (Mount et al., 1992). The second ATG codon of exon 1 (nucleotides 3741-3743) appears to be the *cn* initiation methionine because it is flanked by sequences similar to the *D. melanogaster* translation initiation consensus (Cavener & Ray, 1991). In addition, codon preference analysis (Gribskov, Devereux & Burgess, 1984) of the sequences flanking this ATG revealed it to be coincident with the transition from triplets that poorly correlate with the known *Drosophila* codon bias, to triplets that more closely resemble the bias found in *Drosophila* coding sequences. Sequences similar to the CCAAT (nucleotides 3556-3563), TATA (nucleotides 3600-3605), and mRNA CAP (nucleotides 3683-3689)

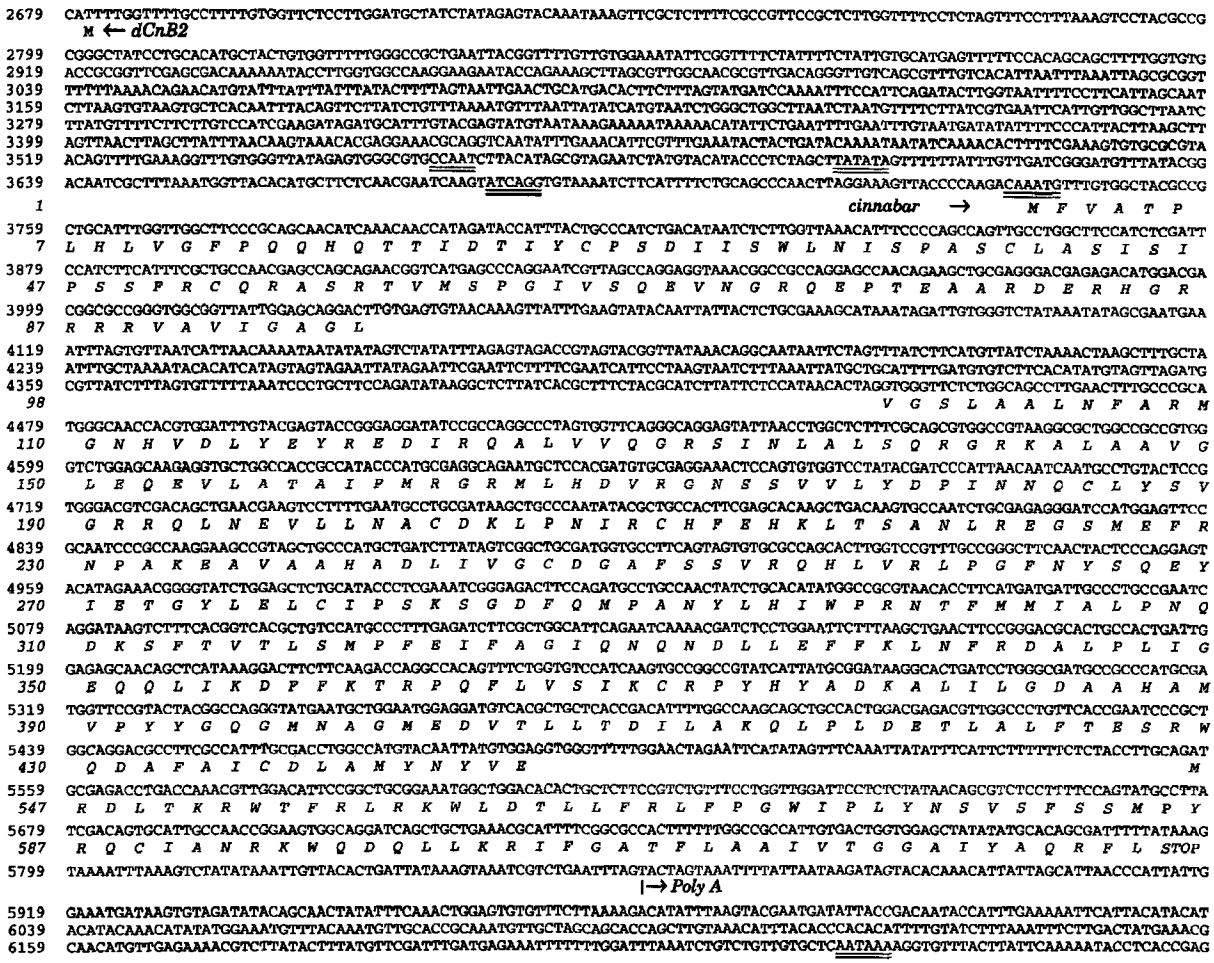

Figure 6. Nucleotide sequence and deduced amino acid translation of the *cinnabar* gene. The sequence shown starts at the ATG initiation codon of the adjacent, divergently transcribed *dCnB2* transcription unit (Warren, Phillips & Howells, 1996). Putative CCAAT (nucleotides 3556-3563), TATA (nucleotides 3600-3605), mRNA CAP site (nucleotides 3683-3689), AUG initiation signal sequences (nucleotides 3738-3743), and polyadenylation signal sequences (nucleotides 6240-6245) are double underlined and the location of the poly A tail determined by cDNA analysis indicated. Nucleotide numbering corresponds to sequences deposited in GenBank accession U56245.

consensus sequences (McKnight & Kingsley, 1982; Hultmark, Klemenz & Gehring, 1986) were also identified in the region upstream of this ATG codon (Figure 6). Analysis of sequences derived from the 3′ end of the *cn* transcript, obtained by RT-PCR amplification of RNA isolated from 56-74 h pupae, revealed a poly A tract commencing after nucleotide 5859. Since the first consensus AATAAA polyadenylation signal sequence downstream of the stop codon at the end of exon 3 occurs at nucleotide 6240 (Figure 6), a cryptic polyadenylation signal must have been used to terminate this transcript. Given the location of the mRNA CAP consensus, the size of the two introns and location of the poly A tail, the full length *cn* transcript was predicted to be approximately 1.8 kb.

Comparison of the cinnabar *protein sequence to other monooxygenases*

Conceptual translation of the *cn* gene, presented below the nucleic acid sequence shown in Figure 6, yields a protein product that is 524 amino acids in length. This sequence was used to search the Swiss-Prot/PIR protein databases as well as translations of the GenBank/EMBL/DDBJ nucleotide sequence databases. Sequence similarity to a number of proteins was detected, including several known monooxygenases that act on aromatic substrates. In addition, homology was

