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# The *oxen* Gene of Drosophila Encodes a Homolog of Subunit 9 of Yeast Ubiquinol-Cytochrome *c* Oxidoreductase Complex: Evidence for Modulation of Gene Expression in Response to Mitochondrial Activity

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ABSTRACT

A *P*-element insertion in the *oxen* gene, $ox^1$, has been isolated in a search for modifiers of *white* gene expression. The mutation preferentially exerts a negative dosage effect upon the expression of three genes encoding ABC transporters involved in pigment precursor transport, *white*, *brown*, and *scarlet*. A precise excision of the *P* element reverts the mutant phenotype. Five different transcription units were identified around the insertion site. To distinguish a transcript responsible for the mutant phenotype, a set of deletions within the *oxen* region was generated. Analysis of gene expression within the *oxen* region in the case of deletions as well as generation of transgenic flies allowed us to identify the transcript responsible for *oxen* function. It encodes a 6.6-kD homolog of mitochondrial ubiquinol cytochrome *c* oxidoreductase (QCR9), subunit 9 of the $bc_1$ complex in yeast. In addition to *white*, *brown*, and *scarlet*, *oxen* regulates the expression of three of seven tested genes. Thus, our data provide additional evidence for a cellular response to changes in mitochondrial function. The *oxen* mutation provides a model for the genetic analysis in multicellular organisms of the effect of mitochondrial activity on nuclear gene expression.

WE are interested in defining a complete set of modifiers that exhibit a dosage effect upon the expression of a single target locus. Our interest in dosage-dependent modifiers of gene expression is centered on the hypothesis that these are responsible for aneuploidy syndromes and various types of dosage compensation (Birchler and Newton 1981; Guo and Birchler 1994; Birchler 1996). It is generally accepted that the expression of a structural gene is directly proportional to gene dose. However, the study of gene expression in dosage series in both maize and Drosophila revealed that this is not always the case. The expression of some genes was found to remain unchanged despite variation in the dosage of the chromosomal segment where they reside. In contrast, some genes, whose dosage has not been altered, were affected by aneuploidy of unlinked chromosomal regions (Birchler 1979; Birchler and Newton 1981; Devlin *et al.* 1982; Guo and Birchler 1994). To resolve this paradox, it was proposed that for any one gene, multiple *trans*-acting dosage-dependent modifiers exist in the genome. When the modifier is present in a varied chromosomal region, the expression of an unlinked target gene is affected. It was further hypothesized that the molecular basis of deleterious effects associated with aneuploid syndromes is due to an imbalance in the system of dosage-dependent modifiers, rather than a disproportion of structural gene products encoded by the varied chromosomal region (Birchler and Newton 1981; Guo and Birchler 1994). Hence, isolation and dissection of the function of these modifiers of gene expression is important for understanding the basis of the dosage effects.

The Drosophila *white* gene represents a particularly convenient model where such dosage effects can be studied. Extensive molecular genetical and biochemical studies of pigment synthesis revealed that *white* together with two other genes, *brown* and *scarlet*, is involved in the uptake of pigment precursors by the cell. Biochemical analysis indicates that *white* and *scarlet* participate in the transport of brown pigment precursors kynurenine, 3-hydroxy-kynurenine, and tryptophan, while *white* and *brown* are responsible for the transport of guanine, a precursor of red pigments (for review, see Ewart and Howells 1998). WHITE (for review, see Hazelrigg 1987), BROWN (Dreesen *et al.* 1988), and SCARLET (Tearle *et al.* 1989) share an extensive homology to each other and belong to a superfamily of ATP-binding cassette (ABC) transporters. ABC transporters are found in both prokaryotes and eukaryotes and their function is to translocate various molecules including sugars, amino acids, peptides, metals, ions, toxins, and antibiotics across the cellular membrane (for review, see Croop 1998). All ABC transporters share a conserved domain of ~200 amino acid residues including an ATP-binding site and a hydrophobic domain comprising five to eight

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transmembrane segments. On the basis of structural organization ABC transporters are subdivided into three broad categories. WHITE, BROWN, and SCARLET define a distinct group of ABC transporters. Their hydrophilic domain contains an ATP-binding motif followed by the hydrophobic domain. Genetical, molecular, and biochemical studies indicate that these proteins form heterodimers to transport pigment precursors (for review, see Ewart and Howells 1998). In this model, heterodimers between WHITE and SCARLET perform an active transport of tryptophan while WHITE and BROWN form a heterodimer responsible for guanine uptake (Dreesen *et al.* 1988; Tearle *et al.* 1989; Ewart and Howells 1998). Thus, *white* mutants will have defects in transport of both pigments. A leaky allele, *white-apricot*, retains some *white* activity, which results in a light eye color. This allows one to detect a wide range of modulation of eye pigmentation, which in turn reflects changes in *white* expression.

Using *white* as a target a number of modifiers of gene expression have been isolated. Among them are *Wow* (Birchler *et al.* 1994), *Mow* (Bhadra *et al.* 1997a), *Ufo* (Bhadra *et al.* 1997b), *sugarless* (Benevolenskaya *et al.* 1998), *Regena* (Frolov *et al.* 1998), and others. Interestingly, a majority of the modifiers exert an effect not only on *white* but also on *brown* and *scarlet* expression (Birchler *et al.* 1994; Bhadra *et al.* 1997a,b; Benevolenskaya *et al.* 1998; Frolov *et al.* 1998). These observations indicate the existence of coordinated regulation of the expression of three ABC transporters in Drosophila.

In this article we describe the isolation and molecular characterization of the *oxen* gene, a dosage-dependent modifier involved in regulation of gene expression that is distinct in function from the previously identified ones. Loss-of-function *oxen* alleles affect the steady-state mRNA level of all three ABC transporters as well as some other unrelated genes, *rudimentary*, α*Gpdh*, and *P0*. The gene encodes a 6.6-kD protein homologous to the yeast subunit 9 of mitochondrial ubiquinol cytochrome *c* oxidoreductase ($bc_1$ complex). The yeast homolog is essential for the assembly of a functional $bc_1$ complex and its deletion perturbs mitochondrial function (Phillips *et al.* 1990). Recent evidence indicates that modulations of mitochondrial functions will affect nuclear gene expression (Parikh *et al.* 1987; Poyton and McEwen 1996). Our data establish an additional example of an alteration of mitochondrial biogenesis that leads to changes in nuclear gene expression, thus suggesting a possible mechanism for the cell to adapt to variation in mitochondrial activities.

