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# Mutations in *calphotin*, the Gene Encoding a Drosophila Photoreceptor Cell-Specific Calcium-Binding Protein, Reveal Roles in Cellular Morphogenesis and Survival

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ABSTRACT

Calphotin is a Drosophila photoreceptor cell-specific protein expressed very early in eye development, at the time when cell-type decisions are being made. Calphotin is a very hydrophobic and proline-rich protein which lacks obvious transmembrane domains. The cDNA encoding Calphotin was mapped to a region removed by a set of existing chromosomal deletions. Mutations that alter photoreceptor cell structure and development were isolated that fail to complement these deletions. These mutations fall into two classes. Class I mutations alter the structure of the rhabdomere, a photoreceptor cell organelle specialized for phototransduction. Class II mutations have rough eyes, due to misorientation of the rhabdomeres and photoreceptor cell death. Transformation rescue of these phenotypes in transgenic flies bearing *calphotin* genomic DNA indicates that both classes of mutations are in the *calphotin* gene. Analysis of these mutations suggest that Calphotin plays important roles in both rhabdomere development and in photoreceptor cell survival.

THE photoreceptor cell is specialized for light absorption and for transmitting visual sensory information on for further information processing. As the photoreceptor cells of the Drosophila compound eye begin to differentiate morphologically, they express proteins common to many neuronal cells, the pan-neuronal proteins (*e.g.*, molecules recognized by monoclonal antibody (mAb) 22C10 and anti-horseradish peroxidase (ZIPURSKY *et al.* 1984; TOMLINSON and READY 1987). At about the same time they also begin to express Calphotin, a photoreceptor cell-specific calcium binding protein (BALLINGER *et al.* 1993). About 24 hr later in development, they express the photoreceptor cell-specific adhesion molecule Chaoptin (ZIPURSKY *et al.* 1984; REINKE *et al.* 1988; VAN VACTOR *et al.* 1988; KRANTZ and ZIPURSKY 1990). Mature Drosophila photoreceptor cells contain a number of other cell-specific proteins. Many of these function in the phototransduction process and are initially expressed during late pupal development (*e.g.*, opsin) (Zuker *et al.* 1985). Opsin expression begins about 5–6 days after the initial expression of Calphotin and the pan-neural proteins.

Photoreceptor cell development in Drosophila appears to involve a number of progressive cell-type restrictions. First a precursor cell becomes a neuron, expressing pan-neural proteins, then it becomes a photoreceptor neuron, and finally is restricted to a particular photoreceptor cell subclass through interactions with its neighboring cells (ZIPURSKY *et al.* 1984; TOMLINSON and READY 1987; RENFRANZ and BENZER 1989). The Calphotin protein is expressed very near the morphogenetic furrow in the developing eye imaginal disc, indicating that expression begins at about the same time as overt morphological differentiation of the photoreceptor cells. Calphotin is expressed in all photoreceptor cells at the same time as the pan-neural proteins, when cell fate decisions are being made. However, unlike the pan-neural proteins, Calphotin is highly specific to photoreceptor cells (BALLINGER *et al.* 1993; MARTIN *et al.* 1993).

Calphotin expression is not altered in *glass* mutant eye discs (BALLINGER *et al.* 1993). The Glass transcription factor is expressed in all cells of the eye imaginal disc, though its activity is restricted to photoreceptor cells. Glass activity appears to be specifically repressed in non-sensory cells to prevent them from adopting the photoreceptor cell fate (MOSES *et al.* 1989; MOSES and RUBIN 1991; ELLIS *et al.* 1993). The early expression of pan-neural antigens is not altered in *glass* mutant eye discs, while *glass* mutations block the later expression of other photoreceptor cell-specific antigens, including Chaoptin (ZIPURSKY *et al.* 1984; RENFRANZ and BENZER 1989; ELLIS *et al.* 1993). This feature distinguishes *calphotin* from other known photoreceptor cell-specific genes, and may indicate a role for the gene in the determinative process or in photoreceptor cell differentiation, perhaps by either directly or indirectly influencing the photoreceptor cell-specific activation of the Glass transcription factor.

To assess Calphotin function in photoreceptor cells, we describe the isolation and analysis of mutations in the

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gene. Mutations causing disruption of rhabdomere structure, misorientation of rhabdomeres and photoreceptor cell death can be rescued by genomic DNA containing the *calphotin* gene. Thus, Calphotin appears to play important roles in rhabdomere development and photoreceptor cell survival.

## MATERIALS AND METHODS

**Preparation of DNA and RNA:** Genomic DNA was isolated from adult flies according to the procedure of MEYEROWITZ *et al.* (1980). Total Drosophila head RNA was prepared according to the procedure of CHIRGWIN *et al.* (1979). Radioactive probes were prepared from the nearly full length 2.8-kb *calphotin* cDNA (BALLINGER *et al.* 1993) subcloned in Bluescript (Statagene). Plasmid DNA was purified using the Qiagene plasmid purification column and labeled by random priming (Boehringer Mannheim).

**Southern and Northern blots:** About 1 mg of genomic DNA was digested with different restriction endonucleases, separated on 1% agarose gels and processed for Southern blot analysis. For Northern analysis, about 30 mg total head RNA were run on 1% agarose gels in 2.2 M formaldehyde. The RNA samples were incubated for 15 min at 65° before loading. Gels were run at 3 V/cm for 5 hr and then processed for Northern blot analysis according to the procedure suggested by the manufacturer of the transfer membrane (Amersham Corp.).

**Mosaic analysis:** To determine the possible function of Calphotin, mosaic analysis was performed with both of the two small deficiencies that remove the *calphotin* gene [*Df(3R)P29* and *Df(3R)E-229*–see Figure 1]. Clones of cells homozygous for these deficiencies generated by γ-ray-induced mitotic recombination in *white* mutant flies heterozygous for the deficiency and for a chromosome carrying the $white^+$ gene inserted at 86C. Clones homozygous for the deficiency were recognized by their lack of pigment. Photoreceptor defects found in the mosaic clones resemble those shown for *calphotin* mutations (data not shown). These phenotypes were used to devise a screen for candidate *calphotin* mutations (see below). Similar mosaic analyses were performed with each preexisting lethal complementation group within *Df(3R)P29* (*ck10, ck12* and *ck13*, mutant alleles of *ck11* are no longer available).