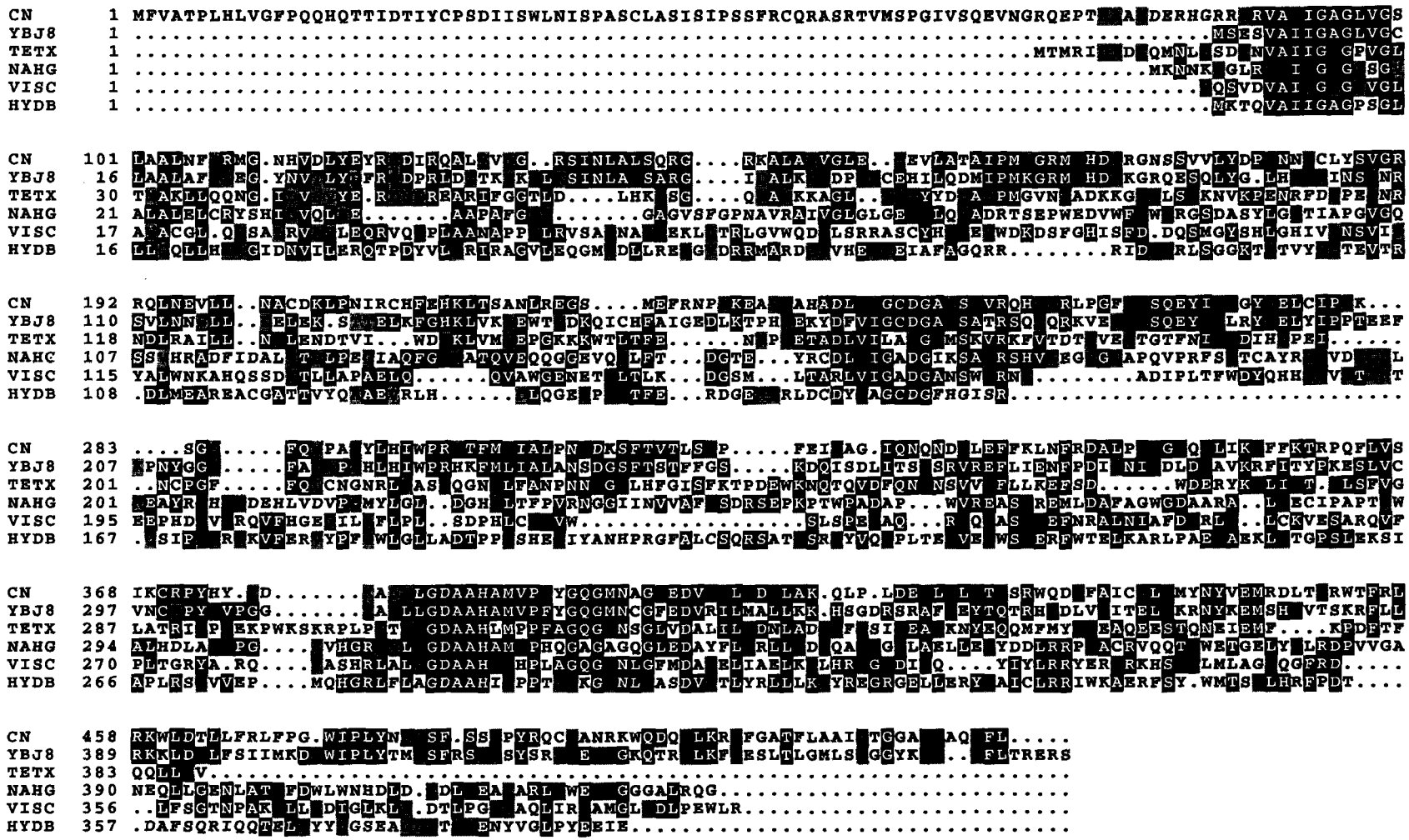

Figure 7. Alignment of the *cn* protein to related oxidoreductase sequences. Amino acid identities are shown as white letters on a black background and amino acid similarities as black letters on a gray background. Sequences shown are CN, *cinnabar* protein; YBJ8, hypothetical protein in ATP1-ROX3 intergenic region of *Saccharomyces cerevisiae* chromosome 2 (GenBank accession Z35859); TETX, transposon Tn4351 tetracycline resistance protein from *Bacteroides fragilis* (Speer, Bedzyk & Salyers, 1991); NAHG, salicylate 1-monooxygenase encoded by the *Pseudomonas putida nahG* gene (You, Ghosal & Gunsalus, 1991); VISC, *visC* protein from *E. coli* (Nakahigashi et al., 1992); HYDB, 4-hydroxybenzoate 3-monooxygenase from *Pseudomonas fluorescens* (Weijer et al., 1983).

observed between sequences near the N-terminus of the *cn* protein and a large number of FAD binding enzymes. An alignment of the *cn* protein to a number of these homologous proteins is shown in Figure 7. Two conserved domains were clearly identified; the first, spanning residues 89-117, are involved in the binding of FAD (Wierenga, Terpstra & Hol, 1986) and the second, spanning residues 383-411, are implicated in the formation of a substrate binding cavity and NADPH binding site (Weijer et al., 1983; Sejlitz et al., 1990). The extended sequence similarity to several known monooxygenases and the presence of FAD and NADPH/substrate binding motifs in the proposed *cn* protein indicates that *cn* encodes a flavin containing aromatic monooxygenase.

