The *cyclope* Gene of Drosophila Encodes a Cytochrome c Oxidase Subunit VIc Homolog

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ABSTRACT

Cytochrome c oxidase is the terminal enzyme of the mitochondrial electron transfer chain. In eukaryotes, the enzyme is composed of 3 mitochondrial DNA-encoded subunits and 7–10 (in mammals) nuclear DNA-encoded subunits. This enzyme has been extensively studied in mammals and yeast but, in Drosophila, very little is known and no mutant has been described so far. Here we report the genetic and molecular characterization of mutations in *cyclope* (*cype*) and the cloning of the gene encoding a cytochrome c oxidase subunit VIc homolog. *cype* is an essential gene whose mutations are lethal and show pleiotropic phenotypes. The 77-amino acid peptide encoded by *cype* is 46% identical and 59% similar to the human subunit (75 amino acids). The transcripts are expressed maternally and throughout development in localized regions. They are found predominantly in the central nervous system of the embryo; in the central region of imaginal discs; in the germarium, follicular, and nurse cells of the ovary; and in testis. A search in the Genome Annotation Database of Drosophila revealed the absence of subunit VIIb and the presence of 9 putative nuclear cytochrome c oxidase subunits with high identity scores when compared to the 10 human subunits.

CYTOCHROME c oxidase (COX) is the terminal enzyme in both the eukaryotic and prokaryotic respiratory chain complex that catalyzes the conversion of redox energy to ATP (reviewed in Capaldi 1990; Poyton and McEwen 1996; Lenka *et al.* 1998). The electron-transport and oxidative phosphorylation (OXPHOS) chain (reviewed in Saraste 1999) contains five multisubunit complexes and is located on the inner mitochondrial membrane. Different substrates are metabolized, producing reducing equivalent via complex I (NADH dehydrogenase) and II (succinate dehydrogenase). Ubiquinone (coenzyme Q) shuttles electrons from complexes I and II to complex III (ubiquinol-cytochrome c oxidoreductase), which in turn reduces cytochrome c. Cytochrome c transfers electrons to complex IV (cytochrome c oxidase) driving ATP generation in complex V (ATPase). In human the OXPHOS system contains 83 polypeptides of which 70 are nuclear-DNA encoded, whereas the other 13 are encoded by mtDNA (Leonard and Schapira 2000a; Schon 2000).

Cytochrome c oxidase is a complex heme-copper containing metalloprotein embedded in the mitochondrial inner membrane as either a monomer or a dimer. The enzyme catalyzes the reaction: 4 ferrocytochrome c + 8 H^+ + O_2 = 4 ferricytochrome c + 4 H^+ + 2 H_2O. In mammals, the enzyme is composed of 13 subunits. The 3 major subunits (I, II, and III) are encoded by the mitochondrial DNA and form the catalytic core. They are synthesized inside the mitochondrion and are homologous to the three major subunits found in purple bacteria as *Paracoccus denitrificans*. The other subunits are specific to eukaryotes and are nuclear-DNA encoded. Their numbers vary according to the organism: 10 subunits are found in mammals and 8 in yeast. They are synthesized on cytosolic ribosomes, mostly as precursors carrying N-terminal basic presequences for mitochondrial targeting and import. Although the exact structural and functional role of these small subunits remains unclear, they are believed to modulate the overall activity of the complex. Some of the nuclear-encoded subunits are expressed as tissue- and developmental-specific isoforms. In different vertebrates, subunits IV, Va, Vb, VIb, VIIb, VIIc, and the mammalian liver isoforms (L), VIa, VIIa, and VIII are detected in all tissues and are classified as ubiquitous, although the mRNA levels for individual subunits vary in different tissues. The mammalian tissue-specific H isoforms, VIa, VIIa, and VIII, are specific to heart and skeletal muscle.

In yeast (*Saccharomyces cerevisiae*), null mutants for subunits I, II, III, IV, Va, Vb, VIc, and VIIa (nomenclature of Kadenbach and Merle 1981) do not grow on nonfermentable carbon sources and either fail to assemble cytochrome c oxidase or yield a cytochrome c oxidase with diminished activity. In contrast, a strain deleted for the gene encoding subunit VIIc has 80% wild-type levels of cytochrome c oxidase activity and grows on nonfermentable carbon sources. Subunit VIIc is required, however, for the production of stable cytochrome c oxidase

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dimers and affects the low-affinity electron-transfer site from cytochrome c.

A number of human diseases with various clinical symptoms have been attributed to defects in the cytochrome c oxidase complex (reviewed in Taanman 1997; Poyton 1998; Leonard and Schapira 2000a,b; Schon 2000). Partial cytochrome c oxidase deficiency in the adolescent or the adult is often associated with specific point mutations in tRNA as well as rearrangements in subpopulations of mtDNA. For instance, defective muscle fiber in Kearns-Sayre syndrome, as well as ocular myopathy and progressive external ophthalmoplegia, are attributed to single large or multiple deletions or duplications in mtDNA. Fatal infantile and benign myopathies as well as cardiomyopathy are associated with deficiency of subunit VIIa and/or VIIb with, moreover, in the case of benign myopathy, a lack of subunit II. In Leigh syndrome a severe encephalopathy is attributed to a decrease in COX activity due to incomplete assembly of the enzyme complex. The sequence of nuclear-encoded subunits reveals no defect, but mutations in the nuclear gene *Surf 1*, encoding a mitochondrial protein involved in COX assembly and maintenance, have been found in many patients.

In Drosophila, several genes encoding mitochondrial proteins have been identified. Two of them encode essential components of the OXPHOS chain, the ATPase α-subunit of complex V (Jacobs *et al.* 1998) and the ubiquinol-cytochrome c oxidoreductase subunit 9 of complex III (Frolov *et al.* 2000). The sequences of the mitochondrial DNA including COX subunits (I, II, III) genes (de Bruijn 1983) and of the nuclear COX subunit Va (Caggese *et al.* 1999) gene of complex IV have also been identified, but no functional information and no COX mutants have been described so far. Identification of genes encoding nuclear COX subunits in Drosophila and analysis of corresponding mutations will provide tools to study the modulation of their role during development and will contribute to our understanding of the human diseases that result from alteration of COX activity. We report here the identification of a previously undescribed Drosophila lethal locus, *cyclope*, whose mutations lead to pleiotropic phenotypes and the generation and molecular characterization of new alleles. We show that germline *cype* clones are lethal and that somatic clones lead to inhibition of cell growth. We have cloned the gene and show that *cype* encodes a cytochrome c oxidase subunit VIc homolog. A search in the Genome Annotation Database of Drosophila identified nine putative nuclear COX subunits.

MATERIALS AND METHODS

Fly stocks and mutagenesis: Flies were raised on standard media and all crosses were performed at 25° unless otherwise specified. *l(2)03771* (Karpen and Spradling 1992; Spradling *et al.* 1999), renamed here $cype^{1}$, *FLP/FRT*, and *Δ2-3* stocks were provided by the Bloomington Stock Center. $cact^{99}$ is described in Roth *et al.* (1991), $ea^{161.13}$ in Jin and Anderson (1990), and dpp^{hr4} and dpp^{hr27} in Spencer *et al.* (1982). Balancer chromosomes and other stocks are described in Lindsley and Zimm (1992) and in FlyBase (1997). To perform a secondary *P*-element mutagenesis, the $cype^{1}$ *PZ[ry]* element was mobilized by providing the *Δ2-3* external source of transposase (Robertson *et al.* 1988). $cype^{1}$*/CyO;* ry^{506}/ry^{506} females were crossed to *w/Y; Sp/CyO;* ry^{506} *Dr P[*ry^{+} *Δ2-3]/TM6 Ubx* males to generate $cype^{1}$*/CyO;* ry^{506} *Dr P[*ry^{+} *Δ2-3]/*ry^{506} males. They were crossed individually to $cype^{1}$*/CyO* females to select ry^{-}*/CyO* individuals that were crossed to $cype^{1}$*/CyO* to test for lethality. Embryonic cuticles were prepared as described in Wieschaus and Nüsslein-Volhard (1986). Wings were dissected, collected in 70% ethanol, and mounted in Euparal (Labosi).

Clonal analysis: Mitotic clones were generated using the *FLP/FRT* technique (Golic and Lindquist 1989; Xu and Rubin 1993). $cype^{1}$ was recombined onto a *P[*ry^{+} *hs-neo FRT]40A* second chromosome. To generate germline clones $cype^{1}$ *P[*ry^{+} *hs-neo FRT]40A/CyO* females were crossed to *w P[*ry^{+} *hs FLP]1/Y; P[*w^{+} ovo^{D1}*] P[*ry^{+} *hs-neo FRT]40A/CyO* males and clones were analyzed in *w P[*ry^{+} *hs FLP]1/+; P[*w^{+} ovo^{D1}*] P[*ry^{+} *hs-neo FRT]40A/* $cype^{1}$ *P[hs-neo-FRT]40A* female progeny (Chou *et al.* 1993). To generate somatic clones in adult *y w/y w;* $cype^{1}$ *P[*ry^{+} *hs-neo-FRT]40A/CyO* females were crossed to *y P[*ry^{+} *hs FLP]1/Y; P[y+] P[*ry^{+} *hs-neo FRT]40A/Bc* males and *yellow* clones were analyzed in *y P[*ry^{+} *hs FLP]1/y w; P[*y^{+}*] P[*ry^{+} *hs-neo FRT]40A/* $cype^{1}$ *P[*ry^{+} *hs-neo FRT]40A* female progeny. Clones were heat-shock induced at third larval instar by 1-hr exposure to 38°. To generate mitotic clones in the eye we used the *EGUF/hid* method (Stowers and Schwarz 1999). *y w/y w; P[*ry^{+} *hs-neo FRT]40A GMR-hid l(2)CL-L1/CyO; ey-GAL4 UAS-FLP/ey-GAL4 UAS-FLP* females were crossed to *y w/Y;* $cype^{1}$ *P[*ry^{+} *hs-neo FRT]40A/CyO* males at 29°. To observe eye phenotypes in the progeny, flies were fixed in 3% glutaraldehyde (2 hr at RT and then 24 hr at 4°), dehydrated in ethanol series and then amyl acetate series. Electron microscopy was performed on a JEOL 6100 scanning electron microscope.