## MATERIALS AND METHODS

**Fly stocks:** Flies were raised on standard Drosophila media at 25°. Genetic markers used here can be found in Lindsley and Zimm (1992).

*ms(2)00815*, a single *P*-element insertion on chromosome *2* (49C1-D3), was identified in a screen for dominant autosomal mutations affecting the eye color of *white-apricot* flies. The same insertion was previously isolated as a male-sterile mutation and named $oxen^1$ ($ox^1$; Castrillon *et al.* 1993). To mobilize the *P* element, $ox^1/CyO; ry^{506}/ry^{506}$ males were crossed to the *Sp/CyO; Δ2-3, Sb/TM6, Ubx* strain (Robertson *et al.* 1988). The $F_1$ $ox^1/CyO$; $\Delta 2\text{-}3, Sb/ry^{506}$ males were crossed individually to three $Sp/CyO$; $ry^{506}/TM6, Ubx$ females. The *Cy*, non-*Sb*, non-*Sp* progeny ($ox^1/CyO; ry^{506}/ry^{506}$) were screened for $rosy^-$ flies, which were mated to *Gla/SM6a, Cy* balancers to establish a stock. In total, 211 derivatives were generated.

Three *oxen* alleles, $ox^{44\text{-}2}$, $ox^{107\text{-}2}$, and $ox^{157\text{-}1}$, were recombined with $P[ry^+; hs\text{-}neo; FRT]42D$. Presence of both the *FRT* and *oxen* mutation was confirmed by complementation tests and G418 resistance. To produce mitotic clones in the adult eye, $y\ w\ P[ry^+; hsFLP]12; P[ry^+; hs\text{-}neo; FRT]42D\ P[mw^+\ NM]46F/CyO$ males were crossed to $y\ w; ox^-\ P[ry^+; hs\text{-}neo; FRT]42D/SM6a$ and the progeny were heat-shocked during the first and second instars for 1 hr at 37° in a water bath (Xu and Harrison 1994). Mitotic mutant clones in $y\ w\ P[ry^+; hsFLP]12; P[ry^+; hs\text{-}neo; FRT]42D\ P[mw^+\ NM]46F/; ox^-\ P[ry^+; hs\text{-}neo; FRT]42D$ were $w^-$.

To produce germline clones, first $ox^{107\text{-}2}$ and $ox^{44\text{-}2}$ were recombined with $P[mw; FRT]^{2R\text{-}G13}$. Four independent stocks carrying both *FRT* and the *oxen* allele were generated for each deletion. $w\ P[ry^+; hsFLP]12; P[mw; FRT]^{2R\text{-}G13}\ ox^-/ovo^D\ P[mw; FRT]^{2R\text{-}G13}$ females were heat-shocked twice for two hr at the late second to early third larval instar and crossed to $ox^-/SM6a$ males (Chou and Perrimon 1996).

For the developmental Northern analysis, $ox^1/SM6a, Cy$ females were crossed to *T(2;3)CyO, Cy Tb ch* translocation males. The $F_1$ males containing this translocation heterozygous with $ox^1$ were mated to Canton-S females. The *Tb* marker allows discrimination between +/+ and $ox^1/+$ classes at the pupal stages, while the *Cy* marker allows this distinction in adults.

For *P*-element transformation, the cDNAs containing complete open reading frames (ORFs) of *QCR9* and α*NAC* were cloned into the pHT4 vector that has a *rosy* marker gene (Schneuwly *et al.* 1987) to generate $P[ry^+; hsp\text{-}QCR9]$ and $P[ry^+; hsp\text{-}\alpha NAC]$, respectively. The constructs were injected together with the *wings-clipped* helper plasmid into $ry^{506}$ embryos (Spradling and Rubin 1982). One transformed line was established carrying the α*NAC* transgene and 11 transformants were established for the *QCR9* transgene. The *DGK*ε cDNA was cloned into the pUAST vector (Brand and Perrimon 1993) and injected with *wings-clipped* helper plasmid into $y\ w^{1118}$ embryos. Two transformed lines with $P[mw^+; UAS\text{-}DGK]$ on chromosome *2* were generated. The $P[mw^+; UAS\text{-}DGK]$ transgenes were then mobilized by supplying the stable source of transposase, *Δ2-3*, to obtain three independent insertions on chromosome *3*. $P[mw^+; UAS\text{-}DGK]$, $P[ry^+; hsp\text{-}QCR9]$, and $P[ry^+; hsp\text{-}\alpha NAC]$ transgenes on the third chromosome were separately recombined with $ox^{107\text{-}2}$ and $ox^{44\text{-}2}$ alleles for rescue experiments.

**DNA and RNA techniques:** All standard DNA manipulations were performed as described in Sambrook *et al.* (1989).

The P1 phage 05-71, containing wild-type DNA from the 49D1-2 region on the cytological map (Hartl *et al.* 1994), was used to obtain an overlapping set of DNA fragments for cDNA library screens and sequencing.

The cDNA library was prepared from 2-week-old male and female wild-type adults (Canton-S) in the λZAP II vector (Stratagene, La Jolla, CA). About 600,000 phage have been screened as described in Sambrook *et al.* (1989). To clone the *QCR9* cDNA, the rapid amplification of cDNA ends (RACE) protocol was performed as described (Frolov *et al.* 1998). For PCR the following gene-specific primer was used: 5′-GGAACAAATCGGACGTCTTT-3′.

RNA blots, RNA probes, and Northern hybridization were performed according to Frolov *et al.* (1998).

For sequencing, DNA fragments were cloned into the pSP72 (Promega, Madison, WI) or Bluescript II SK (Stratagene) vector. To obtain nested clones for sequencing, a γ-δ transposon-based system was used (Strathmann *et al.* 1991). Sequencing was performed on a Sequi-Gen GT nucleic sequencing cell (Bio-Rad, Hercules, CA) using the Sequenase (v.2.0) kit (Amersham, Arlington Heights, IL). Homology searches were performed at the National Center for Biotechnology Information's BLAST WWW Server.