In order to investigate aspects of the secondary structure of the polypeptide encoded by *cn*, hydropathy predictions were made using the method of Kyte and Doolittle (1982). With the exception of the extreme N- and C-terminal regions, the hydropathy of the *cn* protein is consistent with a globular cytosolic structure (data not shown). The N- and C-terminal regions are hydrophobic and could be involved in membrane anchoring. Because other outer mitochondrial membrane targeted enzymes have been proposed to contain an amphiphilic alpha helix at their N-terminus (von Heijne, 1986), this region of the *cn* protein was examined in detail for such structures. Although the extreme N-terminus has very few charged residues and appears unlikely to form an alpha helix, a region of probable helical structure begins at around residue 70. Given that the FAD binding motif, found within the first 20 or so amino acids in all the other known monooxygenase sequences belonging to subclass EC 1.14.13, is located just over 90 amino acids from the N-terminus of the *cn* protein, it seems likely that the first 70 amino acids are involved in mitochondrial targeting and/or anchoring.

Northern blot analysis of cinnabar *expression*

To determine the transcriptional characteristics of *cn*, a 0.8 kb partial cDNA clone spanning sequences from nucleotides 3887 to 5068 was hybridized to polyA$^+$ RNA extracted from larvae, pupae, and newly emerged adults. Two transcripts of about 1.8 kb and 2.2 kb were detected in varying amounts in mid- and late-larvae and in early- and mid-pupae with the highest levels being present in mid-pupae (Figure 8). Low levels of both transcripts were observed in RNA from late pupae, but neither could be detected in newly emerged adults.