DNA and RNA analysis: Standard molecular biology experiments were performed as described in Sambrook *et al.* (1989). Genomic DNA was extracted from adult flies. About 50 individuals were ground in 200 μl of 0.1 M Tris pH 9.2, 0.1 M EDTA pH 8, 1% SDS and incubated 30 min at 65°. After centrifugation, proteins were precipitated 30 min on ice after addition of K acetate to 1 M final and DNA was recovered after isopropanol precipitation. Southern blots were hybridized with [^{32}P]dCTP random primed DNA probes in 0.25 M Na_2HPO_4 pH 7.2, 7% SDS and washed in 20 mM Na_2HPO_4 pH 7.2, 1% SDS at 65°. PCR amplifications were performed with 0.25–0.5 μg of genomic DNA. 5′*P* primer (reverse) was 5′-CCTCTCAACAG CAAACGTGTACTG-3′ and 3′*P* primer (forward) was 5′-TCT CTTGCCGACGGGACC-3′. Genomic primer positions were established from cDNA GH04604 start (position 1): primer 1 (forward) 5′-ATCTGCCATACTTGAATC-3′ (−2080/−2062), primer 2 (reverse) 5′-GATATGGCCAATGGTATG-3′ (141/122), primer 3 (reverse) 5′-TAGACTACTTGTGTTTGGG-3′ (597/578), primer 4 (reverse) 5′-TCAATACAGAAGCGCA ACTC-3′ (640/621). PCR products were cloned into pGEM plasmid (Promega, Madison, WI) and sequenced using primer 2, SP6 or T7 primers, or directly sequenced using primer 2. RNAs were extracted with pH 5 hot phenol at 60° (Sato *et al.* 1982), enriched for poly(A)$^{+}$ RNAs by oligo(dT) cellulose chromatography (Pharmacia, Uppsala, Sweden), and run in 1% formaldehyde agarose gels. Northern blots were hybridized with [^{32}P]dCTP random primed DNA probes in 50% formamide, 5× SSPE, 5× Denhardt's, 0.1% SDS at 42° and washed at high stringency.

cDNA library screening and sequencing: λ57 phage genomic

DNA (Canton-S), isolated from a previous walk (George and Terracol 1997), was subcloned in pBluescript II KS+ vector. Oregon-R cDNAs were isolated from 1.5- to 5-hr embryonic λgt10 library (a gift from M. Goldschmidt-Clermont). Canton-S cDNAs were isolated from 0- to 24-hr embryonic and late third larval instar λZap II libraries (gifts from C. S. Thummel). λgt10 cDNA were sequenced directly using λgt10 primers. Forward primer was 5′-GCAAGTTCAGCCTGGTTAAG-3′ and reverse primer was 5′-AGGTGGCTTATGAGTATTTC-3′. λZap II cDNAs were excised (Stratagene rapid excision kit, La Jolla, CA) and sequenced using T3 and T7 primers. The sense and antisense genomic strands were sequenced by the dideoxy-chain termination method (Sanger *et al.* 1977) using the United States Biochemicals sequencing kit (Pharmacia). cDNA and PCR product sequencing was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequences were compiled using the Genetics Computer Group software (GCG; Devereux *et al.* 1984) and compared to the Drosophila database using the Berkeley *Drosophila* Genome Project (BDGP; http://www.fruitfly.org/blast/) Blast Searches program (Altschul *et al.* 1990). Protein alignments were performed on the Blast servers of the National Center for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov/blast/) and of the BDGP.

Whole mount *in situ* hybridizations and *lacZ* staining: Whole mount *in situ* hybridizations in embryos were performed according to Tautz and Pfeifle (1989) and Mével-Ninio *et al.* (1991). Purified restriction fragments or pBScDNA19 were used to synthesize digoxigenin-labeled DNA or antisense RNA probes (Boehringer Mannheim labeling kit, Mannheim, Germany). *lacZ* staining was as described in Mlodzik and Hiromi (1992).

Transformation experiments: The 3.8-kb *Bgl*II-*Xba*I genomic fragment including *cype* cDNAs was cloned at the *Bgl*II-*Xba*I sites of *Pw6* mini *white P*-element vector (Klemenz *et al.* 1987). The construct was co-injected with the pUChΔ2-3 helper plasmid in pole cell region of w^{1118} preblastoderm embryos (Spradling and Rubin 1982). A transgene on the X chromosome was used for the rescue experiments.

RESULTS

Secondary *cyclope* mutagenesis: The original lethal mutation, *l(2)03771*, was recovered after a *PZ* (Mlodzik and Hiromi 1992) element insertion mutagenesis (Karpen and Spradling 1992) and is renamed here $cype^{1}$. The *P* element was localized as a single insertion site in the 25D4-6 interval (Spradling *et al.* 1999). Prior to analysis, the $cype^{1}$ chromosome was cleaned up by crossing several times to the ry^{506}/ry^{506} strain, to eliminate the other potential lethal mutations that occurred on the second chromosome. To obtain null or stronger alleles we performed a secondary *P*-induced mutagenesis by excision of the *PZ[ry]* transposon of $cype^{1}$. Starting with 20 $cype^{1}/CyO;\ ry^{506}\ Dr\ P[ry^{+}\ \Delta 2\text{-}3]/ry^{506}$ individual males (materials and methods), we recovered 20 viable (9 independent) and 12 lethal (6 independent) *ry* lines. The excision of the *P* element leads to viability indicating that the initial lethality was due to the *P* insertion. Six independent *ry* lethal lines were retained for further analysis.

***cype* embryonic phenotypes and germline clones:** With all the alleles, *cype/cype* individuals from a *cype/+ inter se* cross die at the embryo stage (25%). However, $cype/Df(2L)tkv^{Sz2}$ individuals die at first larval stage. This is true with the $cype^{1}$ allele and the six new revertant alleles ($cype^{1R}$). This was observed in both directions of the $cype/+ \times Df(2L)tkv^{Sz2}/+$ cross. However, from this cross, we found 6% dead embryos when the females were $cype^{1}/+$ and 2% when the females were $Df(2L)tkv^{Sz2}/+$, a difference that is at the limit of significance. This deficiency deletes the entire *cype* gene (George and Terracol 1997), and this result was also observed with other deficiencies covering *cype*. No significant embryonic lethality was observed either way with the cross $cype^{1}/+ \times +/+$ (2% when females were $cype^{1}/+$ and 1% when females were +/+), showing no dominant maternal effect in this context. With the heterozygous combinations, $cype^{1}/cype^{1R2.5}$ and $cype^{1}/cype^{1R15.1}$, individuals die either in embryo or at the beginning of first larval instar. The slight difference in lethal stage, observed between homozygous, heterozygous, and hemizygous *cype*, is not due to another lethal mutation that had occurred on the second chromosome, since the stocks were cleaned up, and, furthermore, lethality is lost after excision of the *P* element. The lethality of the different alleles, *inter se* and in hemizygotes, was rescued with a transgene including the $cype^{1}$ insertion site.

Most $cype^{1}/cype^{1}$ embryos do not present any strong cuticular alterations except for head defects (Figure 1B). However 10% of the embryos present Filzkörper defects and lateral extension of the ventral denticle belt (Figure 1C). The phenotypes are identical with the six new lethal lines.

Germline $cype^{1}$ clones were performed using the $FLP/ovo^{D1}FRT$ system. No embryos were recovered, indicating that the mutation is either cellular lethal or required for ovarian development. Dissection of clone-bearing female ovaries showed that they were no different from ovo^{D1} ovaries. Therefore $cype^{1}$ clones do not allow correct ovarian development and, unless the phenotype is similar to ovo^{D1}, lead to cell death.