A region between 1.07 and 1.67 kb on the restriction map (Figure 1) was found to be 99% identical to the 3′ untranslated region of the Drosophila G protein β-subunit gene, *Gbe*, (accession no. M76593; Yarfitz *et al.* 1991). *Gbe* has been mapped to 76C on the polytene chromosome map and was proposed to be a single copy gene. Analysis of Drosophila genomic sequence shows that no *Gbe* gene sequences reside within the *oxen* region. Therefore, we concluded that the *Gbe* cDNA, whose sequence was deposited into GenBank, is a cloning artifact comprising both the sequences of the *Gbe* gene and the sequences from the *oxen* region.

***In situ* hybridization:** *In situ* hybridization with whole mount embryos was performed essentially as described in Tautz and Pfeifle (1989). Riboprobes were prepared by *in vitro* transcription with DIG RNA labeling mix and detected with alkaline phosphatase conjugated anti-DIG antibody (Boehringer-Mannheim, Indianapolis).

## RESULTS

**Identification of the *oxen* gene:** A genetic screen was performed to identify the *P*-element insertions dominantly modulating the expression of a target gene, *white*. The hypomorphic *white-apricot* allele that confers an orange-yellow eye color was used. The molecular basis of the *white-apricot* lesion is an insertion of the retrotransposon, *copia*, into the second intron (Bingham and Judd 1981). This sensitized *white-apricot* background allows one to recognize mutations that result in both elevation and reduction of *white* gene expression. As a heterozygote, one of the *P* insertions produced a darker eye color and has previously been identified as a male-sterile mutation, *oxen* ($ox^1$; Castrillon *et al.* 1993). To gain preliminary information about the nature of $ox^1$ interaction with the *white* locus, the effect of $ox^1$ on different *white* alleles was tested (data not shown). In particular, $ox^1$ was found to darken the eye color of point mutations, $w^{crr}$ and $w^{cf}$, implying that *copia* is not required for an interaction to occur. In contrast, the *Adh* promoter-*white* structural gene construct (Birchler *et al.* 1990) did not respond to $ox^1$, which indicates the necessity of the *white* promoter region for the interaction.

The chromosome carrying the $ox^1$ mutation contains a single *P*-element insertion at the cytological position 49C1-4. The $ox^1$ allele is sterile over the deficiency *Df(2)vg135* (FlyBase http://flybase.bio.indiana.edu:82), which uncovers the region from 49A4-13 to 49E7-F1 on the polytene chromosome map (Lindsley and Zimm 1992). In addition, the deficiency as a heterozygote darkens the eye color of *white-apricot* flies as $ox^1$ does, suggesting that $ox^1$ is a loss-of-function allele. To further prove that the insertion is responsible for the mutant phenotype, the *P* element on the $ox^1$ chromosome was mobilized. Out of 211 established stocks, 122 were homozygous viable lines showing precise excision of the *P* element in each case. One line, designated as $ox^{rev}$, was chosen for further study as a control of normal expression of the *oxen* locus. When tested for the interaction with *white-apricot*, $ox^{rev}$ shows a reversion of the eye color phenotype.

We sought molecular evidence that *white* gene expression is affected in the $ox^1$ mutants. Total RNA was isolated from pupae segregating for normal or the $ox^1$ chromosome. Pupae were chosen because the majority of pigment is deposited at this developmental stage. RNA transfers were made in triplicate and hybridized with a *white* antisense probe. The same blots were then probed with rRNA, which served as a gel-loading control. The phosphorimagery data are presented in Table 1. Consistent with the eye color phenotype, the mutation results in elevation of *white* transcript levels in mid- and late pupae. The expression of two related ABC transporters, *brown* (*bw*) and *scarlet* (*st*), was also monitored. It was found that the $ox^1$ mutation affects the expression of both *scarlet* and *brown* but the effect is different. The steady-state level of *st* is increased in females but it is decreased in males. In late pupae, *st* mRNA is increased in females and slightly increased in males. In contrast, the expression of *bw* is mostly increased in pupae of both sexes and the effect is stronger compared to *scarlet* and *white*. In summary, the $ox^1$ mutation exhibits an effect upon the expression of all three genes. The effect has a complex profile depending upon the sex and the developmental stage.

**Transcriptional mapping of the *oxen* gene:** We have previously identified a *DGKε* gene 123 bp from the *P*-element insertion site in the $ox^1$ chromosome (M. V. Frolov, E. V. Benevolenskaya and J. A. Birchler, unpublished results). It encodes a homolog of human diacylglycerol kinase ε isoform. To further characterize the *oxen* region, a set of genomic DNA fragments from a P1 phage, 05-71, (Hartl *et al.* 1994) covering the *P*-element insertion site was used to probe a cDNA library. Isolated clones fall into two transcription units, designated as *tafazzin* and α*NAC* (Figure 1), based upon their homology. TAFAZZIN shows a similarity to a human family of proteins, TAFAZZINs, responsible for Barth syndrome (Bione *et al.* 1996). αNAC encodes a homolog of mouse nascent associated polypeptide complex and coactivator α (Yotov and St-Arnaud 1996).

Two additional transcription units were uncovered from the expressed sequence tag (EST) database made available by the Berkeley *Drosophila* Genome Project/HHMI EST Project. The ESTs are located in the immediate vicinity of the *P*-element insertion site and the corresponding transcription units were referred to as *HL*

**TABLE 1**

**Effect of *ox*[1] on *white*, *scarlet*, and *brown* transcripts**

| | Mid-pupae | | Late pupae | |
|---|---|---|---|---|
| Gene | Males | Females | Males | Females |
| *white* | 1.12 ± 0.22 | 1.38 ± 0.05* | 1.34 ± 0.07* | 1.55 ± 0.09* |
| *scarlet* | 0.76 ± 0.06* | 1.47 ± 0.08* | 1.14 ± 0.11 | 1.53 ± 0.10* |
| *brown* | 2.43 ± 0.09* | 0.91 ± 0.07 | 1.97 ± 0.10* | 1.79 ± 0.06* |

Northern blots containing total RNA from the same isolation were performed in triplicate. After hybridizations, the blots were quantified with a Fuji phosphorimager. Each value was normalized to the corresponding value obtained for the *rRNA* gel-loading control. The numbers reported are the means and standard errors of ratios obtained by dividing the values for the $ox^1/+$ class by those obtained for the sibling $+/+$ class. *Ratios significantly different from 1 at the 95% confidence level in a *t*-test.