**Isolation of candidate *calphotin* mutants:** Over 15,000 progeny of males mutagenized with γ-rays (3,000 to 4,000 Rads) and heterozygous for *Df(3R)P29* were screened for defects in eye structure using the deep pseudopupil technique (FRANCESCHINI and KIRSCHFELD 1971). This technique allows for the inspection of photoreceptor cells in live anesthetized flies. Moreover, the deep pseudopupil image depends critically on the relative orientation of photoreceptors in a set of seven adjacent unit eyes, and thus provides a very sensitive assay for the overall structure of the compound eye, as well as for the integrity of the rhabdomeres of individual photoreceptor cells (FRANCESCHINI and KIRSCHFELD 1971).

**Histology:** Preparation of samples for transmission electron microscopy was essentially as described in ROGGE *et al.* (1991). Thick sections (2 μm) of the Epon/Araldite embedded samples were stained with methylene blue and observed by light microscopy. For each genotype or staged sample, at least four heads were sectioned, and at least four thin sections per eye were analyzed by transmission electron microscopy.

**Isolation and transformation of the *calphotin* gene:** Two cosmid clones containing the *calphotin* gene (each with an insert of about 30 kb) were obtained by screening a cosmid library (a gift from J. W. TAMKUN) of Drosophila genomic DNA cloned in the NotBamNot-CoSpeR cosmid vector (PIRROTTA *et al.* 1985) using a *calphotin* cDNA probe (BALLINGER *et al.* 1993). An 8-kb *Xho*I-*Xho*I fragment from one of the two cosmid clones was subcloned into the pW8 vector (KLEMENZ *et al.* 1987). Transgenic flies were isolated by injecting these genomic clones into *y w ; Ki P[ry⁺ Δ2-3]* embryos, and selecting $w^+$ transformants.

**Behavior characterization:** Phototactic behavior of the mutant flies were determined using a countercurrent apparatus as described (BENZER 1967; BALLINGER and BENZER 1988).

## RESULTS

**Genetic mapping of the calphotin cDNA:** The *calphotin* cDNA maps by *in situ* hybridization to the border between 87A and 87B on the right arm of chromosome *3* (BALLINGER and BENZER 1989; BALLINGER *et al.* 1993; MARTIN *et al.* 1993). Because of its close proximity to the hsp 70 gene at 87A7, this region of the third chromosome has been well characterized genetically (GAUZ *et al.* 1981). A partial map of the region around 87A/B is shown in Figure 1. The deficiencies shown, as well as several others in the region, were checked for restriction fragment length polymorphisms (RFLPs) by genomic Southern analysis, and two contained RFLPs when probed with the *calphotin* cDNA (those with adjacent asterisks in Figure 1, data not shown). These data indicate that the cDNA lies at least partially within the region bounded by these two deficiencies. There are two small deficiencies that each remove this region, *Df(3R)P29* and *Df(3R)E-229* (the top two deficiencies depicted in Figure 1).

**Isolation of candidate *calphotin* mutations:** To assess the possible phenotype of mutations in the *calphotin* gene, genetic mosaic experiments were performed. Genetic mosaics can indicate the function in a particular cell type of a mutation that is lethal to the whole organism by creating patches of cells that are homozygous for the mutation in an otherwise heterozygous animal. Mosaic patches were made that were homozygous for each of the two small deficiencies [*Df(3R)P29 and Df(3R)E-229*], and the three lethal complementation groups they contain (see MATERIALS AND METHODS for details).

These mosaic experiments indicated that the two deficiencies tested remove a gene required for the normal structure of photoreceptor cells. Given that at least part of the *calphotin* gene is removed by these deficiencies, and that it is specifically expressed in photoreceptor cells, it was reasonable to assume that *calphotin* was this gene. None of the lethal complementation groups tested show significant phenotypic effects in mosaic photoreceptor cells, indicating that the gene within the deficiencies required for photoreceptor cell development is unlikely to correspond to an existing lethal complementation group (see MATERIALS AND METHODS for details).

The phenotype observed in mosaic patches suggested a possible screen for mutations in the gene responsible

