Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1531–1535, February 1993
Neurobiology

Calphotin: A *Drosophila* photoreceptor cell calcium-binding protein

(calcium compartment/invertebrate eye/monoclonal antibody/leucine zipper)

JOEL H. MARTIN*, SEYMOUR BENZER†, MARIA RUDNICKA*, AND CAROL A. MILLER*

*Department of Pathology, University of Southern California School of Medicine, Los Angeles, CA 90033; and †Department of Biology, California Institute of Technology, Pasadena, CA 91125

Contributed by Seymour Benzer, October 22, 1992

ABSTRACT **Monoclonal antibody 23E9 identifies a calcium-binding protein, calphotin, in photoreceptor cells of the *Drosophila melanogaster* compound eyes and ocelli. The antigen is restricted to a defined cytoplasmic region; it is not present in the rhabdomeres, nuclei, mitochondria, or rough endoplasmic reticulum. A corresponding cDNA recognizes a 3-kb mRNA with retinal specificity similar to the antigen and maps to band 86E/F–87A/B on chromosome 3. An open reading frame of 2595 bp encodes an estimated 85-kDa protein of unusual amino acid composition, with >50% proline, alanine, and valine and very few basic residues. The C-terminal segment contains a leucine zipper motif uninterrupted by prolines. We found no significant similarities with the GenBank or National Biomedical Resource Foundation data bases. The location of the protein within a distinct cytoplasmic region suggests that it might function as a calcium-sequestering "sponge" to regulate the amount of free cytoplasmic calcium.**

The intracellular level of free Ca^{2+} is crucial to many developmental and metabolic functions and, in particular, signal transduction. Intracellular Ca^{2+}-binding proteins fall mainly into two groups—the annexins and the EF-hand homolog family (reviewed in ref. 1). Originally described in vertebrates, several homologs have been found in *Drosophila* (2, 3). Drosocalbin, a fly homolog of calbindin-D28 and calretinin, is expressed in many neurons in the *Drosophila* brain, as well as in two small thoracic muscles (K. Zinzmaier, personal communication).

In photoreceptor cells of *Drosophila* and other invertebrates, light induces release of calcium from undefined intracellular stores via the action of inositol 1,4,5-trisphosphate (IP_3), leading to light-activated depolarization as well as light adaptation (reviewed in refs. 4 and 5). The trp (transient receptor potential) protein (6) is thought to be involved in the release of Ca^{2+} from small cisternae at the base of the rhabdomere (J. Pollock, personal communication). Walz (7) has shown that specific parts of the smooth endoplasmic reticulum in the retina of the blowfly *Calliphora* can sequester Ca^{2+}. Baumann and Walz (8) described calcium-sequestering cisternae in the honey bee compound eye. In vertebrate nonmuscle cells, some Ca^{2+} may be stored in similar organelles called calcisomes (9), which may be intracellular targets of IP_3.

The protein we describe is different from those previously reported. It is identified by monoclonal antibody (mAb) 23E9 in the photoreceptor cell cytoplasm within a structure that extends along the length of the cell, parallel to but separated from the rhabdomere, and into the axon. As described in an accompanying paper, Ballinger *et al.* (10) using a different mAb, 72H5 (11), have independently cloned what is clearly the same *Drosophila* gene; both have the same chromosomal location and only minor differences in sequence.‡

MATERIALS AND METHODS

Antibody and *Drosophila*. mAb 23E9 is an IgG produced by a hybridoma obtained using *Drosophila* head homogenates as immunogen (12). *Drosophila melanogaster* wild-type (Canton S), white-eyed mutants, and eyes absent (*eya*) mutant (13) were raised at 25°C on cornmeal medium. Cryostat sections (10 μm) were stained with mAb 23E9 as described (12).

Electron Microscopy. Fly heads were fixed in 1% glutaraldehyde/1% paraformaldehyde in phosphate-buffered saline (PBS) (0.15 M NaCl/27 mM KCl/12 mM NaH_2PO_4, pH 7.2), embedded in LR White (Polyscience), sectioned at 0.7 μm, and stained on grids using mAb 23E9 at a 1:1 dilution of hybridoma supernatant with PBS. After several washes with PBS and 0.05% Tween 20, they were incubated with goat anti-mouse IgG conjugated with either horseradish peroxidase or 10-nm gold particles. Diaminobenzidine was used to develop the horseradish peroxidase. Sections were rinsed and postfixed in 2% glutaraldehyde/1% osmium tetroxide. In a second method, samples were prepared by Kent McDonald and Mary Morphew (University of Colorado, Boulder) by quick-freezing under high pressure, freeze substitution, and embedding in Lowacryl (14). For detecting intracellular calcium, an oxalate precipitation (15) method was used.

cDNA Libraries. Poly(A)$^+$ mRNA was prepared as described (16). Fly head cDNA libraries were constructed in Lambda ZAP II [oligo(dT) primed] and pcDNA II (random primed) (16). The λ expression library was screened with mAb 23E9 (16). A clone containing a 1550-bp insert was isolated and used to probe for longer clones. Clones corresponding to sequences at the 5′ end of the mRNA were isolated from the random-primed library by colony filter hybridization (16).