Adult phenotypes and somatic clones: Mutant phenotypes were also observed, at low penetrance, in the $cype^{1}/CyO$ flies. In the most frequent phenotype (18%), flies have atrophic tergites. Defects are also observed (6%) in legs (Figure 2A) and in wings (Figure 2B). Less frequently (1/250) flies with half (Figure 2C) or split thorax or rough or deformed eyes (Figure 2D) are found. Lethality is observed in 35% of the $cype^{1}/CyO$ pupae, *CyO/CyO* individuals dying during the early L1 larval stage. Flies with abnormally close eyes (Figure 2E) or, exceptionally, fused eyes (Figure 2F) are observed at low frequency. This phenotype is sublethal and these flies generally die in pupae. This phenotype is not due to another dominant or recessive mutation that had occurred in the stock, since, when fused eye flies were crossed *inter se*, the frequency of this phenotype was not increased in either the progeny or the second generation. This is also true for the other phenotypes. Dead

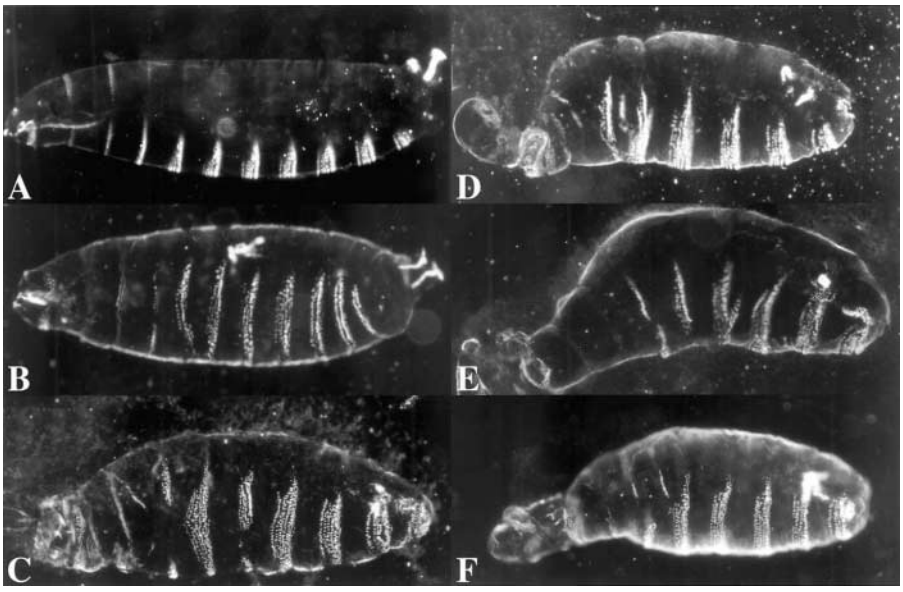

FIGURE 1.—*cyclope* embryonic phenotypes. The embryos are oriented with anterior to the left and dorsal up. (A) Wild-type larvae. (B) $cype^{1}/cype^{1}$ with head defects and ventral denticle compressed. (C) $cype^{1}/cype^{1}$ embryo with dorsoventral polarity defects (moderately ventralized V3). (D) V3 embryo from $cype^{1}/+$; $ea^{161.13}/+$ females crossed to wild-type males. (E) V3 embryo from $cype^{1}+/+$ $cact^{99}$ females crossed to wild-type males. (F) Moderately ventralized embryo (V3) from $cype^{1}/CyO$ females crossed to dpp^{hr27}/CyO males. Classification of phenotypes is described in Table 1 according to the nomenclature of ROTH *et al.* (1991).

fused eye pupae were also observed in the progeny of the new alleles crossed to dpp^{hr27}. Most of these phenotypes (except fused eyes) were also observed with deficiencies of the region. Therefore these phenotypes are probably due to the haploinsufficiency of *cype*. This was confirmed by the fact that both $cype^{1R2.5}/CyO$ and $cype^{1R15.1}/CyO$ new lines obtained after the secondary mutagenesis have a 2-day emergence delay at 25° when compared to $cype^{1}/CyO$ flies. This result also indicates that $cype^{1}$ is probably not a null allele and that these new lines, unless they alter other genes, are stronger alleles than $cype^{1}$. However, the study of the embryonic phenotypes and the stage of the lethal phase of the different alleles did not show a stronger phenotype in the lethal revertant lines.

Somatic clones were induced with the FLP/FRT technique and were recovered and observed in adults. As shown in Figure 2G, *y* $cype^{1}$ clones present atrophic or missing bristles. This phenotype is observable on the whole adult cuticle. At the wing margin, bristles are shorter and thinner due probably to the reduction in cell size (Figure 2H). $cype^{1}$ clones were induced in the eye with the *EGUF/hid* method (STOWERS and SCHWARZ 1999). This technique makes it possible to generate eyes that are composed exclusively of clones, because all other genotypes are eliminated. This is done by using the dominant photoreceptor cell lethal *GMR-hid* and a recessive cell death mutation. $cype^{1}/cype^{1}$ eyes have a rough aspect (Figure 3C), disorganized ommatidia with an abnormal morphology, and duplicated or missing bristles (Figure 3D).

***cype* enhances mutant phenotypes of dorsoventral patterning genes:** The $cype^{1}$ embryonic defects are usually observed in weakly ventralized embryos. To determine whether these phenotypes were the result of an alteration in dorsoventral polarity, we searched for possible interactions with genes implicated in this process (TERRACOL and LENGYEL 1994). We tested the effects of the maternal genes *easter* (*ea*) and *cactus* (*cact*) of the *dorsal* pathway, as well as those of the zygotic gene *decapentaplegic* (*dpp*) of the *dpp* pathway (reviewed in MORISATO and ANDERSON 1995).

cype was associated with *ea* maternally (Table 1). Of the embryos from $cype^{1}/+$; $ea^{161.13}/+$ females crossed to wild-type males, 99% die with ventralized phenotypes varying from V5 (very weakly ventralized) to V3 (moderately ventralized) with 80% V4 (weakly ventralized; Figure 1D). The detail of the nomenclature used (ROTH *et al.* 1991) is presented in Table 1. With the control $ea^{161.13}/TM3$, 62% of the embryos die and are 100% V5. The same type of cross was performed with *cact*. *cype* $+/+$ $cact^{99}$ females crossed to wild-type males produce 6% dead embryos of which 80% are V4 and 20% V3 (Figure 1E). In the $cact^{99}/CyO$ control cross, 6% of the embryos also die but are 100% V4. These results show that altering *cype* maternally enhances the maternal ventralizing effect of *ea* and *cact* mutations.

The effect of *cype* was tested on two different *dpp* alleles. $cype^{1}/CyO$ females crossed to dpp^{hr27}/CyO (strong allele) males yield only 4% of the expected + $cype^{1}/dpp$ + progeny, while 38% are recovered with dpp^{hr4} (weaker allele). In the cross performed the other way the expected progeny is recovered with dpp^{hr27} and 71% with dpp^{hr4} (Table 2). The lethality is embryonic and the embryos are ventralized with phenotypes varying from V4 to V3 (Figure 1F). The haploinsufficient effect of dpp^{hr27} gives only V5 embryos. These results show a maternal enhancement of *dpp* mutant ventralizing embryonic phenotypes by the alteration of *cype*. The same results were observed with the other *cype* alleles obtained after the secondary mutagenesis.

***cype* deficiency mapping:** The *PZ* element (*P-lacZ-rosy*$^{+}$) from the lethal line *l(2)03771*: $cype^{1}$ (KARPEN and SPRADLING 1992) was localized by *in situ* hybridization in the 25D4-6 interval, by the BDGP (http://www.fruit

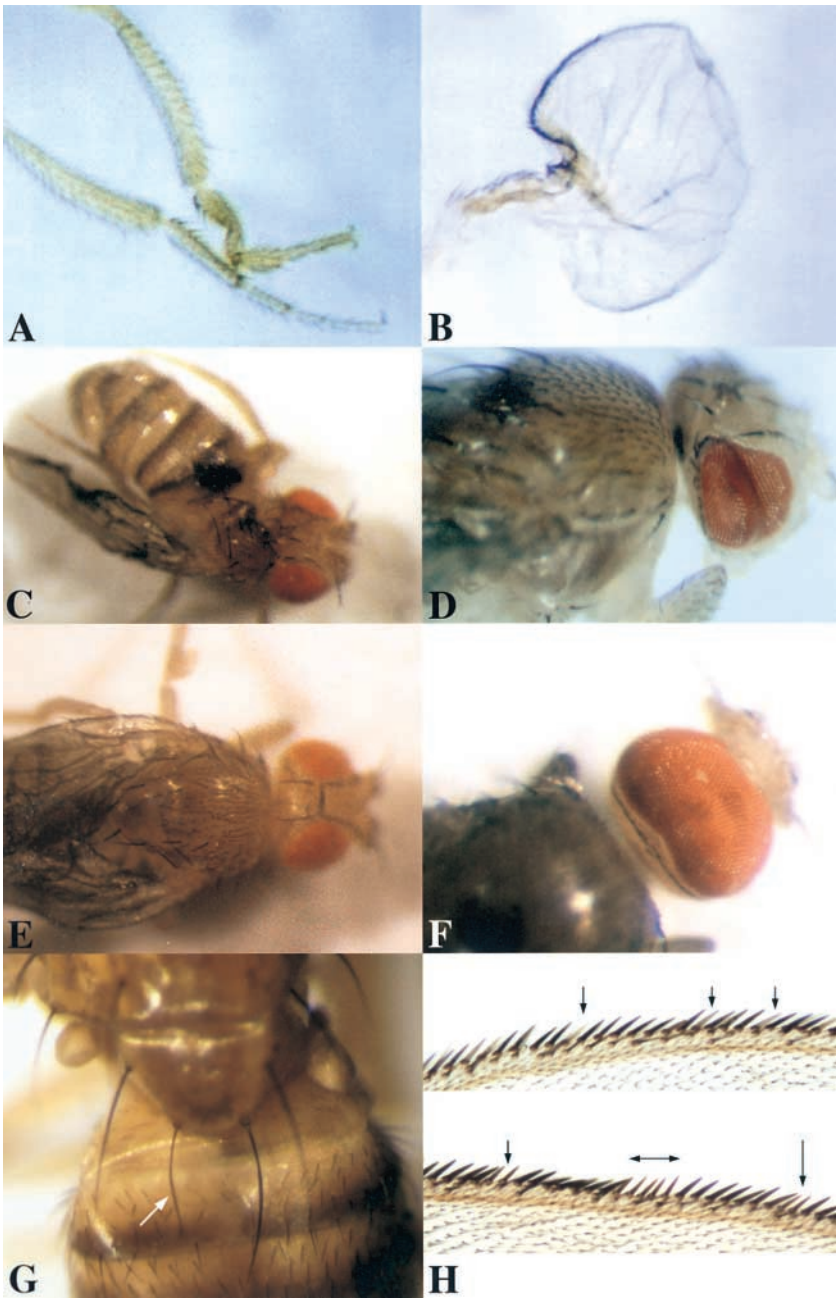

FIGURE 2.—*cype*^{1} adult phenotypes. (A–F) *cype*^{1}/*CyO* adult flies. (A) Crooked legs. (B) Atrophic wing. (C) Fly with half a thorax. (D) Anterior part of the eye flattened. (E) Close eyes. (F) Fused eyes. (G and H) Mitotic *y cype*^{1}/*y cype*^{1} clones performed with the *FLP/FRT* technique (MATERIALS AND METHODS). (G) Clone on the thorax with thinner and smaller *y* machrochaete. (H) Clones with thinner and smaller *y* bristles at the wing margin.