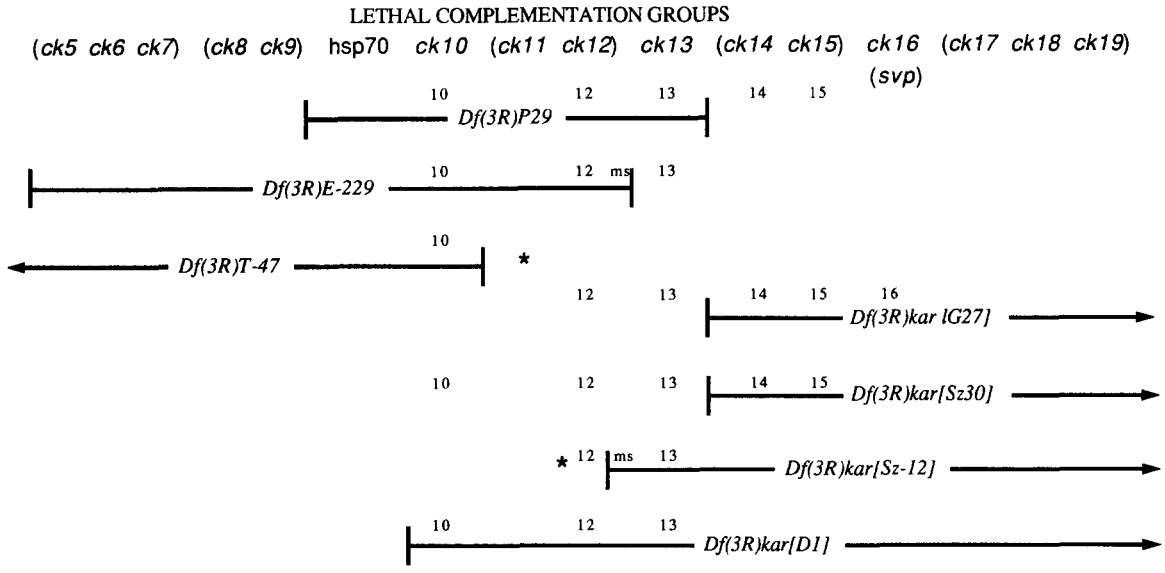


FIGURE 1.—Physical mapping of the *calphotin* gene. Lethal complementation groups (*ck*) and deficiencies around 87A/B on chromosome 3R are shown. Regions removed by the deficiencies are indicated by solid lines. The numbers above these lines indicate lethal mutations for which we repeated complementation tests. ms indicates male sterility associated with flies of the genotype $Df(3R)E\text{-}229/Df(3R)kar^{Sz\text{-}12}$. *svp* indicates that *ck16* corresponds to the *sevenup* gene (MLODZIK *et al.* 1990). Southern blots probed with the *calphotin* cDNA reveal restriction fragment length polymorphisms in $Df(3R)T\text{-}47$ and $Df(3R)kar^{Sz\text{-}12}$ (marked by asterisks, data not shown). *Df(3R)P29* and *Df(3R)E-229* at least partially uncover the *calphotin* gene (see text for details).

for the photoreceptor phenotype. The deep pseudopupil technique allows for the direct observation of photoreceptor cell structure in the Drosophila compound eye. Moreover, the deep pseudopupil image depends critically on the relative orientations of photoreceptors in a set of seven adjacent unit eyes, and thus provides a very sensitive assay for the overall structure of the compound eye, as well as for the integrity of the rhabdomeres of individual photoreceptor cells (FRANCESCHINI and KIRSCHFELD 1971).

Over 15,000 progeny of males mutagenized with γ-rays were screened for defects in eye structure using the deep pseudopupil technique. Nine mutations were isolated (in an isogenic $ry^{506}$ mutant background) that failed to complement *Df(3R)P29*. These mutations fall into two general classes (Table 1). Because of the transformation rescue by the *calphotin* gene of these mutations (see below), they are designated *cap* (*calphotin*) mutations. Both mutant classes have defects revealed by the deep pseudopupil technique, while class II mutations also have a semidominant roughened eye surface.

All of the mutations appear to fall into a single complementation group. That is, the disorganized deep pseudopupil phenotype of each class I allele was not complemented when heterozygous with either *Df(3R)P29*, *Df(3R)E229* or a class II allele. Thus, it is likely that the two classes of mutations represent different types of defects in a single gene. None of the lethal complementation groups within *Df(3R)P29* (*ck10*, *ck12* or *ck13*) appear to be allelic to *cap* mutations because all tested pairwise combinations show full complementation of recessive *cap* mutant phenotypes.

**TABLE 1**

**Isolation of *calphotin* mutations**

| Mutation | Mutant phenotype, class |
|---|---|
| $ry^{506}\ cap^{36}$ | II |
| $ry^{506}\ cap^{51}$ | I |
| $ry^{506}\ cap^{58}$ | I |
| $ry^{506}\ cap^{65}$ | II |
| $ry^{506}\ cap^{68}$ | I |
| $ry^{506}\ cap^{85}$ | II |
| $ry^{506}\ cap^{97}$ | II |
| $ry^{506}\ cap^{107}$ | I |
| $ry^{506}\ cap^{115}$ | I |

Mutations were isolated that fail to complement *Df(3R)P29*, a deletion that removes at least part of the *calphotin* gene (Figure 1). These mutations fall into two classes (see text for details). Both class I and class II mutants have rhabdomere defects. Class II mutants also have rough eyes.

Class I alleles appear to be hypomorphic, their phenotype is worse over a deficiency than when homozygous. Class II mutations may be semidominant antimorphic alleles of the *calphotin* gene because flies heterozygous for class II mutations and *Df(3R)P29* have less severely rough eyes than homozygous class II mutations, and extra copies of the normal *calphotin* gene restore a more normal phenotype. Flies homozygous for strong class II mutations ($ry^{506}\ cap^{65}/ry^{506}\ cap^{65}$) have very rough eyes and lack pigment in part of the eye, which is similar to the *glass* mutant phenotype (MOSES *et al.* 1989).

**Phenotypic analysis of candidate *calphotin* mutations:** Class I *cap* mutations have a partially penetrant defect in rhabdomere structure (compare Figure 2, A