(EST HL07956) and *QCR9* (EST GH22548; Figure 1). The ORF derived from the *HL* cDNA does not share significant homologies to proteins with known function. To clone the corresponding cDNAs for the *HL* and *QCR9* transcripts, RACE was performed. We were not able to generate a cDNA for *HL*. In the case of *QCR9*, a 0.35-kb product was amplified by PCR using a primer complementary to the sequence from the 5′ region of EST GH22548.

**Generation and analysis of deletions in the *oxen* region:** About 10% of $ox^1$ homozygotes survive to the adult stage, suggesting that the $ox^1$ allele might retain a substantial level of function. Indeed, the *P* element apparently does not abolish the transcription of any mRNA. To generate stronger *ox* alleles we looked for imprecise excisions of the *P* element, which would remove flanking sequences. Southern analysis revealed that in 22 excision events, the sequences outside of the *P*-element insertion site are deleted. The deletions ranged from 0.3 to 2.6 kb and can be grouped into three classes (Figure 1). Two deletions, $ox^{68\text{-}1}$ and $ox^{86\text{-}1}$, remove the structural sequences of four genes, *tafazzin*, *HL*, *QCR9*, and *DGKε*, and thus presumably represent null alleles. The second class comprises 11 deletions, which eliminate *QCR9* and various portions of both *DGKε* and *HL*, while leaving the 5′ sequences of *tafazzin* unaffected. Two representative examples from the second class, $ox^{44\text{-}2}$ and $ox^{38\text{-}1}$, are shown in Figure 1. Finally, 9 deletions, such as $ox^{12\text{-}1}$, $ox^{107\text{-}2}$, and $ox^{157\text{-}1}$, are associated with the loss of a structural portion of *DGKε* and *QCR9* and presumably do not affect *HL* and *tafazzin*. No deletions spanning into α*NAC* were isolated.

The new *ox* alleles were tested for the lethality, male sterility, and eye color phenotypes. With no exceptions, all 22 deletions exhibited an interaction with $w^a$ and were lethal as homozygotes. To determine the stage of

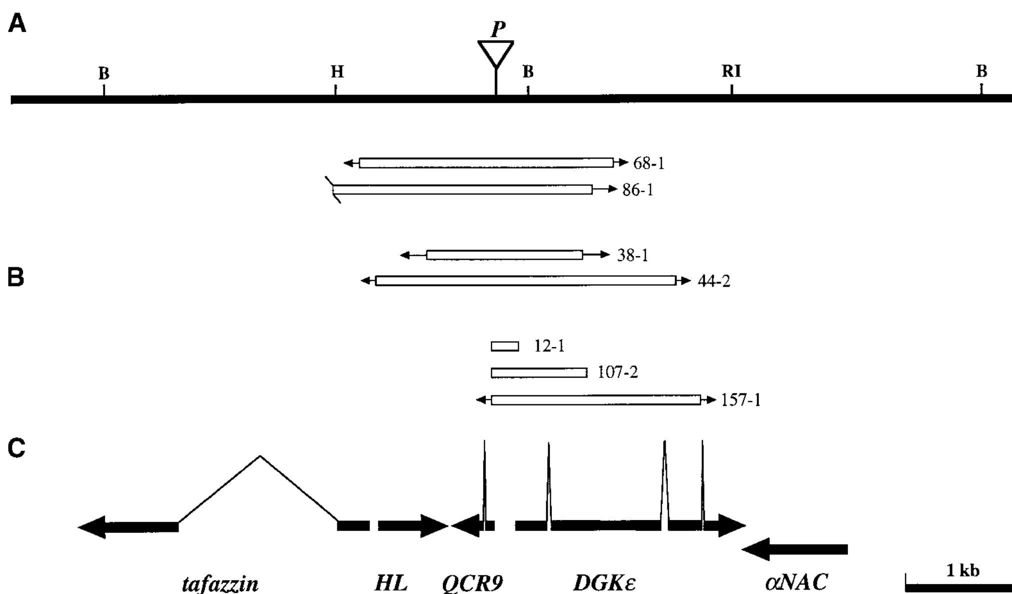


Figure 1.—Molecular map of the *oxen* region. (A) Restriction map of the *oxen* locus. Sites for endonucleases *Eco*RI (RI), *Bam*HI (B) and *Hin*dIII (H) are shown. Site of the *P*[*lacZ*, *rosy*$^+$] insertion in the $ox^1$ allele is designated by a triangle. (B) Positions of several *oxen* deletions. The sequences deleted in different *oxen* alleles are shown by open boxes while the positions of the endpoints are denoted with arrows. In the case of deletion $ox^{86\text{-}1}$ the location of the left breakpoint was not determined. (C) The intron-exon structure and direction of the transcripts identified in the *oxen* region. For the *HL* transcription unit, no cDNA was isolated. The position of the 5′ end is therefore based upon the sequence of the EST HL07956, while the 3′ end is not mapped. Using a genomic fragment containing the sequences of *HL* as a probe a single 0.7-kb transcript was found on Northern blots.

**TABLE 2**

**Effect of deletions $ox^{44\text{-}2}$ and $ox^{107\text{-}2}$ on the expression of *white* mRNA and transcripts from the *oxen* region in heterozygous (*ox*/+) adults**

| Transcript | $ox^{44\text{-}2}$ males | $ox^{44\text{-}2}$ females | $ox^{107\text{-}2}$ males | $ox^{107\text{-}2}$ females | $ox^{rev}$ males | $ox^{rev}$ females |
|---|---|---|---|---|---|---|
| *white* | 0.99 ± 0.08 | 1.29 ± 0.03* | 1.32 ± 0.07* | 1.32 ± 0.04* | 1.01 ± 0.02 | 0.88 ± 0.08 |
| *DGK*ε | 0.56 ± 0.04* | 0.54 ± 0.03* | 0.61 ± 0.02* | 0.57 ± 0.05* | 1.00 ± 0.08 | 0.99 ± 0.03 |
| *tafazzin* | 0.70 ± 0.00* | 0.92 ± 0.04 | 1.34 ± 0.06* | 1.30 ± 0.07* | 1.05 ± 0.05 | 0.95 ± 0.06 |
| *HL* | 0.52 ± 0.08* | 0.64 ± 0.02* | 1.11 ± 0.11 | 0.94 ± 0.04 | 1.05 ± 0.05 | 0.95 ± 0.06 |
| *QCR9* | 0.50 ± 0.05* | 0.65 ± 0.04* | 0.68 ± 0.05* | 0.59 ± 0.04* | 1.17 ± 0.07 | 0.94 ± 0.03 |
| α*NAC* | 1.11 ± 0.12 | 1.42 ± 0.08* | 2.07 ± 0.14* | 1.47 ± 0.06* | 1.03 ± 0.09 | 0.95 ± 0.14 |