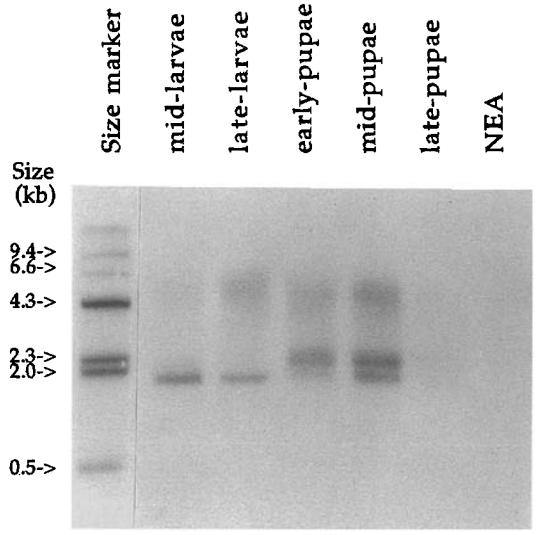

Figure 8. Northern blot analysis of *cn* mRNA. RNA extracted from 50–70 h larvae (mid larvae), 72–92 h larvae (late larvae), 24–32 h pupae (early pupae), 40–58 h pupae (mid-pupae), 72–78 h pupae (late pupae), and newly emerged adults (NEA) was hybridized with a radiolabelled plasmid clone containing *cn* cDNA sequences. Each lane contained equal quantities of polyA+ RNA as judged by ethidium bromide staining of the gel prior to blotting. The leftmost lane shows size standards (*Hin*dIII fragments of λ phage DNA).

This developmental profile is consistent with the developmental variation in kynurenine 3-monooxygenase enzyme activity, which shows peaks in both larval and pupal life with the highest activity being present in mid- to late-pupae (Sullivan, Kitos & Sullivan, 1973). Although both transcripts are found during larval and pupal development, the 1.8 kb transcript appears to be the dominant *cn* mRNA in larvae, whereas the 2.2 kb transcript is the dominant *cn* mRNA found in pupae. The size of the 1.8 kb mRNA is consistent with the *cn* gene structure detailed in Figure 6, while the exact nature of the 2.2 kb mRNA has not yet been determined.

Discussion

Four independent lines of evidence indicate that we have cloned and sequenced the *cn* gene: (i) the cloned sequences encompass a region that displays altered restriction patterns in nine different *cn* mutants; (ii) an 8 kb restriction fragment from this region is capable of supplying *cn* function when introduced into *cn*$^-$ mutants; (iii) the deduced *cn* protein displays amino acid similarity to other proteins known to encode aro-

matic monooxygenases, and (iv) the cloned sequences hybridize to RNA transcripts with developmental profiles consistent with the developmental variations seen in kynurenine 3-monooxygenase activity.

The molecular analysis of *cn* mutants shows several unexpected features. Whereas a high proportion of spontaneous mutations in a number of *D. melanogaster* genes are caused by the insertion of transposable elements (Berg & Howe, 1989), only cn^2 and cn^4 appear to contain transposable element insertions. The 7.5 kb insertion detected in a cn^3 strain obtained from the Bowling Green Stock Center may be a transposable element, but it is unlikely to be associated with the loss of *cn* activity because it lies about 6 kb upstream from the *cn* gene and is not present in the cn^3 strain obtained from Indiana University. The molecular analysis of *cn* mutants also revealed a number of seemingly identical lesions, including four mutants (cn^1, cn^{rbr}, cn^{MR1} and cn^{88d}) that have similar 1.5 kb deletions. The finding that cn^{rbr} is associated with a deletion is difficult to reconcile with the reported instability of this mutant and of its ability to induce reversions of cn^1 and cn^2 alleles in *trans* (Valade del Rio, 1974; 1982). One possible explanation for these results is that the four alleles simply represent copies of the cn^1 lesion resulting from cross contamination of strains. For the cn^{88d} and cn^{MR1} alleles, which were isolated relatively recently as single mutant individuals in separate MR mutagenesis experiments, strain contamination seems less likely. Although the details of cn^{MR1} isolation are uncertain, the cn^{88d} allele was selected over a chromosome containing the cn^1 allele (M.M. Green, unpublished). Since MR chromosomes have been shown to induce P-element mobilization (Green, 1986) and therefore must also induce P-transposase activity, cn^{88d} and cn^{MR1} may have been derived from cn^1 by gene-conversion following a P-element excision and subsequent double-stranded break repair, as described by Engels et al. (1990).