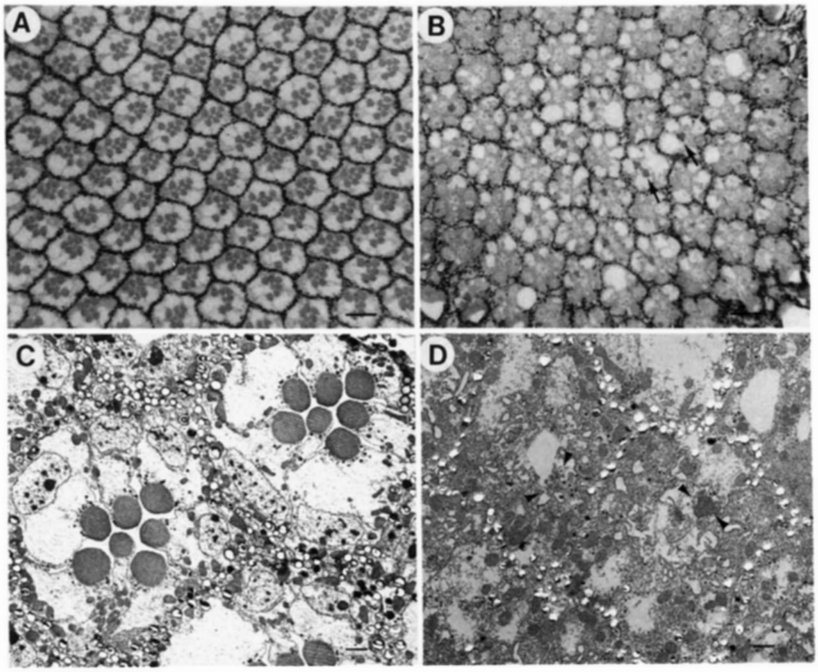


FIGURE 2.—Phenotypic characterization of *cap*$^{68}$. (A and B) Light microscopic analysis of thick tangential sections; (C and D) electron microscopic analysis of thin tangential sections. (A and C) Wild type (O-R). The ommatidia are arranged in a regular trapezoid. Rhabdomeres of seven photoreceptor cells are visible: six outer, and the inner R7 rhabdomere. The rhabdomere of the R8 photoreceptor lies more proximal in the retina, and is not visible in these sections. The photoreceptor cells are surrounded by a regular lattice of pigment cells. (B and D) *ry*$^{506}$*cap*$^{68}$*/Df(3R)P29*. Ninety-eight percent of the photoreceptor cells lack intact rhabdomeres (arrows in B point to cells that retain rhabdomeres). All photoreceptor cell bodies are present and morphologically normal except the disorganization of rhabdomere microvilli (arrowheads in D). The pigment cell lattice is unaffected. Scale bars = 10 μm (in A and B) and 1 μm (in C and D). Anterior to the top in A, to the right in B, C and D.

and B). The rhabdomere of an insect photoreceptor cell is a tightly packed array of microvilli, specialized for the phototransduction process (WADDINGTON and PERRY 1960; VAN VACTOR *et al.* 1988). The rhabdomeres of approximately 70% of photoreceptors are disorganized in flies homozygous for the class I *cap*$^{68}$ mutation. However, when *cap*$^{68}$ is heterozygous over *Df(3R)P29*, the rhabdomeres of about 98% of photoreceptor cells are disorganized. This indicates that *cap*$^{68}$ is a hypomorphic allele. The higher magnification micrographs include a pair of normal or mutant unit eyes (Figure 2, C and D). All eight photoreceptor cells are clearly visible in each *cap*$^{68}$ unit eye, though most of them have disorganized rhabdomeres. The normal tightly packed microvillar array is replaced by disorganized and unarrayed microvilli, a phenotype very similar to null mutations of the photoreceptor cell-specific adhesion molecule encoded by *chaoptin* (VAN VACTOR *et al.* 1988). These rhabdomere defects are not the result of light-induced rhabdomere degeneration because *cap*$^{68}$ mutant flies raised in dark also have disorganized rhabdomeres (data not shown).

Micrographs of compound eyes from flies hemizygous for one of the class II semidominant rough mutations [*ry*$^{506}$*cap*$^{65}$*/Df(3R)P29*] are shown in Figure 3. Class II *cap* mutations are plieotropic. Some photoreceptor cells entirely lack rhabdomeres, while others project their rhabdomeres toward the pigment cells that surround the unit eye rather than toward its center (the arrowheads in Figure 3D point to two photoreceptor cells with misoriented rhabdomeres). Other photoreceptor cells have more than one rhabdomere with at least one of them oriented in the wrong direction. This type of rhabdomere misorientation has not been previously reported and may represent a relatively subtle developmental phenotype in which the photoreceptor cell is unable to orient itself within the developing unit eye, resulting in a misdirection of its rhabdomere. Alternatively, the process of directed membrane trafficking during rhabdomere development or maintenance may be disrupted.

In addition, some unit eyes of class II mutants have fewer than 8 photoreceptor cells. In sections from pupal eyes, most ommatidia have the correct photoreceptor cell number, but some photoreceptor cells appear electron dense, with characteristics of apoptotic cell death (WYLLIE *et al.* 1980; KERR *et al.* 1987; CLARKE 1990). Such dying photoreceptor cells were observed in the eyes of late pupae and adults of class II mutant flies (Figures 3 and 4). Homozygous *ry*$^{506}$*cap*$^{65}$ mutant eye discs are nearly normal. About one in six homozygous mutant discs have a single area of apparent pattern disruption (monitored by staining with mAb 22C10). This pattern disruption is often associated with a cell or cells which appear to be dying (highly refractive cells observed by differential interference contrast optics–data not shown). Thus, while rare early defects are seen in eye discs, most pupal ommatidia have the normal cellular composition. However, pupal photoreceptor cells in *cap*$^{65}$ homozygotes show all defects associated with adult mutant flies, in approximately the same proportions.

The phenotypes of severe class II *calphotin* mutants indicate that impairment of *calphotin* function can cause defects in rhabdomere orientation and photoreceptor cell death. The two classes of mutant *calphotin* alleles may represent defects in two separable functions of the *calphotin* gene. For example, the rhabdomere defects of class I mutations may represent a structural requirement for *calphotin* function in rhabdomeres,