fly.org/p_disrupt/; SPRADLING *et al.* 1995, 1999). We previously mapped the insertion site by plasmid rescue within a genomic walk in the 25D region (GEORGE and TERRACOL 1997). The sequence at the junction between the genomic DNA and the 5′*P* end is 5′-GAATTTG CAAATGTGAGTAC/CATGATGAAA-3′, which is identical to the sequence provided by the BDGP (AQ025616). The genomic sequence downstream from the 3′*P* end was obtained by PCR amplification. The sequence at the junction between the 3′*P* end and the genomic DNA is 5′-TTTCATCATG/GTGAGTACATAA CAAGGTTTTT-3′. This sequence shows an 8-bp duplication (underlined) of genomic DNA present, in the wild-type DNA, upstream from the 5′*P* end (underlined). The 8-bp duplication is characteristic of *P*-element insertions and is excised with the *P* element in precise excision process (O'HARE and RUBIN 1983).

Southern blot analysis was performed with *cype*^{1} and six lethal independent new *cype* lines. Genomic DNA was *Eco*RI digested and the blot was hybridized with the 4.6-kb *Eco*RI fragment (E4.6, Figure 5D) extending upstream from the 5′*P*-insertion site. A 4.6-kb band was generated in the wild-type Oregon-R and *CyO* chromosomes while a 5.1-kb band was generated in *cype*^{1} by the insertion of the *P* element (Figure 4). These results indicate that there are new patterns in four lines and that deletions have occurred in the genomic region flanking the 5′*P* end. The same new bands were ob-

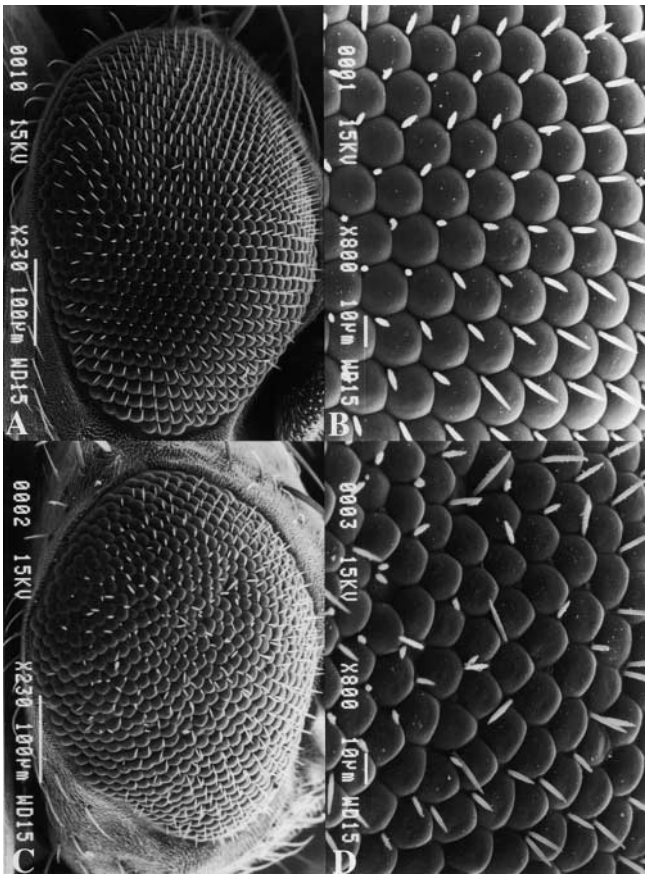

FIGURE 3.—Scanning electron micrographs of *cype*^{1}/*cype*^{1} clonal eye. Clones were generated by the *EGUF/hid* method at 29°. Photoreceptor cells bearing the dominant photoreceptor cell lethal transgene *GMR-hid* die during metamorphosis. The *l(2)CL-L1* recessive cell lethal mutation leads to lethality at an earlier stage of eye development. (A and B) *cype*^{1}*[ry*$^{+}$ *hs-neo FRT]40A/CyO; ey-GAL4 UAS FLP/+*, wild-type eye. (C and D) *cype*^{1}/*cype*^{1} clonal eye from a *cype*^{1}*[ry*$^{+}$ *hs-neo FRT]40A/[ry hs-neo FRT]40A GMR-hid l(2)CL-L1; ey-GAL4 UAS FLP/+* fly. (C) Clonal eye with a rough aspect. (D) The ommatidia are disorganized and present an abnormal morphology, bristles are missing or duplicated.

served with the 2.2-kb *Bgl*II-*Eco*RI internal probe (BE2.2, Figure 5D) indicating that the deletions do not reach the *Bgl*II site. In the excised lines, new fragments of 4.2 kb (*cype*$^{1R2.5}$), 5 kb (*cype*$^{1R4.5}$), 7.5 kb (*cype*$^{1R5.7}$), and 3.6 kb (*cype*$^{1R15.1}$) replace the 5.1-kb *Eco*RI band. Hybridization of the same blot with the 2.2-kb *Eco*RI fragment (E2.2, Figure 5D) mapping downstream from the *P*-element 3′ end insertion site gave a single 2.2-kb *Eco*RI band in all cases, indicating that this fragment was not altered by the insertion or the excisions.

PCR amplifications were performed to determine the extent of the deletions. Positions of the primers are shown in Figure 5B and the results are summarized in Table 3. *cype*$^{1R7.1}$ and *cype*$^{1R9.1}$ lines produced amplified DNA with both primer 1-primer 5′*P* and primer 3′*P*-primer 3 or 4 pairs giving the same bands, 2.2 and 0.6 kb long, respectively, as the control, *cype*^{1}. These results

TABLE 1

Maternal enhancement of *easter* and *cactus* phenotypes by *cyclope*

Female genotype	Dead embryos (%)	Phenotypes (%) V5	V4	V3
ea$^{161.13}$/*TM3*	62	100	0	0
cype^{1}/+; *ea*$^{161.13}$/+	99	10	80	10
cact^{99}/*CyO*	0	100	0	0
cype^{1} +/+ *cact*^{99}	6	0	80	20

In all cases, females were crossed to wild-type males. V5: very weakly ventralized; head involution does not occur and some embryos have a tail up phenotype, probably due to defective retraction of the germband; Filzkörper are normal. V4: weakly ventralized; the head is not involuted and the posterior segments are internalized; Filzkörper are disorganized. V3: moderately ventralized; the ventral denticle belt is extended laterally; Filzkörper are reduced and disorganized; the head is not involuted and head and thorax are extended and convoluted. The phenotypic categories are according to ROTH *et al.* (1991).

indicate that the *ry* phenotype of these lines is due to an internal rearrangement within the *rosy* gene. *cype*$^{1R4.5}$ and *cype*$^{1R5.7}$ lines produced only amplified DNA with primer 3′*P*-primer 3 or 4 pair giving the same band (0.6 kb) as the control, *cype*^{1}. This indicates that a deletion of the 5′*P* region had occurred. It was not possible to recover any PCR product with these lines using various primers within the *ry* gene and, therefore, the exact extent of the deletions could not be determined. Finally, *cype*$^{1R2.5}$ and *cype*$^{1R15.1}$ DNAs were not amplified with either pair of primers. Both mutant line DNAs were amplified, however, with the primer 1-primer 3 or 4 pair giving a 2.3-kb band with *cype*$^{1R2.5}$ and a 1.7-kb band with *cype*$^{1R15.1}$. These results indicate that the *P* element was excised leading to a deletion in the genomic adjacent region (Figure 5B). The DNA sequence resulting from the *cype*$^{1R2.5}$ excision is 5′-TTGCTATATT/ATATATATTATA TATATGTTATTTCATCATG/GTGAGTACATAACAA GGT-3′. The excision has generated a 402-bp deletion in the genomic DNA and the elimination of most of the transgene. An insertion of 14 nucleotides (AT) not present

TABLE 2

Maternal enhancement of *dpp* lethality by *cype*^{1}

Allele	From	% of expected + *cype*^{1}/*dpp* + adult progeny *dpp*^{hr27}	*dpp*^{hr4}
cype^{1}	Females	4	38
	Males	104	71

The values given are percentages of + *cype*^{1}/*dpp* + adult flies compared to the quantity of *cype*^{1}/*CyO* control progeny. At least 300 flies were counted in each experiment.