Northern blot analysis revealed two *cn* mRNAs of about 1.8 and 2.2 kb, the former being consistent with the anticipated size of the *cn* transcript as inferred from the DNA sequence data. Although we have no definitive data on the 2.2 kb mRNA, the simplest explanation is that this transcript contains an additional 400 bp of 3′ untranslated sequences resulting from polyadenylation following the signal found at nucleotide 6240 instead of the cryptic signal used for generating the 1.8 kb mRNA. The developmental profile of *cn* transcript levels is consistent both with the biology of eye pigmentation (Summers, Howells & Pyliotis, 1982) and with the developmental variation in levels of kynurenine 3-monooxygenase enzyme activity (Sullivan, Kitos & Sullivan, 1973).

Comparison of the *cn* polypeptide to other flavin-containing monooxygenases that act on aromatic substrates led to the identification of two conserved sequence motifs corresponding to the FAD binding site and to the putative NADPH binding active site regions. Given the biochemical similarites of this class of enzymes (requiring NADPH to catalyse the incorporation of one atom of oxygen into an aromatic substrate) the lack of more general sequence similarity seems surprising. A comparison of the sequences of kynurenine 3-monooxygenase from a number of other species would provide insight into the level of sequence variation resulting from the different substrate specificities of these enzymes and their mitochondrial targeting signal sequences. Towards this end we have commenced the characterization of the *D. virilis cn* homologue (C. Patterson, S.P. & A.J.H., unpublished).

The unusual position of the FAD binding motif in the *cn* protein, found within 20 residues of the N-terminus of all of the other known flavin-containing monooxygenase-like sequences shown in Figure 7, is consistent with the proposal that the first 80 N-terminal residues of the *cn* protein contain sequences necessary for mitochondrial targeting. However, the nature of sequence motifs involved in targeting proteins to the outer mitochondrial membrane is still in considerable doubt. Monoamine oxidase B is another nuclear-encoded FAD binding enyzme targeted to the outer mitochondrial membrane (Greenawalt & Schnaitman, 1970), which in contrast to the *cn* protein, has its FAD binding domain located extremely close to the N-terminus (Ito et al., 1988). Similarly, the 42 kD and 38 kD outer mitochondrial membrane proteins, involved in the mitochondrial protein import mechanisms in yeast and Neurospora respectively (Baker et al., 1990; Kiebler et al., 1990), do not contain N-terminal targeting sequences of the type employed by other well-characterized outer membrane proteins such as porin or the 70 kD protein (Hase et al., 1984; Mihara & Sato, 1985; Kleene et al., 1987).

The work presented in this paper provides a firm basis for future studies of the structure, function, and regulation of the *cn* gene. A comparison of cDNA and genomic DNA sequences from this region has also led to the characterization of a divergently transcribed gene, *dCnB2*, located immediately upstream of *cn* (coordinates +1.1 to +2.7), which encodes a protein homologous to the Ca^{2+} binding regulatory sub-

unit of the protein phosphatase, calcineurin (Warren, Phillips & Howells, 1996). The identification of initiation codons for both *cn* and for the divergently transcribed *dCnB2* gene defines a 1 kb regulatory region that probably contains all of the *cis*-acting sequences involved in the regulated expression of both *dCnB2* and *cn*. Comparison of this region with upstream regions in the other genes involved in xanthommatin production may provide valuable information about the *cis*-elements and *trans*-acting factors that act to coordinate the expression of genes involved in this common metabolic process in the terminally differentiated pigment cells of the developing adult eye. Such information should contribute to a greater understanding of the molecular mechanisms that regulate the activity of genes in time and space during metazoan development.

References

Alexandrov, I.D., 1984. Comparative genetics of neutron- and gamma-ray-induced lethal *b*, *cn* and *vg* mutations in *D. melanogaster*. Dros. Inf. Serv. 60: 45–47.

Alexandrov, I.D. & M.V. Alexandrov, 1991. Cytogenetics of the *cinnabar* mutations induced by different quality radiations. Dros. Inf. Serv. 70: 16.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers & D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Ames G.F., C.S. Mimura & V. Shyamala, 1990. Bacterial periplasmic permeases belong to a family of transport proteins operating from *Escherichia coli* to human: Traffic ATPases. FEMS Micro. Rev. 6: 429–46.

Baglioni, C., 1960. Genetic control of tryptophan peroxidase-oxidase in *Drosophila melanogaster*. Nature 184: 1084–1085.