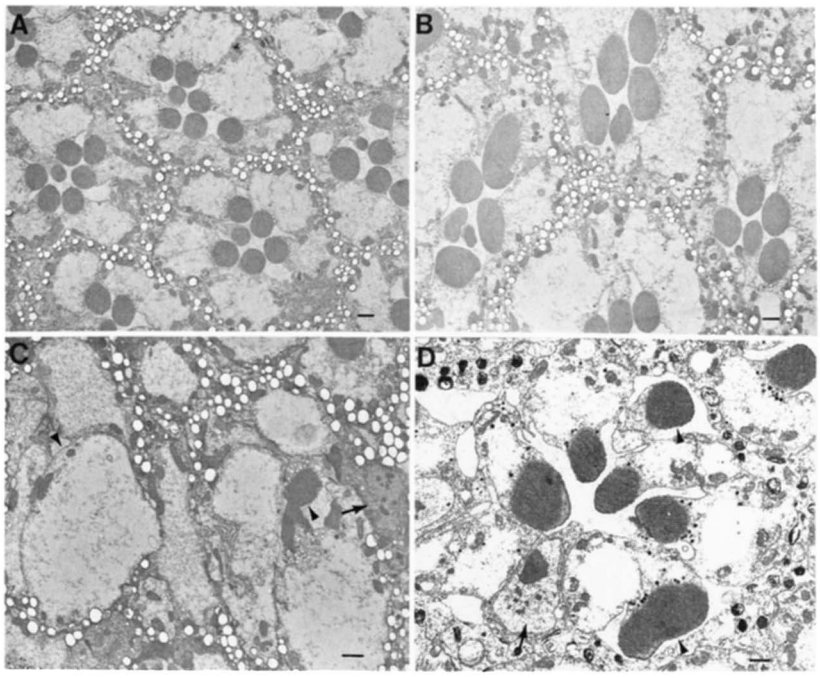


FIGURE 3.—Phenotypic characterization of $cap^{65}$. Tangential sections of eyes from flies carrying allele $cap^{65}$ heterozygous with chromosome deficiency *Df(3R)P29* are shown. Representatives of each of the three phenotypic categories are shown: (A) slightly rough, (B) moderately rough and (C and D) severely rough. Some rhabdomeres are missing, deformed or misoriented (arrowheads). Some photoreceptor cells are dying (arrows). The proportion of photoreceptor cells with defects increases with the severity of the mutant phenotype. Scale bars = 1 μm. Anterior to the bottom in A, to the right in B, C and D.

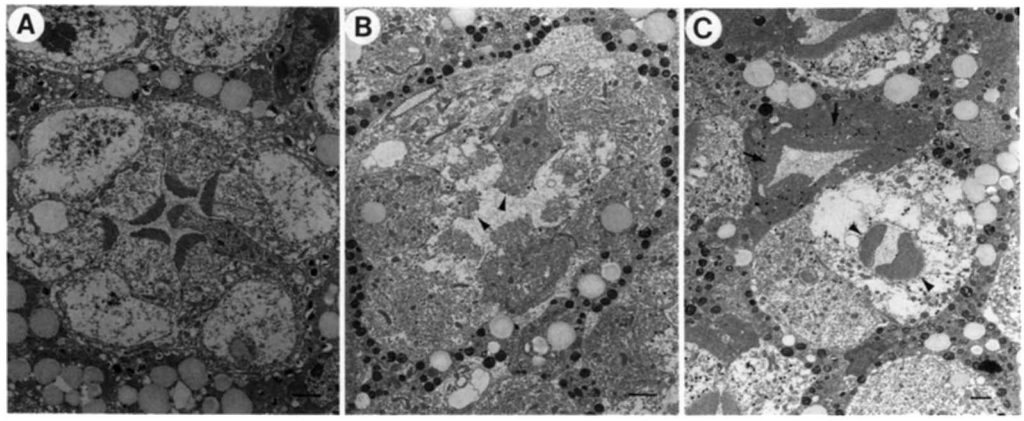


FIGURE 4.—Rhabdomere and photoreceptor cell defects during early rhabdomere development. Pupal eyes around 80 hr after pupation are analyzed. (A) Eye of the parental strain $ry^{506}$. All of the microvilli are aligned and tightly packed together. (B) Eye of the $ry^{506}cap^{68}/Df(3R)P29$ mutant, the microvilli fail to align properly, and the density of microvilli is reduced (arrowheads). (C) Eye of the $ry^{506}cap^{65}/Df(3R)P29$ mutant. Although microvilli are tightly packed together, the shape and orientation of the developing rhabdomeres are abnormal. Some photoreceptor cells have more than one rhabdomere with abnormal morphology (arrowheads). Some photoreceptor cells are electron dense and show morphological characteristics of apoptotic cell death (arrows). Scale bars = 1 μm. Anterior to the bottom.

and class II mutant defects may represent more severe defects in *calphotin* function which can lead to cell death.

The defects in mosaic patches homozygous for *Df(3R)P29* resemble those of hemizygotes for the class I allele, $cap^{68}$ (data not shown). Thus, disorganization of rhabdomere structure appears to be the null phenotype of *calphotin*. The phenotype of class II mutants appears antimorphic (see DISCUSSION).

**Defects in developing photoreceptors:** Rhabdomere development begins at about 48 hr post-puparium formation (WADDINGTON and PERRY 1960; WOLFF and READY 1993). The rhabdomere structure, though incomplete, can be clearly seen around 78–87 hr post-puparium formation (Figure 4). We traced the developmental onset of *calphotin* mutant defects by sectioning pupal eyes prior to the completion of rhabdomere development.

Developing pupal rhabdomeres from class I $cap^{68}$ mutants are markedly disorganized (Figure 4B). The membrane microvilli which form the rhabdomere are irregular in length and alignment. These defects are very similar to those caused by *chaoptin* mutations (VAN VACTOR *et al.* 1988). Pupal eye sections of the $cap^{65}$ mutants exhibit properly aligned microvilli. However,