The mean and standard errors were calculated as described in the footnote to Table 1. *Ratios significantly different from 1 at the 95% confidence level in a *t*-test.

lethality, two alleles, $ox^{44\text{-}2}$ and $ox^{107\text{-}2}$, were crossed to the wild-type Canton-S stock. The $F_1$ *ox*/+ progeny were intercrossed, and the numbers of $F_2$ surviving to embryo, larvae, pupae, and adult stages were counted. For both alleles it was found that the lethality occurred at late first instar larvae with no escapers. Homozygous mutant animals do not exhibit any gross abnormalities, although they show a sluggish response to physical contact compared to the *ox* heterozygous larvae. Finally, males transheterozygous for $ox^{1}$ and any of the *ox* deletions are sterile. Thus, there are no differences in the phenotype whether *tafazzin* and/or *HL* transcription units are affected by the deletion.

To further confirm that neither *tafazzin* nor *HL* define the *oxen* phenotype, we examined an effect of deletions $ox^{107\text{-}2}$ and $ox^{44\text{-}2}$ on the expression of the *white* gene and transcripts from the *oxen* region by Northern analysis. As a control for normal expression, the revertant, $ox^{rev}$, was used. Triplicate RNA transfers were hybridized with antisense probes for *white*, *DGK*ε, *tafazzin*, and α*NAC* (Table 2). Consistent with the eye color phenotype, mutant alleles, $ox^{44\text{-}2}$ and $ox^{107\text{-}2}$, mostly upregulate the steady-state level of *white* transcripts. The expression of *DGK*ε and *QCR9* is decreased to one-half in both deletions relative to the respective controls and restored to normal in the revertant. This is in agreement with the Southern data showing that both deletions remove a portion of *DGK*ε and *QCR9*. However, the deletions differ in their effect upon *tafazzin* and *HL* transcripts. Deletion $ox^{44\text{-}2}$ removes most of the sequences upstream of *tafazzin* (Figure 1) and decreases its expression. On the contrary, in the case of the $ox^{107\text{-}2}$ allele, *tafazzin* expression is unaffected. Indeed, the left breakpoint is about 1 kb upstream of the 5′ end of the *tafazzin* gene and therefore its upstream sequences are presumably intact. Expression of *HL* is reduced to one-half in the case of $ox^{44\text{-}2}$, in which most of the *HL* sequences are deleted. On the other hand, the $ox^{107\text{-}2}$ deletion does not extend into the *HL* transcript and the *HL* expression is unaffected (Table 2). Taken together these observations disfavor the possibility that *tafazzin* or *HL* are responsible for the mutant phenotypes.

Northern data indicate that both deletions increase the level of α*NAC* expression (Table 2). One possibility to explain this result is that some regulatory sequences of α*NAC* that might be located within the *DGK*ε gene are eliminated in the mutants. To exclude the possibility that the mutant effects are due to an increased α*NAC* expression, a full-length α*NAC* cDNA was expressed under a heat-shock promoter. The overexpression of α*NAC* after heat shock was confirmed by Northern analysis. Transgenic animals carrying an *ox* mutant or wild-type alleles in a $w^{a}$ background were heat-shocked twice a day until the late pupal stage. The eye color of emerged flies was compared to that of flies without the transgene. The overexpression of the transgene did not exhibit any effect on the eye color. Therefore, we concluded that α*NAC* is not responsible for the mutant phenotype. This was further confirmed by the observation that *Df(2R)vg135*, which deletes α*NAC*, as well as $ox^{107\text{-}2}$ and $ox^{44\text{-}2}$, which retain α*NAC*, have a similar effect on $w^{a}$.

Thus, the above results left *DGK*ε and *QCR9* as remaining candidates. According to the Southern analysis, each of the 22 deletions removes a portion of both *DGK*ε and *QCR9* and therefore potentially represents a null allele for both genes. Hence, the deletion tests do not discriminate between the two transcripts. However, the *P*-element insertion located between the 5′ ends of *DGK*ε and *QCR9* might differ in the effect upon their expression. Indeed, as revealed by sequence analysis, the insertion site is located 15 bp upstream of the 5′ end of *QCR9* and 123 bp upstream of the putative transcription start site of *DGK*ε. Therefore the expression of *DGK*ε and *QCR9* in heterozygotes segregating for the $ox^{1}$ chromosome was monitored. To study the expression in the $ox^{1}$ homozygotes, RNA was isolated from rare surviving flies, which do not carry the *SM6a* balancer marker *Cy* and, therefore, are homozygous for $ox^{1}$. In the case of the mutation, the steady-state *DGK*ε mRNA level is slightly decreased to 0.90 ± 0.02 and 0.81 ± 0.03 in the mutant heterozygotes and homozygotes, respectively, compared to the controls (Figure 2). On the contrary, the effect on *QCR9* is much more profound. The *QCR9* transcripts are decreased to 0.75 ± 0.01 in

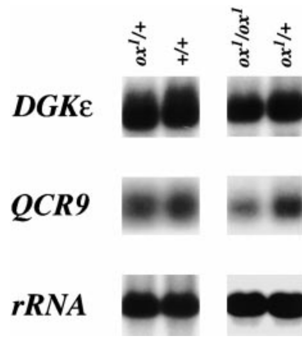


Figure 2.—Northern analysis of *QCR9* and *DGKε*. Hybridization of a blot containing total RNA isolated from flies segregating for mutant ($ox^1$) and wild-type (+) *oxen* alleles with antisense RNA probes for *QCR9* and *DGKε*. *rRNA* was used as a gel-loading control.