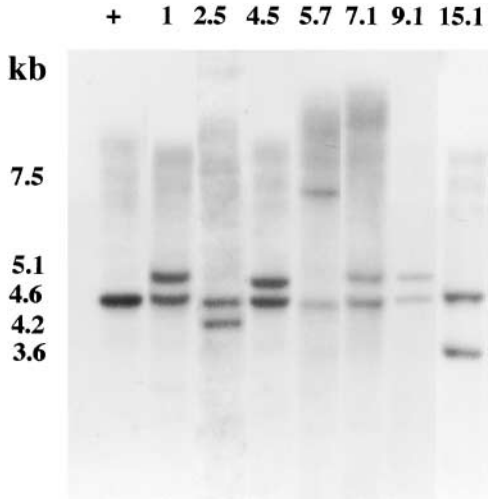

FIGURE 4.—Southern blot analysis of the *cype*^{1} excised lines. About 10 μg of DNA (10 flies) were *Eco*RI digested in 30 μl (20 units overnight) with 10 μg RNAse A. (+) Oregon-R wild-type DNA, compared to the *cype*^{1} (1) and the six excised lines. The blot was hybridized with the 4.6-kb *Eco*RI fragment (E4.6, Figure 5D).

in the genomic DNA and 17 nucleotides (underlined) from the imprecisely excised *P*-element 3′ end separates the two genomic sequences. The DNA sequence resulting from the *cype*$^{1R15.1}$ excision is 5′-TACGGATTTT/TATTT CATCATG/GTGAGTACATAACAAGGT-3′. The excision has generated a 973-bp deletion in the genomic DNA and the elimination of most of the *P* element. Twelve nucleotides resulting from the imprecisely excised *P*-element 3′ end (underlined) remain between the two genomic sequences. The 8-bp duplication is still present in the excised lines.

TABLE 3

***cype* deficiency mapping**

	Primers		
cype allele	1-5′*P*	3′*P*-4	1-4
1	2.2	0.6	—
1R2.5	—	—	2.3
1R4.5	—	0.6	—
1R5.7	—	0.6	—
1R7.1	2.2	0.6	—
1R9.1	2.2	0.6	—
1R15.1	—	—	1.7

Genomic DNA from *cype*^{1} and the six revertant lines was PCR amplified with three pairs of primers. The length of the fragments is given in kilobases and primer positions are shown in Figure 5B.

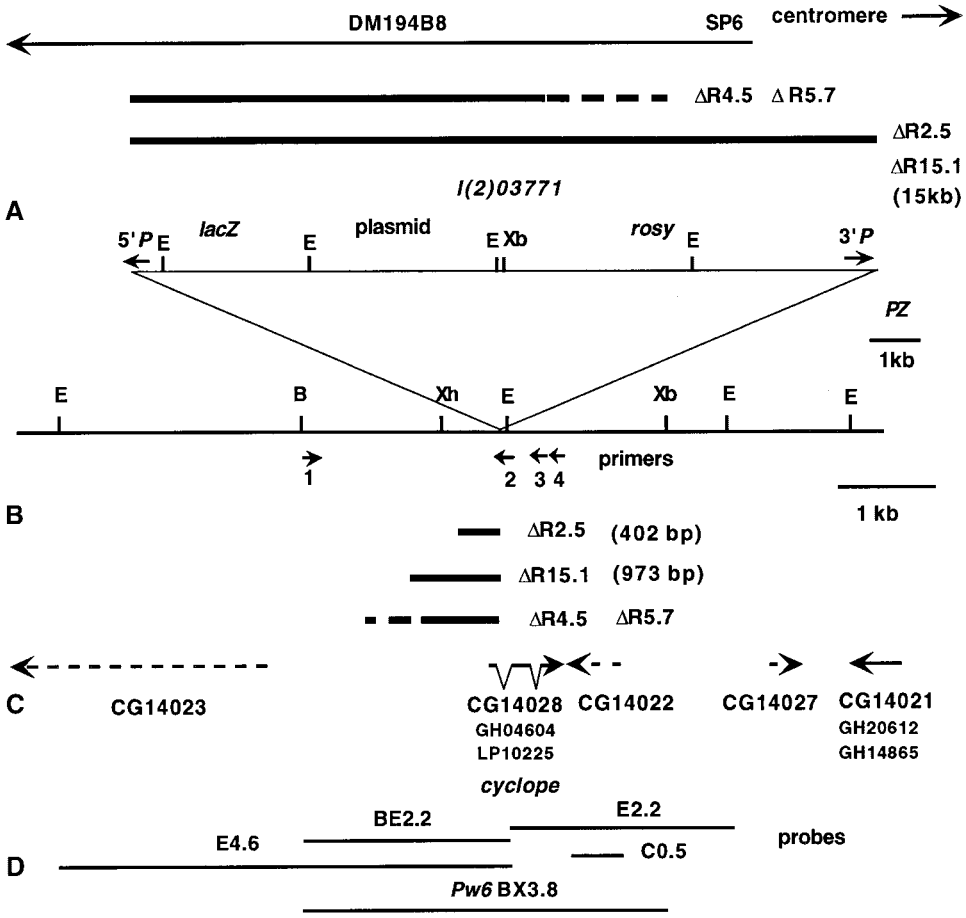

FIGURE 5.—Genomic map of the *cyclope* region. (A) DM194B8: SP6 end of a cosmid isolated by the European Drosophila Mapping Consortium. The extent of the deletions generated in the *PZ* element and the position of the *PZ* element of *cype*^{1} are shown. The scale for the *PZ* element (15 kb) was reduced by half to fit the figure. Only *Eco*RI (E) and *Xba*I (Xb, plasmid rescue cloning site) restriction sites are shown. The position of the 5′ and 3′ *P* primers is shown. (B) The restriction map of the region is indicated (E, *Eco*RI; B, *Bgl*II; Xh, *Xho*I; Xb, *Xba*I). The position of the primers (1, 2, 3, 4) used for PCR amplification and the extent of the deletions generated in the genomic region are shown. (C) The positions and orientations of the *cyclope* cDNAs, putative GadFly genes, CG14023, CG14028 (*cyclope*), CG14022, CG14027, and CG14021, are shown together with the BDGP cDNAs isolated in the region, GH04604 and LP10225 (*cyclope*) and GH20612, GH14865 (CG14021). (D) The extent of the probes used for Southern blot and cDNA screening analyses and of the transgene *Pw6*BX3.8, used for rescue experiments, is indicated.

***cyclope* encodes a cytochrome c oxidase subunit VIc homolog:** cDNA λ-libraries were screened using genomic DNA fragments mapping on both sides of the *cype*^{l} transposon. No cDNAs were found with the 2.2-kb *Bgl*II-*Eco*RI fragment (BE2.2, Figure 5D) mapping upstream from the *P*-element 5′ end while many cDNAs (16) were isolated using the 2.2-kb *Eco*RI fragment (E2.2) mapping downstream from the *P*-element 3′ end (map, Figure 5D). Two cDNAs were isolated from a λgt10 embryonic (1.5- to 5-hr) library, 365 and 431 bp long. Two cDNAs 249 and 171 bp long were isolated from a 0- to 24-hr embryonic λZap II cDNA library. Finally, 12 cDNAs 171 to 449 bp long were isolated from a λZap II late L3 stage library. These cDNAs overlap one another and also overlap with two cDNAs isolated by the BDGP (http://www.fruitfly.org/EST/; Rubin *et al.* 2000), GH04604 (399 bp), isolated from adult head, and LP10225 (371 bp) from larval-early pupal cDNA libraries. They correspond to the CG14028 GadFly gene identified by the Genome Annotation Database of Drosophila (http://www.fruitfly.org/annot/; Adams *et al.* 2000). Two putative genes are proposed in GadFly on the other strand. The 5′ end of the first one (CG14023) maps 2.5 kb upstream from the *P*-element insertion site and the 3′ end of the other (CG14022) maps 60 bp downstream from the 3′ end of the longest cDNA we recovered. Neither the BDGP screen nor our screen identified corresponding cDNAs. Given its position, at the 3′ end of CG14028, and its orientation (opposite), CG14022 is unlikely to be altered by the *cype*^{l} transposon. Furthermore, no transcripts corresponding to CG14022 were detected in developmental Northern blots with a 0.5-kb *Cla*I genomic probe (C0.5, Figure 5D) including this putative transcription unit. Therefore no other cDNAs were identified within the 8 kb surrounding CG14028 (Figure 5). GH04604 is the longest cDNA at the 5′ end, which corresponds to position 1 in the genomic sequence (Figure 6). Most of the cDNAs (13/18) differ slightly at the 5′ end and start at positions 1, 19, 34, 35 (4), 41 (3), 43 (2), 46. Fifteen cDNAs have a poly(A) tail. Eight are identical at the 3′ end (666), and four end at position 670. The others end, respectively, at 672, at 738, and the longest at the 3′ end, at position 743 (1.5- to 5-hr embryonic).