Baillie, D.L. & A. Chovnick, 1971. Studies on the genetic control of tryptophan pyrrolase in *Drosophila melanogaster*. Mol. Gen. Genet. 112: 341–353.

Baker, K.P., A. Schaniel, D. Vestweber & G. Schatz, 1990. A yeast mitochondrial outer membrane protein essential for protein import and cell viability. Nature 348: 605–609.

Bandlow, W., 1972. Membrane separation and biogenesis of the outer membrane of yeast mitochondria. Biochem. Biophys. Acta 282: 105–122.

Beadle, G.W. & B. Ephrussi, 1936. The differentiation of eye pigments in *Drosophila* as studied by transplantation. Genetics 21: 225–247.

Bender, W., P. Spierer & D.S. Hogness, 1983. Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the Bithorax complex in *Drosophila melanogaster*. J. Mol. Biol. 168: 17–33.

Berg, D.E. & M.M. Howe, 1989. Mobile DNA. American Society for Microbiology Publications, Washington DC.

Bingham, P.M. & Z. Zachar, 1985. Evidence that two mutations, w^{Dzl} and z^{1}, affect synapsis dependent genetic behavour of *white* are transcriptional regulatory mutants. Cell 40: 891–827.

Cassady, W.E. & R.P. Wagner, 1971. Separation of mitochondrial membranes of *Neurospora crassa* I. Localization of L-kynurenine-3-hydroxylase. J. Cell Biol. 49: 535–541.

Cavener. D.R. & S.C. Ray, 1991. Eukaryotic start and stop translation sites. Nuc. Acids Res. 19: 3185–3192.

Devereux, J., P. Haeberli & O. Smithies, 1984. A comprehensive set of sequence analysis programs for the VAX. Nuc. Acids Res. 12: 387–395.

Dreesen, T.D., D.H. Johnson & S. Henikoff, 1988. The *brown* protein of *Drosophila melanogaster* is similar to the *white* protein and to components of active transport complexes. Mol. Cell. Biol. 8: 5206–5215.

Engels, W.R., D.M. Johnson-Schlitz, W.B. Eggleston & J. Sved, 1990. High-frequency P element loss in *Drosophila* is homologue dependent. Cell 62: 515–525.

Ewart, G.D., D. Cannell, G.B. Cox & A.J. Howells, 1994. Mutational analysis of the Traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in *Drosophila melanogaster*. J. Biol. Chem. 269: 10370–10377.

Fan, C.L., L.M. Hall, A.J. Skrinska & G.M. Brown, 1976. Correlation of guanosine triphosphate cyclohydrolase activity and the synthesis of pterins in *Drosophila melanogaster*. Biochem. Genet. 14: 271–280.

Ghosh, D. & H.S. Forrest, 1967. Enzymatic studies on the hydroxylation of kynurenine in *Drosophila melanogaster*. Genetics 55: 423–431.

Green, M.M., 1986. Genetic instability in *Drosophila melanogaster*. The genetics of an MR element that makes complete P insertion mutations. Proc. Natl. Acad. Sci. USA 83: 1036–1040.

Greenawalt, J.W. & C. Schnaitman, 1970. An appraisal of the use of monoamine oxidase as an enzyme marker for the outer membrane of rat liver mitochondria. J. Cell. Biol. 46: 173–179.

Gribskov, M., J. Devereux & R.B. Burgess, 1984. The codon preference plot: Graphic analysis of protein coding sequences and prediction of gene expression. Nuc. Acids Res. 12: 539–549.

Grillo, S.L., 1983. The biological and biochemical characterization of L-kynurenine monooxygenase from *Drosophila melanogaster*. Ph.D. thesis, Syracuse University, New York.

Hase, T., U. Muller, H. Riezman & G. Schatz, 1984. A 70-kd protein of the yeast mitochondrial outer membrane is targeted and anchored via its extreme amino terminus. EMBO J. 3: 3157–3164.

Henikoff, S., 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. 155: 156–165.

Howells, A.J. & R.L. Ryall, 1975. A biochemical study of the *scarlet* eye colour mutant of *Drosophila melanogaster*. Biochem. Genet. 13: 273–282.

Hultmark, D., R. Klemenz & W.J. Gehring, 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene *hsp22*. Cell 44: 429–438.

Hyde, S.C., P. Emsley, M.J. Hartshorn, M.M. Mimmack, U. Gileadi, S.R. Pearce, M.P. Gallagher, D.R. Gill, R.E. Hubbard & C.F. Higgins, 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 346: 362–365.