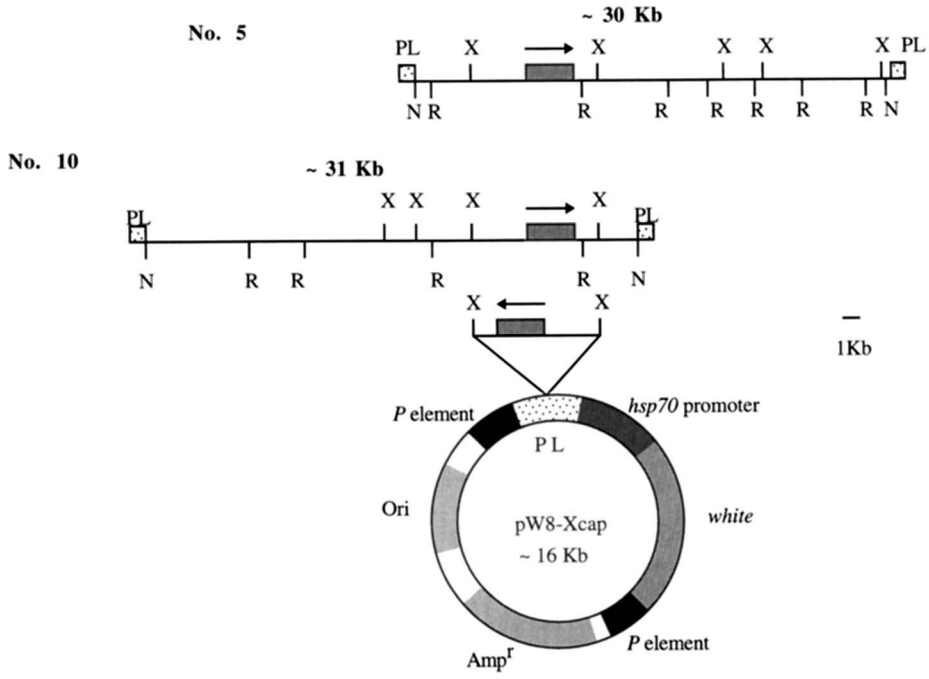


FIGURE 5.—Genomic clones of the *calphotin* gene. Two cosmid clones containing genomic DNA around the *calphotin* gene are diagrammed. An 8-kb *Xho*I-*Xho*I genomic fragment contained by both cosmid clones has both the *cis*-elements sufficient for photoreceptor cell-specific expression of a *lacZ* reporter gene (N. XUE and D. BALLINGER, manuscript in preparation), and the transcribed region of the *calphotin* gene. This fragment was subcloned into the transformation vector pW8. The resulting pW8-Xcap and the two cosmid clones were used to generate transgenic flies.

both the photoreceptor cells and their rhabdomeres have abnormal shapes (Figure 4C). Thus, one *calphotin* function, defective in $cap^{68}$ mutants, is required for the orderly alignment of the rhabdomeric microvilli from the beginning of rhabdomere development. Another aspect of *calphotin* function appears to be involved in the organization of the photoreceptor cell as a whole. The latter function, altered by $cap^{65}$ mutations, can result in defective morphology of both photoreceptor cells and their rhabdomeres, and in photoreceptor cell death.

**Behavioral characterization:** Phototaxis was measured in a countercurrent apparatus (BENZER 1967). The test measures phototactic preference during an agitated-state escape response. The visible light used in the test was strong enough to dominate other stimuli in the complex test environment (HEISENBERG and GOTZ 1975). When given six consecutive trials of phototaxis toward visible light, normal flies and $ry^{506}cap^{68}/Df(3R)P29$ mutant flies choose light over darkness an average of 5 or 6 times, while $ry^{506}cap^{65}/Df(3R)P29$ mutant flies choose light an average of 4 or 5 times. Thus, the pathway triggered by light and leading to phototaxis is capable of functioning, even though the light reception organelle, the rhabdomere is disorganized in $cap^{68}$ and $cap^{65}$ mutants.

**Transformation rescue of *calphotin* mutations:** To prove that the mutations we isolated are mutations in the *calphotin* gene, we carried out transformation rescue experiments. Two cosmid genomic clones containing *calphotin* were isolated (Figure 5). An 8-kb *Xho*I-*Xho*I fragment contained in both cosmids was subcloned into the pW8 vector (KLEMENZ *et al.* 1987), and the resulting plasmid is called pW8-cap (Figure 5). All three constructs were used to generate transgenic flies (see MATERIALS AND METHODS for details). All three constructs can rescue both class I and class II *calphotin* mutations (Figure 6). Some transgenic lines did not rescue as well as others, presumably due to chromosomal position effects of the insertion site on the level of *calphotin* gene expression. In addition, the degree of rescue depended on the copy number of the transgene. One of the pW8-cap transgenic lines, X8-1 was most effective in rescuing *calphotin* mutations (Table 2 and Figure 7), more than 95% of the ommatidia in rescued $cap^{68}$ and $cap^{65}$ flies had normal photoreceptors. The lack of complete rescue may indicate that the transgene is not expressed at normal levels. It may also be a reflection of the semidominant nature of $cap^{65}$. Additionally, $cap^{68}$ heterozygous flies have a small number of defective ommatidia. Thus, the X8-1 transgene may be expressed at fairly normal levels. Northern blot analysis of head RNA indicates that the 8-kb *Xho*I-*Xho*I fragment contains only the *calphotin* gene (data not shown). Thus, both class I and class II mutations are mutations in the *calphotin* gene.

## DISCUSSION

The *calphotin* gene was initially identified by the photoreceptor cell-specific staining of monoclonal antibodies (BALLINGER *et al.* 1993; MARTIN *et al.* 1993). Calphotin protein expression begins right after the morphogenetic furrow, at about the same time as overt morphological differentiation of the photoreceptor cells, and about 24 hr earlier than the earliest photoreceptor cell-specific protein that has been previously characterized, Chaoptin (VAN VACTOR *et al.* 1988). In a previous report (BALLINGER *et al.* 1993), we studied the gene structure and biochemical properties of the *calphotin* gene. Here