Comparison of cDNA and genomic sequences (Figure 6) revealed the presence of two introns. The first (47–262) is 216 bp long and the second (437–503) is 67 bp long. One cDNA (late L3) starts in intron 1 (89) and shows a 152-bp first intron (111–262) due to alternative splicing. In these introns are found the predicted splice donor and acceptor sites (GT at 5′ and AG at 3′ splice sites) that fit the invertebrate splice junction consensus (Mount *et al.* 1992). The splice site predictions from the BDGP program gave, for the three donor sites (GT) observed, the scores 0.91 (47), 0.51 (111), and 0.42 (437). The scores for the acceptor sites (AG) are 0.93 (262) and 0.97 (503). These results are in good agreement with our findings. The *cype*^{l} *P* element is inserted in base position 55, eight nucleotides

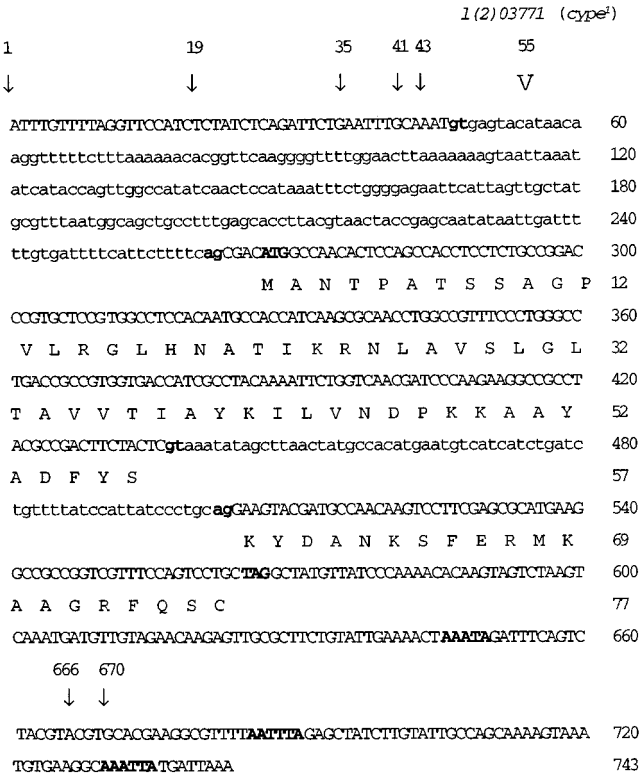

Figure 6.—*cyclope* genomic and predicted amino acid sequences. The sequence corresponding to cDNAs is in capital letters. The start (1) corresponds to the first nucleotide of the longest cDNA (GH04604) at the 5′ end and the end corresponds to the longest cDNA at the 3′ end (743). Position 19 corresponds to cDNA19 start and is the longest 5′ end that we recovered. The most representative cDNA starts and ends are marked by an arrowhead, and the insertion site of the *PZ* element *l(2)03771* at position 55 is shown (V). The two introns are shown (lowercase letters) and the donor (gt), acceptor (ag), the ATG, TAG, and polyadenylation sites are in boldface. The GenBank accession number for the sequence reported here is AY029256.

downstream from the 5′ end of the first intron. The splicing of this first intron is probably defective and it is therefore very likely that the 15-kb *PZ* element insertion of *cype*^{l} alters the function of the gene corresponding to these cDNAs. We found three sequences that fit the consensus polyadenylation site, AATAAA (Proudfoot and Brownlee 1976), preceding most of the cDNA ends: AAATA between positions 646 and 650 followed by the related sequences AATTTA (685–690) and AAATTA (730–735). Between positions −93/−85 we found the sequence TAATATATA that fits the TATA box consensus, and the CAAT sequence that fits the CAT box consensus is present between positions −80/−77. The first ATG in frame is situated at position 267 after the first intron, and a stop codon, TAG, is found at position 565. The sequence flanking the translation start is CGACATG, which fits the Drosophila consensus C/AAAA/CATG (Cavener 1987). There is only one difference, a G, which is a secondary preference, instead of an A at position −3.

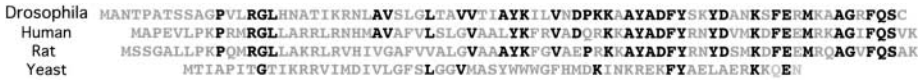

FIGURE 7.—Comparison of the Drosophila COX VIc protein with the homologs in human, rat, and yeast: Drosophila 77 aa (GenBank accession no. AAK32965), human 75 aa (OTSUKA *et al.* 1988; GenBank accession no. NP 004365), rat 76 aa (SUSKE *et al.* 1988; GenBank accession no. P11951), and yeast (*S. cerevisiae*) 59 aa (WRIGHT *et al.* 1986; GenBank accession no. J02633).

This analysis shows that the *cype*$^{1R2.5}$ and *cype*$^{1R15.1}$ deletions, 402 and 973 bp, respectively, eliminate the whole 5′ transcript region upstream from position 55. This includes the first intron 5′ end, the transcript initiation sites, and the CAT and TATA putative boxes. However, among the 18 cDNAs studied one (late L3) starts in the first intron (89) and shows an alternative splicing. The corresponding sequence is not altered in either these lines or *cype*^{1}. Since the coding sequence is also preserved, it is possible that these mutations do not abolish the expression of the gene, but, rather, severely alter its regulation. Alternatively, because this cDNA is unique among the cDNAs studied (18), it is likely that it is not very abundant and does not allow significant protein production. Therefore these alleles could be null alleles.

A 3.8-kb genomic transgene (Figure 5D) including this transcription unit was able to completely rescue, in one dose, the *cype*^{1} and the four new *cype* alleles, in hemizygotes and *inter se*. These results demonstrate that these cDNAs correspond to the *cyclope* gene and also show that the 973-bp *cype*$^{1R15.1}$ deletion does not alter any other vital genes.

The predicted 77-amino acid protein (8.295 kD) is closely related to the mammalian cytochrome c oxidase subunit VIc (Figure 7). The Drosophila protein shares over 65 amino acids (aa), 46% identity, and 59% similarity with the human protein (OTSUKA *et al.* 1988) and 43% identity and 59% similarity with the rat subunit (SUSKE *et al.* 1988). Both the percentages of identity and similarity and the size of the protein, 75 aa in human and 76 in rat, correlate with the hypothesis that *cyclope* encodes the cytochrome c oxidase VIc subunit. The corresponding yeast subunit (WRIGHT *et al.* 1986) is more divergent.

In the higher eukaryotes, cytochrome c oxidase contains 3 mitochondrial DNA-encoded subunits and up to 10 nuclear DNA-encoded subunits in mammals. In Drosophila the 3 mitochondrial DNA-encoded subunits were previously identified (DE BRUIJN 1983). As shown in Table 4, these subunits are highly conserved when compared to the human peptides (ANDERSON *et al.* 1981). Subunit I is the best conserved with 68% identity and 79% similarity. Subunit II is 58% identical and 69% similar and subunit III 61% identical and 75% similar to the human proteins. A search for homologs of the human subunit precursors in the Genome Annotation Database of Drosophila (http://www.fruitfly.org/annot/; ADAMS *et al.* 2000), using the BDGP Blast server, revealed 9 nuclear subunits while 1, VIIb, was not found (Table 4). These subunit putative genes are located on all chromosomes (except chromosome 4) and present a high level of identity with the human subunits, ranging from 29% (VIII) to 59% (VIb). The subunit VIII has the lowest identity score; nevertheless, the size of the putative protein, 68 aa, is in good agreement with the size of the human subunit VIII (69 aa). Four subunits are present in two isoforms. This is the case for subunits, IV, Vb, VIa, and VIIa. Identities between the isoforms is in the range of 40–50%, which is, in most cases, lower than the score obtained for a given unit compared to the corresponding human unit. This finding indicates that the gene duplication is ancient. The two genes encoding the isoforms for subunits Vb and VIIa are both duplicated at the same chromosomal site and the isoforms are also the more divergent (42% identity for both). Subunit Vb genes are duplicated in tandem and are separated by 382 bp, while subunit VIIa genes are duplicated in opposite directions and are separated by 2.7 kb. Only 1 subunit, Va, was previously identified as corresponding to the *CoVa* gene but no mutant has been described (CAGGESE *et al.* 1999). Therefore, *cyclope* is so far the first cytochrome c oxidase mutant to be recovered in Drosophila.

Temporal and spatial expression of *cype* transcripts: Northern blot analysis (Figure 8) shows the presence of transcripts of ~370 bp throughout development. This size is consistent with the length of the cDNAs recovered and with those of the mammalian cytochrome c oxidase VIc transcripts (450–510 bp). There is no major quantitative variation between the different developmental stages when compared with the internal control, *rp49*, except in males where transcripts appear relatively more abundant than in female and in other development stages.

To confirm that the *cype* RNA expression is altered in the different *cype* alleles we performed Northern blots with total RNA from adult heterozygous females and monitored *cype* RNA (Figure 9). *cype* RNA level is decreased to 0.61 ± 0.08 in *Df(2L)tkv*^{5x2}/+, 0.63 ± 0.07 in *cype*^{1}/+, 0.66 ± 0.08 in *cype*$^{1R2.5}$/+, and 0.54 ± 0.06 in *cype*$^{1R15.1}$/+ flies, when compared to +/+ flies. These values are not significantly different and show that the *cype* RNA level is abolished or strongly reduced in the three *cype* alleles.

In situ hybridization performed in embryos (Figure 10, A–D) reveals the presence of a maternal product in preblastoderm embryos (A). Then, at stage 10 (germband extension), RNAs are detectable in neuroblasts (B). In stage 13 embryos (C), RNAs are present in the central nervous system (CNS). At stage 17 the labeling is present only in the condensed CNS (D). *lacZ* staining in *cype*^{1} (Figure 10, E–M) shows, in stage 10 embryos, a labeling in head and neuroblasts (E) and at stage 16 in the CNS (F). *lacZ* staining is detected in larval brain (not shown), in the central region of imaginal discs (G and H), and in

TABLE 4

Putative cytochrome c oxidase subunits encoded by the mitochondrial and nuclear genome of Drosophila

Subunit	GadFly	Location	AA(N) Dro/Hum	I/S(%) Dro/Dro	I/S(%) Dro/Hum	Ref.
I		mtDNA	511/513		68/79	1, 2
II		mtDNA	228/227		58/69	1, 2
III		mtDNA	262/261		61/75	1, 2
IV	CG10664	2L; 38A8	182/169	51/66	39/56	3
	CG10396	2R; 41A3	162/169		38/59	3
Va	CG14727/*CoVa*	3R; 86F10-11	149/150		52/70	4, 5
Vb	CG11015	2L; 26E2	120/129	42/67	51/67	6
	CG11043	2L; 26E2	154/129		34/55	6
VIa	CG17280	2R; 59E3	109/109	45/68	55/69	7
	CG14077	3L; 75E4	118/109		47/60	7
VIb	CG14235	X; 18F1	92/86		59/77	8
VIc	CG14028/*cype*	2L; 25D5-6	77/75		46/59	9
VIIa	CG18193	3R; 84F13-14	106/79	42/65	57/77	10
	CG9603	3R; 84F13	89/79		38/61	10
VIIb	—					11
VIIc	CG2249	2R; 46D7	51/63		46/64	12
VIII	CG7181	3L; 78E1	68/69		29/51	13