Ito, A., T. Kuwahara, S. Inadome & Y. Sagara, 1988 Molecular cloning of a cDNA for rat liver monoamine oxidase B. Biochem. Biophys Res. Comm. 157: 970–976.

Kiebler, M., R. Pfaller, T. Sollner, G. Griffiths, H. Horstmann, N. Pfanner & W. Neupert, 1990. Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. Nature 348: 610–616.

Kleene, R., N. Pfanner, R. Pfaller, T. Link, W. Sebald, W. Neupert & M. Tropschug, 1987. Mitochondrial porin of *Neurospora crassa*: cDNA cloning, in vitro expression and import into mitochondria. EMBO J. 6: 2627–2633.

Kyte, J. & R. F. Doolittle, 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105–132.

Lindsley D.L., & G. Zimm, 1992. The genome of *Drosophila melanogaster.* Academic Press, San Diego.

Linzen, B., 1974. The tryptophan to ommochrome pathway in insects. Adv. Insect Physiol. 10: 117–246.

Maniatis, T., E. Fritsch & J. Sambrook, 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Marchuk, D., D. Drumm, A. Saulino & F.S. Collins, 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nuc. Acids Res. 19: 1154.

McKnight, S.L. & R. Kingsley, 1982. Transcriptional control signals of a eukaryotic protein-coding region. Science 217: 316–324.

Mihara, K. & R. Sato, 1985. Molecular cloning and sequencing of cDNA for yeast porin, an outer mitochondrial membrane protein: a search for targeting signal in the primary structure. EMBO J. 4: 769–774.

Mizusawa, S., S. Nishimura & F. Seela, 1986. Improvement of the dideoxy chain termination method of sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nuc. Acids Res. 14: 1319–1324.

Mount, S., C. Burks, G. Hertz, G. Stormo, O. White & C. Fields, 1992. Splicing signals in *Drosophila*: intron size, informaton content and consensus sequences. Nuc. Acids Res. 20: 4255–4262.

Mount, S. M., 1987. Sequence similarity. Nature 325: 487.

Nakahigashi, K., K. Miyamoto, K. Nishimura & H. Inokuchi, 1992. Isolation and characterization of a light-sensitive mutant of Escherichia coli K-12 with a mutation in a gene that is required for the biosynthesis of ubiquinone. J. Bacteriol. 174: 7352–7359.

Nakazato, Y.F. & M. Edmonds, 1974. Purification of messenger RNA and heterogeneous nuclear RNA containing poly (A) sequences. Methods Enzymol. 29: 431–443.

Okamoto, H., S. Yamamoto, M. Nozaki & O. Hayaishi, 1967. On the submitochondrial localization of L-kynurenine-3-hydroxylase. Biochem. Biophys. Res. Commun. 26: 309–314.

Pepling, M. & S.M. Mount, 1990. Sequence of a cDNA from the *Drosophila melanogaster white* Gene. Nuc. Acids Res. 18: 1633.

Phillips, J.P. & H. S. Forrest, 1980. Ommochromes and pteridines, pp. 542–623, in The genetics and biology of *Drosophila*, edited by M. Ashburner and T.R.F. Wright. Academic Press, London.

Poole, S.J., L.M. Kauvar, B. Drees & T. Kornberg, 1985. The *engrailed* locus of *Drosophila*: Structural analysis of an embryonic transcript. Cell. 40: 37–43.

Roberts, D.B., 1986. Basic Drosophila care and techniques, pp. 1–38 in *Drosophila*, a practical approach, edited by D.B. Roberts. IRL Press, Oxford.

Rubin, G. & A. Spradling, 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218: 348–353.

Ryall, R.L. & A.J. Howells, 1974. Ommochrome biosynthetic pathway of *Drosophila melanogaster*: variations in levels of enzyme activities and intermediates during adult development. Insect Biochem. 4: 47–61.

Sambrook, J., E.F. Fritsch & T. Maniatis, 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Schwabl, G. & B. Linzen, 1972. Formation of eye pigment granules after feeding ommochrome precursors to *Drosophila v* and *cn*. W. Roux Arch. Dev. Biol. 171: 223–227.

Searles, L.L. & R. Voelker, 1986. Molecular characterization of the *Drosophila vermilion* locus and its supressible alleles. Proc. Natl. Acad. Sci. USA. 83: 404–408.