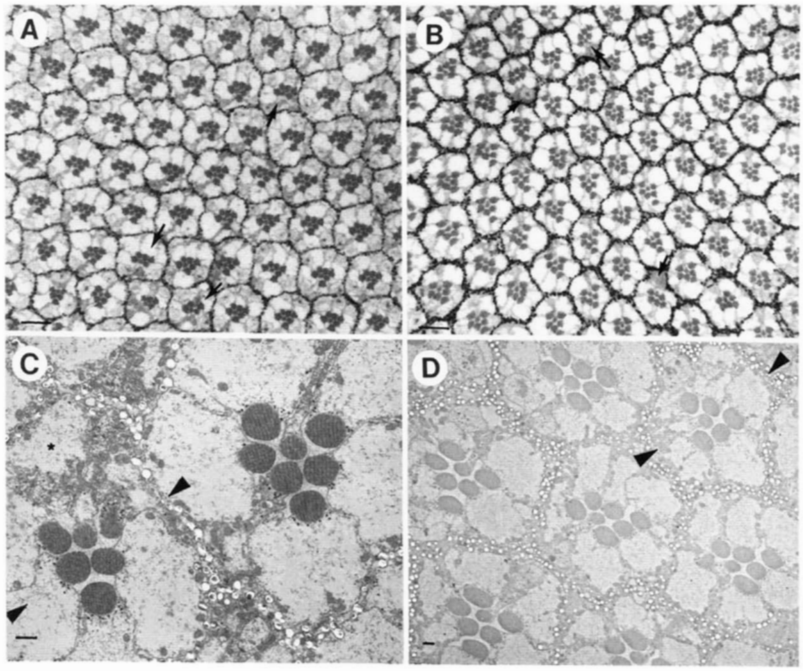


FIGURE 6.—A normal *calphotin* gene can rescue the mutant phenotypes of $cap^{68}$ and $cap^{65}$. (A and B) Light microscopic analysis of thick sections. (C and D) Electron micrographic analysis of thin sections. Arrowheads indicate abnormal unit eyes; in C one cell has no rhabdomere (*), in D one photoreceptor cell is missing. (A and C) Allele $cap^{68}$, heterozygous with chromosome deficiency *Df(3R)P29*, with two copies of the *P[cap⁺, w⁺]*X-8 transgene, made with construct pW8-Xcap (Figure 5). 95% of the photoreceptor cells have normal rhabdomeres. (B and D) Allele $cap^{65}$, heterozygous with chromosome deficiency *Df(3R)P29*, with one copy of the *P[cap⁺, w⁺]*X-8 transgene. Almost all flies have slightly rough eyes (see legend of Figure 2), and 95% of the unit eyes are normal. Scale bars = 10 μm (in A and B), 1 μm (in C and D). Anterior to the lower right in A and D, to the right in B and C.

**TABLE 2**

**Wild-type *calphotin* transgene partially rescues the deep pseudo pupil defects of the class I mutant, $cap^{68}$**

| | Transgenic line | | | | | |
|---|---|---|---|---|---|---|
| | None[a] | X8-1[b] | X8-1[b] | 5.1-2(2)[c] | 5.1-3(3)[d] | 10.200-4(2)[e] |
| Transgene copies | 0 | 2 | 1 | 1 | 1 | 1 |
| Percent rescue[f] | 0 | 95% (19/20) | 76.3% (45/59) | 85.9% (55/64) | 60% (27/45) | 85.7% (30/35) |

[a] No transgene, flies of the genotype $ry^{506}cap^{68}/Df(3R)P29$. Several hundred flies have been observed, none with a normal deep pseudopupil. This mutant genotype was used in all studies included in this table.
[b] X8-1, a transgenic fly line generated with pW8-Xcap (Figure 5).
[c,d] Two transgenic fly lines generated with cosmid no. 5 (Figure 5).
[e] A transgenic fly line generated with cosmid no. 10 (Figure 5).
[f] Percent rescue indicates the percentage of flies which have normal looking deep pseudo pupil images.

we describe the isolation of mutations in the *calphotin* gene, and its function in rhabdomere development and photoreceptor cell survival.

Calphotin has a number of structural domains, including an N-terminal domain involved in calcium binding, and an C-terminal leucine zipper that may be important for interactions with other proteins (BALLINGER *et al.* 1993; MARTIN *et al.* 1993). In addition, the protein is very hydrophobic and proline rich (overall hydrophobic content of 67%, and proline content of 21%), and has a 200-amino acid stretch at its amino terminus that is 82% hydrophobic. Calphotin contains several regions of continuous hydrophobic character sufficiently long to permit membrane spanning; however, each of them is interrupted by two or more proline residues which may prevent their use as such (BALLINGER *et al.* 1993; MARTIN *et al.* 1993). Calphotin is localized within the photoreceptor cell in a poorly defined structure that underlies the rhabdomere and may be bounded by membranes (MARTIN *et al.* 1993). However, we could not detect any specific membrane association of the Calphotin protein when it was expressed in Drosophila tissue culture cells or in rabbit reticulocyte lysates in the presence of dog pancreas membranes (our unpublished data).

We isolated several mutations in the *calphotin* gene, as indicated by their rescue in *calphotin* transgenic flies. The mutations fall into two phenotypic classes. Recessive class I ($cap^{68}$) mutations cause deformed rhabdomeres while semidominant class II ($cap^{65}$) mutations result in misoriented rhabdomeres and photoreceptor cell death. Flies hemizygous for the class I mutations (class I mutations over deficiency) have stronger phenotypes than homozygous mutant flies, thus these alleles represent partial loss of function *calphotin* mutations. Mosaic patches homozygous for *Df(3R)P29* resemble $ry^{506}cap^{68}/Df(3R)P29$ eyes, indicating that the severe class I mutant phenotype is likely to be the null phenotype of *calphotin*. Class II mutations are antimorphic because they have a more severe phenotype as homozygotes than hemizygotes, and additional wild-type *calphotin* copies can restore them to a more normal pheno-