the $ox^1$/+ mutants and to 0.45 ± 0.03 in flies homozygous for the $ox^1$ chromosome (Figure 2). This is consistent with the fact that the *P* element is inserted 15 bp upstream of the the 5′ end of the *QCR9* gene, while the distance between the site of the insertion and the 5′ end of *DGKε* is 123 bp. Thus, the level of *DGKε* expression is higher in $ox^1/ox^1$ homozygotes than in animals heterozygous for the *oxen* deletions, $ox^{44\text{-}2}$ and $ox^{107\text{-}2}$ (see above). This is in apparent contradiction with the stronger phenotype of $ox^1/ox^1$ flies compared to $ox^-$/+, such as semilethality and male sterility. To directly prove that *DGKε* does not define the *oxen* function, a UAS-*DGKε* transgene was constructed, transformed, and expressed using a heat-shock GAL4 driver, *hsp70-GAL4* (Brand and Perrimon 1993). The overexpression of the *DGKε* transgene does not rescue the lethality or male sterility. Because *hsp70* is not efficiently expressed in testes, another GAL4 driver, 32B (Brand and Perrimon 1993), was used. This driver was able to provide a high expression level in testes in the control experiments using a UAS-*lacZ* transgene (data not shown). It was found that UAS-*DGKε* does not rescue the mutant phenotype with the 32B driver. Similar results were obtained with three different lines containing the UAS-*DGKε* transgene at various chromosomal locations.

Thus, the combined data on the expression of the *tafazzin* and *HL* transcripts in the case of the deletions and the facts that the *DGKε* and α*NAC* transgenes do not rescue the mutant phenotype disfavor the possibility that any of them represents the *oxen* gene. On the other hand, *QCR9* is the only transcript whose expression is significantly affected by the *oxen* deletions and the hypomorphic allele $ox^1$.

To unambiguously show that *QCR9* represents the *oxen* locus, the *QCR9* cDNA was expressed under the control of the heat-shock promoter in transgenic animals as described in materials and methods. Six independent insertions on chromosome *3* were tested with the *oxen* mutation, which is on chromosome *2*. The mutant eye color phenotype of the heterozygous $ox^1$ allele was completely rescued by three of six tested *QCR9* transgenes, *hsp-QCR9*#106, *hsp-QCR9*#39, and *hsp-QCR9*#99, when the heat shock was administered once a day at larval and pupal developmental stages. Partial rescue was observed with the three other tested transgenes, *hsp-QCR9*# 1, *hsp-QCR9*#31, and *hsp-QCR9*#19. Consistently, the two strongest transgenes, *hsp-QCR9* #106 and *hsp-QCR9*#99, rescued the lethality of the $ox^{107\text{-}2}$ allele following the heat-shock treatment, while the weaker transgene *hsp-QCR9*#1 did not. In the control experiment without heat shock, no $ox^{107\text{-}2}$ homozygotes were recovered. Taken together these data argue that *QCR9* represents the *oxen* function.

**Requirement of *QCR9* activity in development:** Several experiments were employed to examine the role of *QCR9* in development. As mentioned above, any *QCR9* null allele results in early larval lethality. To determine what the consequences of the loss of *QCR9* might be in adult patterning, a mosaic analysis was performed. We employed the FRT/FLP system (Xu and Harrison 1994) to generate homozygous $ox^{107\text{-}2}$ and $ox^{44\text{-}2}$ mutant somatic clones. Following the induction of FLP recombinase in the first or second instar larvae, numerous duplication clones (+/+) marked with a *mini-white* gene were seen in adult eyes. In contrast, no mutant clones lacking the *mini-white* gene were found. These results indicate that *QCR9* is required for cell viability.

Next we addressed the question of whether *QCR9* is necessary for oogenesis. To generate homozygous $ox^{44\text{-}2}$ and $ox^{107\text{-}2}$ mutant germline clones, the "FLP-DFS" technique was used (Chou and Perrimon 1996). The mosaic females depleted of *oxen* function laid no eggs. Examination of the associated ovarian phenotype revealed that oocyte development is arrested before onset of vitellogenesis. Therefore, the presence of *QCR9* is presumably required either to enter vitellogenesis and/or for cell viability in late oogenesis.

To further elucidate the *QCR9* function, the pattern of its expression in development was investigated by *in situ* hybridization. Wild-type embryos were hybridized to a digoxigenin-labeled RNA probe of *QCR9*. *QCR9* transcripts are ubiquitously present in early embryos at the time of cellularization (Figure 3A) and throughout germ-band extension stages (Figure 3, B and C). By the end of germ-band retraction, expression is enriched in amnioserosa and in midgut (Figure 3D). As dorsal closure proceeds, *QCR9* transcripts are predominantly found in midgut, while the staining in other tissues weakens (Figure 3, E and F). During late development when midgut constrictions appear, the transcripts are present in all four midgut chambers (Figure 3G). At the end of embryogenesis, robust staining persists in the midgut (Figure 3H).

**An effect of *oxen* deletions on gene expression:** The question of whether the *oxen* function is restricted to the the regulation of the expression of Drosophila ABC transporters was addressed. The steady-state mRNA level