Putative cytochrome c oxidase nuclear genes were searched for in the Genome Annotation Database of Drosophila (GadFly) using the human precursor peptide sequence. The nomenclature used is from Kadenbach and Merle (1981). AA(N), comparison of the number of amino acids in Drosophila (Dro) and human (Hum). I/S(%), comparison of percentage of identity (I) and similarity (S) between two Drosophila isoforms or between Drosophila and human peptides. (1) de Bruijn (1983); (2) Anderson *et al.* (1981); (3) Zeviani *et al.* (1987); (4) Caggese *et al.* (1999); (5) Rizzuto *et al.* (1988); (6) Zeviani *et al.* (1988); (7) Fabrizi *et al.* (1989); (8) Taanman *et al.* (1989); (9) Otsuka *et al.* (1988); (10) Arnaudo *et al.* (1992); (11) Sadlock *et al.* (1993); (12) Koga *et al.* (1990); (13) Rizzuto *et al.* (1989).

ovarian germarium (I), follicular and nurse cells (J). A stronger *cype* expression is detected in developing oocytes rather than in follicular cells. High level *lacZ* staining is detected in larval male gonad (K), in adult testis (L), and spermatozoids (M). The strong expression in male reproductive organs is likely to be responsible for the enhanced *cype* expression observed by Northern blot analysis in adult males (Figure 8). The correlation between *in situ* and *lacZ* staining in the embryo and in the other tissues (not shown) is consistent with the implication of the *P* element in the alteration of the cytochrome c oxidase function.

DISCUSSION

***cyclope* is a vital gene with pleiotropic effects:** *cype* is a previously undescribed homozygous lethal gene. The *P* element of the initial allele $cype^1$ was excised leading to viability, showing that the lethality was due to the presence of the *P* element. New alleles were generated by imprecise excision of $cype^1$ *P* element. The mutation causes slight defects in the dorsoventral polarity of the embryo. The defects are enhanced by decreasing simultaneously the maternal activity of *cype* and the activity of maternal or zygotic dorsoventral polarity genes. How do COX alterations cause defects in dorsoventral polarity? Although this may appear rather surprising, it has been observed that changes in the respiratory chain activity are followed by alterations in nuclear gene expression. This is the case, for example, for mutants in the *oxen* gene of Drosophila encoding the ubiquinol-cytochrome c oxidoreductase subunit 9 (Frolov *et al.* 2000). The mutation preferentially exerts a negative dosage effect upon the expression of the *white*, *brown*, and *scarlet* genes. This mechanism, called retrograde regulation, has been hypothesized to monitor and adjust cell functions in response to perturbations in mitochondria (Parikh *et al.* 1987; Poyton and McEwen 1996). *cype* is haploinsufficient, and 35% of the *cype*/+ pupae die. Adult phenotypes are observed in *cype*/+ individuals, mostly due to defects in imaginal disc development. Alteration of imaginal disc derivatives has been described for genes encoding mitochondrial proteins. Mutants in the *colt* gene encoding a mitochondrial carrier exhibit loss of venation and reduction in wing size (Hartenstein *et al.* 1997). Mutations in the tumor suppressor gene, *l(2)tid*, encoding a transmembrane mitochondrial protein lead to imaginal discs that fail to differentiate and grow into lethal tumors (Kurzik-Dumke *et al.* 1992, 1998). Mutations in the *tamas* gene, encoding a mitochondrial DNA polymerase catalytic subunit, induce smaller eyes with a rough aspect and disorganized ommatidia (Iyengar *et al.* 1999). A similar phenotype is observed in $cype^1$ clonal eyes. $cype^1$ germline clones are

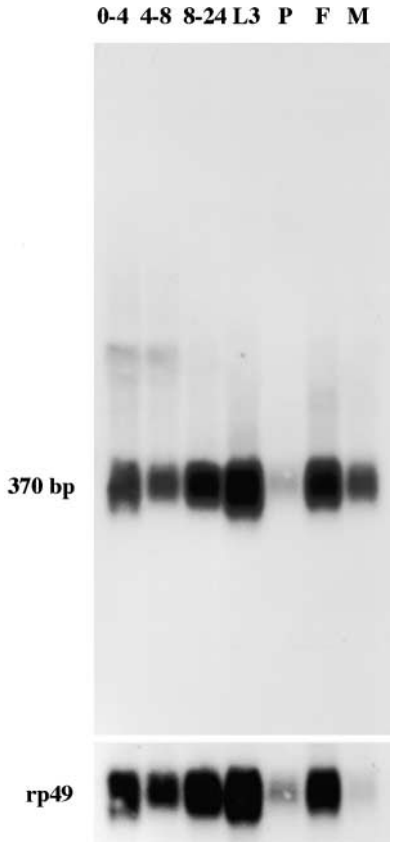

FIGURE 8.—Developmental Northern blot analysis. Poly (A^+) Oregon-R RNA (10 μg) was loaded and run in a 1% formaldehyde agarose gel. The blot was hybridized with cDNA19 from λZap II embryonic (0- to 24-hr) library. cDNA19 is 394 bp long and maps between positions 19 and 670 (Figure 6). The cDNA *rp49* encoding a ribosomal protein was used as a loading control. The developmental stages are as follows: embryonic stages are 0–4 hr, 4–8 hr, 8–24 hr (hours after egg laying); L3, third instar larval stage (3–5 days after egg laying); P, pupal stage (6–7 days after egg laying); F, adult female; and M, adult male.

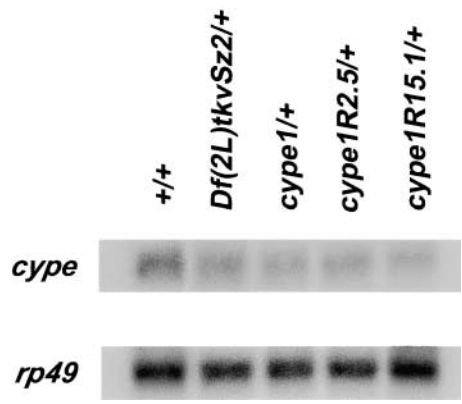

FIGURE 9.—Northern blot analyses of *cype* expression. Total adult female RNA (20 μg) was loaded in each lane and three independent Northern blots were performed using the same RNA extract. Each blot was hybridized with the *cype* cDNA19 and the *rp49* cDNA as a loading control. The blots were quantified with a Storm 860 (Molecular Dynamics) phosphorimager. The *cype* values were normalized to the *rp49* values. The final results are mean values corresponding to fractions of the +/+ values.

lethal while somatic clones in the adult cuticle lead to reduced bristles and cell size. These phenotypes are compatible with a diminished COX activity that is likely to induce cell death and a decrease in cell growth. The *cype* embryonic and adult phenotypes are probably due to a general decrease in COX activity detected preferentially in processes requiring a high energy level. In human, for instance, COX diseases affect mainly brain, eye, and muscle tissue (TAANMAN 1997; POYTON 1998; LEONARD and SCHAPIRA 2000a,b).

CYPE protein is homologous to human cytochrome c oxidase subunit VIc: The gene was cloned by localization of the initial *P*-element allele $cype^1$. The new alleles generated by imprecise excision of $cype^1$ *P* element were analyzed at the molecular level and shown to delete the 5′ end of the gene. Around the insertion site, 16 overlapping cDNAs with a size of ~370 bp were isolated from embryonic and third larval instar libraries. The gene contains two introns and the *P* element was localized in the first one. The gene encodes a 77-aa peptide 46% identical and 59% similar to the human COX VIc subunit. No other types of cDNA were isolated and no other transcripts were detected in the region screened, 2.2 kb upstream and 2.2 kb downstream of the *P*-insertion site. Furthermore, the BDGP screen (RUBIN *et al.* 2000) revealed no other cDNAs within the 8 kb surrounding the COX VIc gene, although they were searched for at all developmental stages and in different types of tissues. The presence of the *P* element in the first intron of the COX VIc cDNA together with the fact that no other transcripts were found in the close vicinity argues in favor of the implication of the *P* element in the alteration of the COX VIc transcript. Furthermore, *cype* RNA level is strongly reduced in *cype* mutants. This was unambiguously demonstrated by the complete rescue of $cype^1$ and the four new mutations with one dose of a 3.8-kb genomic transgene that includes the COX VIc transcript.