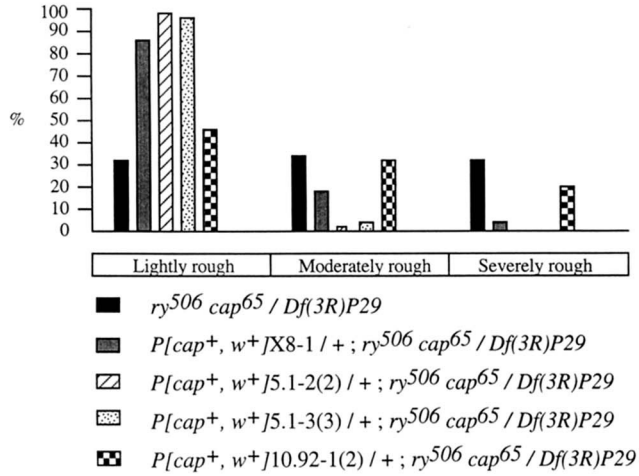


FIGURE 7.—Rescue of the class II *calphotin* mutation, $cap^{65}$. For comparison, flies were separated into three classes based on overall eye roughness (see legend to Figure 3). Flies hemizygous for the $cap^{65}$ mutation [$ry^{506}cap^{65}/Df(3R)P29$] are approximately equally divided between these three categories. Transgenic flies carrying two of the three genomic clones and also hemizygous for $cap^{65}$ are rescued; most of the eyes are only weakly rough, indicating that the roughness is due to a mutation in the *calphotin* gene.

type. Perhaps the class II mutations alter the interaction of Calphotin with another protein. One possible interacting protein co-immunopurifies with Calphotin (BALLINGER *et al.* 1993). Western blot analysis shows that the functional abnormality of both classes of *calphotin* mutations is not due to detectable changes in the level of expression or gel mobility of Calphotin (our unpublished data).

Light activation of rhodopsin, the rhabdomere-localized photopigment can cause retinal degeneration when a $Ca^{2+}$ binding serine/threonine protein phosphatase (rdgC) is mutant (STEELE *et al.* 1992). Since Calphotin can bind $Ca^{2+}$ *in vitro* (BALLINGER *et al.* 1993; MARTIN *et al.* 1993), and light-dependent fluctuations in $Ca^{2+}$ concentrations in part mediate phototransduction (SMITH *et al.* 1991), we tested the possibility that the *calphotin* mutant phenotype was light-dependent. However, $cap^{68}$ had a similar phenotype whether reared in light or dark, indicating that rhabdomere disruptions were not caused by light-induced degeneration. Indeed, the rhabdomeres of $cap^{68}$ mutant flies are abnormal when they begin to develop at the mid pupal stage (Figure 4). Thus, it appears that the rhabdomere defects of class I *cap* mutants reflect a developmental function of the *calphotin* gene.

The developmental function of Calphotin suggests a possible structural role, or a defect in the process of membrane trafficking during rhabdomere development. Rhabdomere development begins during mid pupal stage, which is initially characterized by disorganized membrane ruffling (WADDINGTON and PERRY 1960). Rhabdomeres of wild-type flies are constructed as closely packed and hexagonally arranged stacks of microvilli. Because the rhabdomere of each photoreceptor cell has its own characteristic shape and direction, rhabdomere growth requires directed, highly polarized membrane trafficking (WOLFF and READY 1993). Calphotin may play a critical role in regulating directed membrane trafficking during rhabdomere development. This role may involve specific protein-protein or protein-membrane interactions via the leucine zipper and hydrophobic domains of Calphotin, or its modulation of intracellular calcium level.

Mutations in the *calphotin* gene affect both the regularity and the direction of rhabdomere growth. Developing $cap^{68}$ rhabdomeres have fewer microvilli and they fail to align properly. This could result from an insufficient level of membrane trafficking in class I *calphotin* mutant flies. This interpretation is in consistent with the genetic data that $cap^{68}$ is a partial loss of function allele of *calphotin*. Photoreceptor cells with rhabdomeres pointing toward the pigment cells instead of toward the center of the unit eye and cells with two rhabdomeres pointing in different directions are seen in semidominant class II *calphotin* mutant flies. These defects may result from a misdirection of membrane trafficking in $cap^{65}$ mutant. The incomplete penetrance of both $cap^{68}$ and $cap^{65}$ mutants may reflect a partial redundancy of function. Molecular characterization of *calphotin* mutations may shed more light on possible Calphotin functions in the membrane transport process.

In class II *calphotin* mutant flies, some photoreceptor cells undergo cell death. This could result from the severely impaired development or function of the dying photoreceptor cells. The observed rhabdomere abnormality may be only one visible indication of a more general impairment of photoreceptor cell function. Alternatively, the class II mutations appear to be antimorphic, and may interfere with the function of another protein that interacts with Calphotin. If so, cell death may reflect the normal function of this interacting protein.

While rare dying cells are seen in $ry^{506}$ $cap^{65}$ homozygous discs, the bulk of *calphotin* mutant phenotypes are not manifest until the beginning of rhabdomere development. This is about 4–5 days after the onset of Calphotin expression, This phenotypic delay is similar to *chaoptin* mutations (VAN VACTOR *et al.* 1988). It may represent fortuitous early expression of Calphotin or functional redundancy at early developmental stages.

Calphotin may also alter photoreceptor development indirectly by modulating Glass protein activity. Calphotin expression is not altered by *glass* mutations (BALLINGER *et al.* 1993). Glass is a zinc-finger containing transcription factor whose activity is restricted to the photoreceptor cells (ELLIS *et al.* 1993). It is possible that some Glass target genes may function in the membrane trafficking pathway. Glass is highly modified (ELLIS *et al.* 1993) suggesting that its activity could be regulated by post-translational modification in response to differentiation signals. The intriguing early expression of Calphotin, 24 hr prior to Chaoptin expression and 3–4 days

prior to the development of rhabdomere, may suggest the involvement of Calphotin in the modification of Glass, or some other regulatory protein, at an early stage of photoreceptor cell development. In *glass* mutants, when the photoreceptors are prevented from attaining their photoreceptor identity, they die (MOSES *et al.* 1989). Indeed, some ommatidia in the severe class II $cap^{65}$ mutant appear similar to those in *glass* mutants. Future genetic and biochemical dissection of the pathway which leads to the modification of Glass, and an analysis of Glass protein in *calphotin* mutants may indicate a link between Calphotin and Glass modification or function.

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