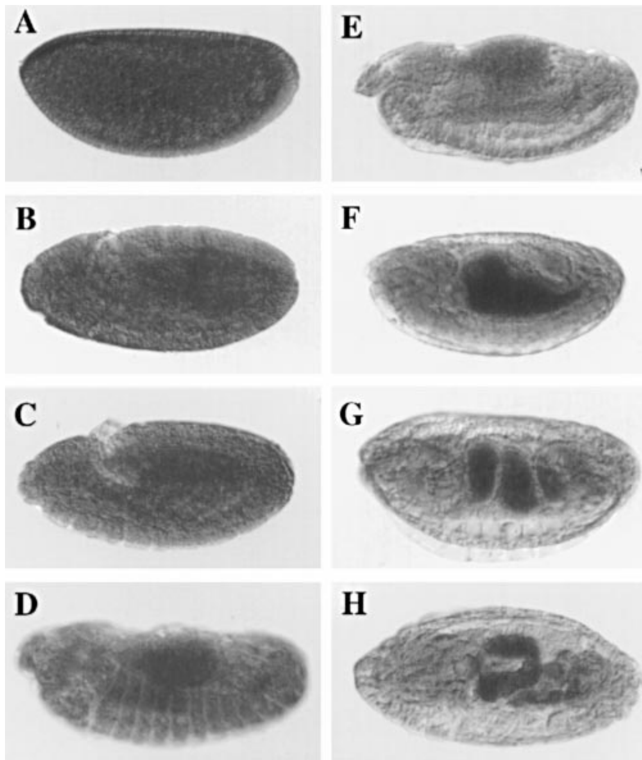


FIGURE 3.—*QCR9* expression pattern in embryogenesis. (A–C) *QCR9* is ubiquitously expressed at early stages of development. Uniform expression in the yolk of early stage 5 (A) and during germ-band extension at stage 9 (B) and stage 11 (C) embryos. At stage 13, staining is present in amnioserosa and in the midgut (D). From the time of dorsal closure and until the end of embryogenesis, *QCR9* transcripts are strongly expressed in the midgut at stage 14 (E), at stage 15 (F), at stage 16 (G), and at stage 17 (H) embryos.

of seven unrelated genes, β*1-tubulin at 56D* (β-*tubulin*), *Glucose-6-phosphate dehydrogenase* (*Zw*), α-*Glycerol-3-phosphate dehydrogenase* (α*Gpdh*), *rudimentary* (*r*), *Alcohol dehydrogenase* (*Adh*), *ribosomal protein P0* (*P0*), and *ribosomal protein 49* (*rp49*), was examined by Northern analysis in $ox^{107\text{-}2}$ mutants (Table 3). As a control of normal expression, the $ox^{rev}$ allele was used. The expression of two genes, α*Gpdh* and *P0*, is increased in the case of mutation in both sexes and is restored to normal in the revertant. On the contrary, the *oxen* deletion results in elevation of *r* transcripts in females and their reduction in males. The steady-state *Adh* mRNA level was found to be elevated in mutant males. However, examination of the *Adh* expression in $ox^{rev}$ flies revealed a similar effect suggesting that the observed effect on *Adh* transcripts is not caused by the $ox^{107\text{-}2}$ allele. Finally, three genes, β-*tubulin*, *Zw*, and *rp49*, are not affected by the *oxen* deletion. The same set of genes was affected by deletion $ox^{44\text{-}2}$ (data not shown).

***QCR9* encodes a component of the mitochondrial $bc_1$ complex:** Sequence analysis of the *QCR9* mRNA revealed that it encodes a 6.6-kD protein that shares extensive similarity throughout the whole sequence with the smallest protein of the mitochondrial ubiquinol-cytochrome *c* oxidoreductase complex ($bc_1$ complex) from bovine (SCHAGGER *et al.* 1983) and yeast (PHILLIPS *et al.* 1990; Figure 4). Drosophila QCR9 is 57 and 36% identical to bovine and yeast homologs, respectively. Inspection of the first 15 amino acids of the *QCR9* product shows features necessary for the protein to be transported to mitochondria (VON HEIJNE 1986). Indeed, there is a high content of Arg, Lys, Leu, Ser, and Thr, while no acidic residues are present.

## DISCUSSION

The *P*-element mutation in the *oxen* gene was isolated in a screen for *trans*-acting modifiers exhibiting a dominant effect on the *white-apricot* phenotype. Our and previously published data (CASTRILLON *et al.* 1993) demonstrate that the *oxen* gene is essential for cell viability, spermatogenesis, and oogenesis. Mutations in the locus are haplo insufficient for the expression of a subset of Drosophila genes, in particular the ABC transporters, *white*, *brown*, and *scarlet*. We provide evidence that the *oxen* locus encodes a structural homolog of the smallest subunit of mitochondrial ubiquinol cytochrome *c* oxidoreductase ($bc_1$ complex).

In the course of transcriptional mapping, mRNAs

**TABLE 3**

**Effect of *oxen* deletion, $ox^{107\text{-}2}$, on gene expression**

| Gene | $ox^{107\text{-}2}$ males | $ox^{107\text{-}2}$ females | $ox^{rev}$ males | $ox^{rev}$ females |
|---|---|---|---|---|
| *rudimentary* | 0.89 ± 0.03* | 1.25 ± 0.03* | 0.98 ± 0.06 | 1.05 ± 0.02 |
| *Adh* | 0.90 ± 0.02* | 0.95 ± 0.08 | 0.91 ± 0.02* | 0.95 ± 0.04 |
| *Zw* | 1.06 ± 0.04 | 1.01 ± 0.03 | 0.90 ± 0.11 | 1.03 ± 0.10 |
| α*Gpdh* | 1.40 ± 0.06* | 1.65 ± 0.07* | 1.07 ± 0.11 | 0.99 ± 0.06 |
| β-*tubulin* | 0.93 ± 0.09 | 1.09 ± 0.02 | 1.06 ± 0.02 | 1.03 ± 0.03 |
| *P0* | 1.34 ± 0.07* | 1.22 ± 0.04* | 1.06 ± 0.06 | 1.04 ± 0.02 |
| *rp49* | 1.01 ± 0.04 | 0.98 ± 0.05 | 1.11 ± 0.13 | 1.09 ± 0.11 |

The mean and standard errors were calculated as described in footnotes for Table 1. *Ratios significantly different from 1 at the 95% confidence level in a *t*-test.

```
drosophila   MKV--IYNTLFKRTSTYAVAIIASAFFFERALDVTSVAIFEGINKGKLWKDIKGKY-------E     55
bovine       ARL---YSLIFRRTSTFALTIVVGALFFERAFDQGADAIYEHINEGKLWKHIKHKY-------ENK   62
yeast        MSFSSLYKIFFKRNAVFVGTIFAGAFVFQTVFDTAITSWYENHNKGKLWKDVKARIAAGDGDDDDE   66
```

Figure 4.—Multiple alignment of the amino acid sequences of Drosophila QCR9 and subunit 9 ofm yeast and subunit 11 of bovine $bc_1$ complexes. Amino acids that are identical between QCR9 and one or more of other proteins are shaded. The alignment was made by use of the computer program MegAlign from DNA STAR.