Different initiation sites were identified in *cype* cDNAs as well as a TATA and a CAT box upstream from the transcription starts. Multiple initiation sites are generally observed in nuclear DNA-encoded COX subunits. In mammals, for instance, a single transcription initiation site is observed in rat and bovine subunit VIII genes only (LENKA *et al.* 1998). Transcription initiation sites of diverse tissue-specific COX and ubiquitous genes fall into two categories, those containing a TATA box and the others. Generally, genes that lack the TATA box are of the housekeeping type and show 5′ heterogeneity. However, TATA and CAT boxes were identified upstream from the multiple initiation sites of subunits VIa (H) and VIc (rat) genes. A TATA box was also found upstream from the single initiation site of rat and bovine subunit VIII (H) genes. Subunit VIc gene from rat resembles *cype* gene with multiple initiation sites and TATA and CAT boxes. Most

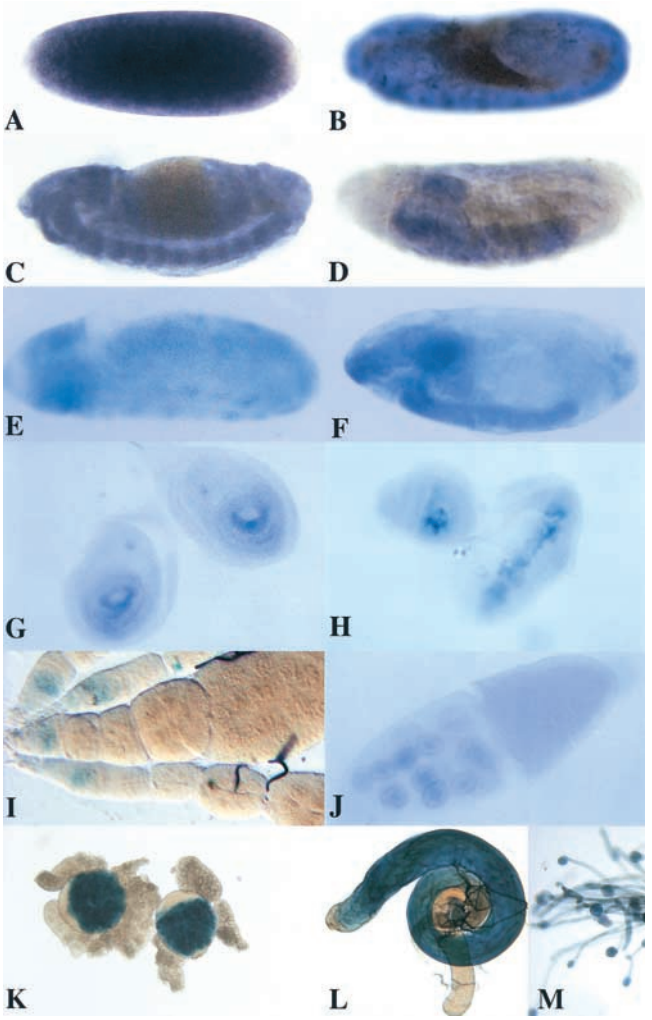

Figure 10.—*In situ* hybridizations and *lacZ* staining. (A–D) *In situ* hybridizations in wild-type embryos performed with the digoxigenin-labeled DNA fragment E2.2 (Figure 5D). The results are identical when using the antisense RNA probe made from pBScDNA19. Stages are according to Campos-Ortega and Hartenstein (1985). (A) Preblastoderm embryo showing a maternal contribution. (B) Extended germband stage with a segmental pattern. (C) Stage 13 embryo with expression in the CNS. (D) Stage 17 embryo showing labeling exclusively in the condensed CNS. (E–M) *lacZ* staining in the $cype^{1}$ allele. (E) Stage 10 embryo with labeling in neuroblasts. (F) Stage 17 embryo showing labeling in CNS. (G and H) Staining in the central region of leg (G) and eye-antennal imaginal discs (H). (I and J) Staining in ovaries with labeling in germarium (I) and in follicular and nurse cells at stage 10 (J). *cype* RNA expression is higher in developing oocyte than in follicular cells. (K–M) Staining in male reproductive organs. (K) Larval (L3) gonads are stained except at the anterior end (germ cells). Adult testis (L) and spermatozoids (M) strongly labeled.

(eight) of the mammalian nuclear-encoded COX subunits contain N-terminal mitochondrial targeting sequence that is cleaved off during importation. Presequence size ranges from 12 aa (VIa) to 41 aa (Va) and is not found in COX VIb and COX VIc. It is therefore likely that CYPE does not contain either presequence. This hypothesis was confirmed by the study of the *COX9* gene of yeast encoding the COX VIc (VIIa in yeast nomenclature) homolog showing that the subunit lacks a leader peptide (Wright *et al.* 1986). There is, however, a precursor longer by one amino acid (M) at the NH_3 terminus and four amino acids at the COOH terminus. In the mutant strain with deletion of the *COX9* gene, cells lack a functional cytochrome c oxidase, which suggests that the subunit VIc is an essential component of the holoenzyme. In yeast the three smallest subunits (VIc, VIIa, and VIIc) are transmembranous and play a role in assembly and stability, which is consistent with the fact that mutants in *COX9* also lack many of the other COX subunits.

***cype* is expressed in localized domains during development:** Study of spatial and temporal transcripts showed that *cype* is expressed throughout development (Northern) with no significant quantitative modulation, except in adult male where transcripts are more abundant. *In situ* hybridization in preblastoderm embryos revealed the presence of a maternal product. This finding confirms the genetic analysis that demonstrates the maternal effect of the gene. Transcripts were preferentially observed in embryonic neuroblasts and CNS, which was confirmed by *lacZ* staining. This correlation confirms the role of the *P* element insertion in *cype* expression. Later in development, *lacZ* staining is observed in larval brain, in imaginal discs, in the germarium, nurse, and follicular cells of the ovary, and in male reproductive organs. Therefore *cype* appears to be expressed in specific tissues and in localized regions, unlike the ubiquitous mammalian COX VIc gene. Only a small number of tissues have been studied in mammals, essentially heart and liver tissues, and expression during development has not been studied as yet. These localized transcripts may reflect a greater energy requirement. It is noteworthy that mutations in numerous mitochondrial proteins result in nervous system defects. Behavioral defects (bang sensitivity) are observed in mutants in *tko* with altered ribosomal protein S12 (Royden *et al.* 1987) and in the *stress sensitive B* gene encoding an adenine nucleotide translocase (Zhang *et al.* 1999). The *sluggish-A* gene encoding a proline oxidase is predominantly expressed in the embryonic central nervous system and depletion of its activity induces locomotory defects (Hayward *et al.* 1993). Mutants in the *colt* gene encoding a mitochondrial carrier exhibit a sluggish response to physical contact (Hartenstein *et al.* 1997). Similar defects are observed in mutants in the *oxen* gene encoding the ubiquinol-cytochrome c oxidoreductase subunit 9 (Frolov *et al.* 2000). Finally, mutations in the *tamas* gene, encoding a DNA polymerase catalytic subunit, disrupt the development of the visual system and larvae present a reduced response to light due to locomotory deficiency (Iyengar *et al.* 1999).

Nine putative cytochrome c oxidase nuclear subunits are found in Drosophila: Comparison of the three mitochondrial DNA-encoded subunits I, II, and III with the corresponding human peptide shows a high degree of

conservation in size and sequence (about 65% identity). This is not surprising because these subunits are found in all species from bacteria to human and constitute the catalytic core of the enzyme. A search in the Genome Annotation Database of Drosophila (Adams *et al.* 2000) revealed the presence of nine putative nuclear subunits homologous to human peptides. Except for subunit VIIb, we found a Drosophila homolog in all cases. We searched for these subunits, however, using the human sequences and the VIIb peptide is perhaps too divergent to be found. Cytochrome c oxidase subunits have been studied essentially in mammals and no sequences from species closer to Drosophila are available. Alternatively, it is possible that this subunit does not exist in Drosophila. In yeast, for example, there are only eight nuclear subunits, VIIb and VIII being absent (Capaldi 1990). Drosophila subunit VIII is the most divergent (29% identity) and was not identified in the database as a COX VIII homolog. However, the size of the peptide (68 aa) is very close to that of the human (69 aa). These subunits are found on all chromosomes (except four) and four have isoforms, IV, Vb, VIa, and VIIa. Two forms of subunits VIa and VIIa have also been found in mammals while two forms of subunit IV have been identified in yeast. Two forms of subunit VIc have been found in Dictyostelium but only one in Drosophila. The presence of isoforms reflects the different energy requirements depending on the tissues, developmental stages, or growth conditions.

Human OXPHOS diseases affect mainly brain and muscles: In the human, OXPHOS diseases (reviewed in Poyton 1998; Wallace 1999; Leonard and Schapira 2000a,b; Schon 2000) predominantly affect brain and muscles, both types of tissue having a high energy requirement. OXPHOS mtDNA defects induce myopathies in progressive external ophthalmia and Kearns Sayre syndrome (KSS), encephalomyopathies in mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) and myoclonus epilepsy with ragged-red fibers (MERRF) syndromes, and neuropathies in Leber's heredity optic neuropathy. OXPHOS neurodegenerative disorders are also found in nuclear gene defects. Mutations have been found in subunits of complex I, including NADH binding site and in succinate dehydrogenase (SDH complex II) in Leigh syndrome. COX deficiences, due to mutations in the *Surf1* gene involved in COX assembly and maintenance, are also found in Leigh syndrome. In Freidreich's ataxia, deficiencies of complexes II and III due to mutations in the nuclear gene frataxin are observed. Dystonia, parkinsonism, and psychiatric illness are observed in Wilson's disease due to mutations in the P type ATPase. In Huntington's disease there is loss of medium spiny neurons. The defect is due to mutations in the huntingtin gene and results in deficiencies in complexes II, III, and IV (COX). In Parkinson's disease, complex I defect is observed. Nevertheless, although cells in mitochondrial diseases are disabled, in many cases they do not die. For example, muscle necrosis is rarely found in mitochondrial myopathies and brain regions in KSS and MELAS syndromes show little neuronal loss.

It will be therefore interesting to research, in *cyclope* mutants, the occurrence of defects in the nervous system and behavior. Furthermore, isolation and study of other mutations in the OXPHOS chain and, principally, in the cytochrome c oxidase (complex IV) of Drosophila will probably contribute to our understanding of, not only the human degenerative OXPHOS diseases, but also the aging process.

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