Searles, L.L., R.S. Ruth, A.M. Pret, R.A. Fridell & A.J. Ali, 1990. Structure and transcription of the *Drosophila melanogaster vermilion* gene and several mutant alleles. Mol. Cell. Biol. 10: 1423–1431.

Sejlitz, T., C. Wernstedt, A. Engstroem & H.Y. Neujahr, 1990. Amino acid sequences around the pyridoxal-5′-phosphate-binding sites of phenol hydroxylase. Eur. J. Biochem. 187: 225–228.

Speer, B.S., L. Bedzyl & A.A. Salyers, 1991. Evidence that a novel tetracycline resistance gene found on two bacteroides transposons encodes an NADP-requiring oxidoreductase. J. Bacteriol. 173: 175–183.

Stratakis, E., 1981. Submitochondrial localization of kynurenine 3-hydroxylase from ovaries of *Ephestia kuhniella*. Insect Biochem. 11: 735–741.

Sullivan, D.T., 1984. Tryptophan metabolism in Drosophila, pp. 697–710 in Progress in Tryptophan and Serotonin Research, edited by. H. G. Schlossberger, W. Kochen, B. Linzen and H. Steinhart. Walter de Gruyter & Co., Berlin.

Sullivan, D.T. & M.C. Sullivan, 1975. Transport defects as the physiological basis for the eye color mutants of *Drosophila melanogaster*. Biochem. Genet. 13: 603–613.

Sullivan, D.T., R.J. Kitos & M.C. Sullivan, 1973. Developmental and genetic studies on kynurenine hydroxylase from *Drosophila melanogaster*. Genetics 75: 651–661.

Sullivan, D.T., S.L. Grillo & R.J. Kitos, 1974. Subcellular localization of the first three enzymes of the ommochrome synthetic pathway in *Drosophila melanogaster*. J. Exp. Zool. 188: 225–234.

Sullivan, D.T., W.T. Carroll, C.L. Kanik-Ennulat, Y.S. Hitti, J.A. Lovett & L.V. Kalm, 1985. Glyceraldehyde 3-phosphate dehydrogenase from *Drosophila melanogaster*. J. Biol. Chem. 260: 4345–4350.

Summers, K.M., A.J. Howells & N.A. Pyliotis, 1982. Biology of eye pigmentation in insects. Adv. Insect Physiol. 16: 119–166.

Tearle, R.G., 1987. Genetic and molecular biological analysis of the ommochrome biosynthetic pathway in *Drosophila melanogaster.* Ph.D. thesis, Australian National University, Canberra.

Tearle, R.G., J.M. Belote, M. McKeown, B.S. Baker & A.J. Howells, 1989. Cloning and characterization of the *scarlet* gene of *Drosophila melanogaster*. Genetics 122: 595–606.

Tsang, T.C. & D.R. Bentley, 1988. An improved sequencing method using sequenase that is independant of template concentration. Nuc. Acids Res. 16: 6238.

Valade del Rio, E., 1974. cn^{rbr}: rojo brillante. Dros. Inf. Serv. 51: 22.

Valade del Rio, E., 1982. Unstable genetic system in *Drosophila melanogaster*: I. Instability at the *cinnabar* locus. Experientia 38: 790–792.

von Heijne, G., 1986. Mitochondrial targeting sequences may form amphiphilic helicies. EMBO J. 5: 1335–1342.

Walker, A.R., A.J. Howells & R.G. Tearle, 1986. Cloning and characterization of the *vermilion* gene of *Drosophila melanogaster*. Mol. Gen. Genet. 202: 102–107.

Warren, W.D., A.M. Phillips & A.J. Howells, 1996. *Drosophila* contains both X-linked and autosomal homologues of calcineurin B. Gene (in press).

Weijer, W.J., J. Hofsteenge, J.J. Beintema, R.K. Weirenga & J. Drenth, 1983. *p*-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* 2. Fitting of the amino-acid sequence to the tertiary structure. Eur. J. Biochem. 133: 109–118.

Wierenga, R.K., P. Terpstra & W.G.J. Hol, 1986. Prediction of the occurrance of the ADP-binding $\beta\alpha\beta$-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. 187: 101–107.

Wustmann, G., J. Szidonya, H. Taubert & G. Reuter, 1989. The genetics of position-effect variegation modifying loci in Drosophila melanogaster. Mol. Gen. Genet. 217: 520–527.

You, I.-S, D. Ghosal & I.C. Gunsalus, 1991. Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nahG*) and it's 3′-flanking region. Biochemistry 30: 1635–1641.