from five closely arranged genes, *tafazzin*, *HL*, *QCR9*, *DGKε*, and α*NAC*, were identified in the *oxen* region. Several lines of evidence indicate that the effects seen in the *oxen* mutants are due to a lesion in *QCR9*. First, the hypomorphic allele $ox^1$, which has a *P*-element insertion upstream of *QCR9*, can be reverted by precise excision of the *P* element. In addition, *Df(2R)vg135*, which uncovers this region, exhibits a similar effect on *white-apricot*. Second, the mutant phenotype is retained whether *tafazzin* and/or *HL* sequences are uncovered in various deletions within the region. This excludes both *tafazzin* and *HL* as a source for *oxen* activity. Third, α*NAC* is upregulated in all three tested *oxen* mutant alleles; however, overexpression of the α*NAC* transgene does not cause the mutant phenotype. Neither does it exhibit an effect on the eye color in a *white-apricot* background. This leaves *DGKε* and *QCR9* as possible candidates. Indeed, all of the *oxen* deletions eliminate at least the 5′ portion of both genes. This is confirmed by a Northern analysis, which shows a twofold reduction of *DGKε* and *QCR9* expression in the case of two *oxen* deletions. Unlike the deletions, the *P*-element insertion in the $ox^1$ allele preferentially affects the level of *QCR9* rather than *DGKε* transcripts. This difference is most likely due to the fact that the insertion site is 15 bp upstream of the putative 5′ end of *QCR9* while the distance to the *DGKε* 5′ end is 123 bp. Finally, the *QCR9* transgene completely rescues the eye color phenotype and lethality associated with the *oxen* mutations. On the contrary, *DGKε* transgenic flies do not show the effect upon the eye color or reversion of lethality. Taken together, these data indicate that *QCR9* defines the *oxen* function.

The predicted product of *QCR9* shows a sequence similarity to the smallest subunit of mitochondrial $bc_1$ complex from yeast and bovine. The cytochrome $bc_1$ is one of the three major respiratory enzyme complexes residing in the inner mitochondrial membrane. The enzyme oxidizes ubiquinol, which reacts from the membrane phase, reduces cytochrome *c* in the intermembrane space, and uses the free energy change to transport two protons across the membrane from the matrix to the intermembrane space, and releases two additional protons there (Saraste 1999). The bovine and yeast $bc_1$ complexes contain 11 and 9 subunits, respectively. Only 3 subunits carry the redox centers. These key polypeptides are cytochrome *b*, the Rieske FeS protein and cytochrome *c1* (Saraste 1999). Most of the other subunits are small proteins and their role is not well understood. Genetic studies in yeast identified those essential for assembling a fully functional complex. It was found that deletion of a yeast homolog of Drosophila *QCR9* results in a 20-fold reduction of the activity of the $bc_1$ complex (Phillips *et al.* 1990). The generation of *QCR9* mutant Drosophila lines provides a tool for examining the consequences of the loss of *QCR9* in a multicellular organism. Our data are consistent with the proposed indispensability of the yeast homolog in the cell. Flies homozygous for *QCR9* null alleles die as first instar larvae. Moreover, no homozygous mutant clones were observed in the eye indicating that the *QCR9* is required for cell viability. It is possible that the survival of homozygous animals to the first instar larval stage is due to a maternal contribution of *QCR9* from heterozygous mothers. However, because of the requirement of the *QCR9* function for oogenesis, the possibility of recovering homozygous individuals from a homozygous female germline lineage is eliminated and does not permit a test of this potential explanation. Oocyte development is arrested before vitellogenesis if the germline is depleted of *QCR9*. Thus, our data suggest that *QCR9* is an essential gene in Drosophila and its function is required in every examined organ.

Several genes encoding mitochondrial proteins have been identified in Drosophila (Hayward *et al.* 1993; Hartenstein *et al.* 1997; Iyengar *et al.* 1999; Zhang *et al.* 1999). Interestingly, mutations in three of them result in nervous system defects. The *sluggish-A* gene encodes a mitochondrial proline oxidase involved in proline biosynthesis and depletion of its activity causes locomotory abnormalities (Hayward *et al.* 1993). The *tamas* gene was isolated in a screen for mutations affecting larval response to light. The *tamas* product shows a significant similarity to the mitochondrial DNA polymerase catalytic subunit (Iyengar *et al.* 1999). Larvae homozygous for the *colt* mutation exhibit a sluggish response to physical contact. *colt* encodes a member of the mitochondrial carrier family (Hartenstein *et al.* 1997). It is worth noting that *oxen* mutant homozygous larvae show defects similar to *colt* mutants. This may indicate that the nervous system is more sensitive to perturbations in mitochondrial function, reflecting a higher energy requirement. Indeed, altered mitochondria were found in all cases of hereditary motor and sensory neuropathy with optic atrophy and in several cases in a larger group of unselected neuropathies (Schroder 1993).

Why was *oxen* isolated in a screen for modifiers of *white* gene expression? As mentioned above, *white* together with *brown* and *scarlet* encode ABC transporters. Yeast mitochondrial ABC transporter *ATM1* was pro-

posed to be involved in the signaling from the mitochondria to the cell (Leighton and Schatz 1995), so a modulation of this gene family is potentially occurring. There are a growing number of observations that changes in mitochondria and, in particular, in the respiratory chain are followed by alterations in nuclear gene expression. This retrograde regulation has been hypothesized to be a general mechanism for the cell to monitor and adjust its functions in response to perturbations in mitochondrial biogenesis (Parikh *et al.* 1987; for review, see Poyton and McEwen 1996). One of the best-studied examples of retrograde regulation is the induction of the peroxisomal isoform of citrate synthase, *CIT2*, in cells with dysfunctional mitochondria (Liao *et al.* 1991). Remarkably, *CIT2* induction was observed after addition of antimycin A, which inhibits the respiratory chain (Liao *et al.* 1991). Given an absolute requirement of yeast *QCR9* for assembling a functional $bc_1$ complex and cell lethality caused by the loss of *QCR9* in yeast and Drosophila, it is likely that mitochondrial respiratory functions are compromised in *oxen* mutants. Although there is increasing evidence in favor of retrograde regulation, the molecular mechanism underlying this phenomenon remains largely unknown. Because the changes in gene expression occur when the *oxen* mutation is heterozygous, it is likely that mitochondrial perturbations contribute to aneuploid syndromes. The *oxen* mutational effects provide additional evidence for the existence of signal communication between mitochondrial and nuclear genomes and provide a model genetic system in a multicellular organism to study this phenomenon.

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