***In Situ* Hybridization.** This was as described by Hafen and Levine (17).

Northern Blots. These were done with ^{32}P-labeled 23E9 cDNA and washed at high stringency (16).

Sequence Analysis. Both cDNA strands were sequenced by dideoxynucleotide chain termination (18) using T7 DNA polymerase (United States Biochemical). Analysis used the Genetics Computer Group programs (19). The Swiss-Prot and GenBank Genpept data bases were searched for homologies by using the program FASTA (20).

Immunoblots. Fly heads were homogenized at 1 g per 5 ml of buffer [5 mM Tris·HCl, pH 7.5/2.5 mM KCl/5 mM $MgCl_2$/protease inhibitors as follows: 1 mM EDTA, 1 mM leupeptin, aprotinin (0.5 μg/ml), pepstatin (5 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 μM antipain] and centrifuged at 100,000 × *g* for 30 min. The supernatant solution was solubilized with SDS loading buffer, boiled 5 min, and applied to a 3–12% gradient SDS/polyacrylamide gel. A fusion protein was made using the pMAL-cRI expression vector (New England Biolabs) that contained the full

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: mAb, monoclonal antibody.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L02111).

polypeptide except for 100 N-terminal amino acids. The fusion protein was expressed in *Escherichia coli* and a crude cell lysate run with SDS/PAGE. The proteins were transferred to nitrocellulose, reacted with mAb 23E9 at 4°C overnight, and visualized by horseradish peroxidase-conjugated anti-mouse IgG.

Calcium-Binding Assays. As described by Maruyama *et al.* (21), the membrane was soaked in buffer (60 mM KCl/5 mM $MgCl_2$/10 mM imidazole·HCl, pH 6.8) for 1 hr, incubated in the same buffer with $^{45}CaCl_2$ (New England Nuclear; 25 mCi/mg; 1 Ci = 37 GBq) at 1 μCi/ml for 10 min, rinsed with 50% ethanol for 5 min, and dried. Autoradiographs were on Amersham Hyperfilm MP x-ray film exposed for 24–48 hr.

RESULTS

Cellular Localization of Calphotin. Fig. 1*A* shows the distribution of 23E9 antigen as seen by immunofluorescence. The retina of the compound eye stained intensely, with lobulated streaks extending along the lengths of the photoreceptor cells and their axons entering the optic lobes. The photoreceptor layers of the ocelli as well were strongly positive (Fig. 1*B*). The larval photoreceptor (Bolwig's) organ was negative (data not shown).

With conventional processing for electron microscopy using glutaraldehyde and paraformaldehyde, one sees a region that is relatively low in electron density, occupying part of the cell proximal to the rhabdomere (Fig. 2*A*). The immunoperoxidase-positive antigen was in a corresponding region (Fig. 2*B*). In longitudinal sections parallel to the rhabdomere, a band of immunoreactivity could be seen along the length of the cell (Fig. 2*C*). All the photoreceptor cells of the compound eye, including R7 and R8, were positively stained. No antigen was detected in the rhabdomeres, the cell nuclei, the mitochondria, or the intraretinular space central to the ommatidium. Cone and pigment cells were likewise negative. The distribution was very different from that seen with antibody specific for the trp antigen, which stains a thin band straddling the base of each rhabdomere (J. Pollock, personal communication).

Particularly sharp localization was seen by staining samples prepared by rapid freezing under high pressure, followed by freeze substitution, provided by Kent McDonald and Mary Morphew (University of Colorado High Voltage Electromicroscopy Laboratory) (13). Fig. 3 shows a section of the eye stained with immunogold. The antigen was not associated with multivesicular bodies or the rough endoplasmic reticulum but was clearly confined to a circumscribed region slightly less electron dense than the surrounding cytoplasm. The antigen-positive region was relatively devoid of membranous structures, although they are visible elsewhere in the cell. Under the conditions used, no membrane bounding the region was evident.

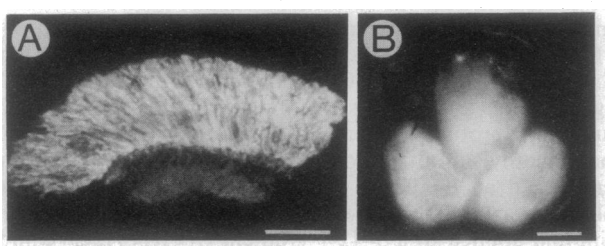

FIG. 1. Calphotin in the *Drosophila* compound eye and ocelli. Immunofluorescence labeling with mAb 23E9 using white-eyed flies. (*A*) Cryosection perpendicular to the compound eye surface. Note labeled axons extending into the lamina. (*B*) Whole mount of the three ocelli. Labeled photoreceptor layer is seen as a bright arc under each lens. (Bars = 50 μm.)

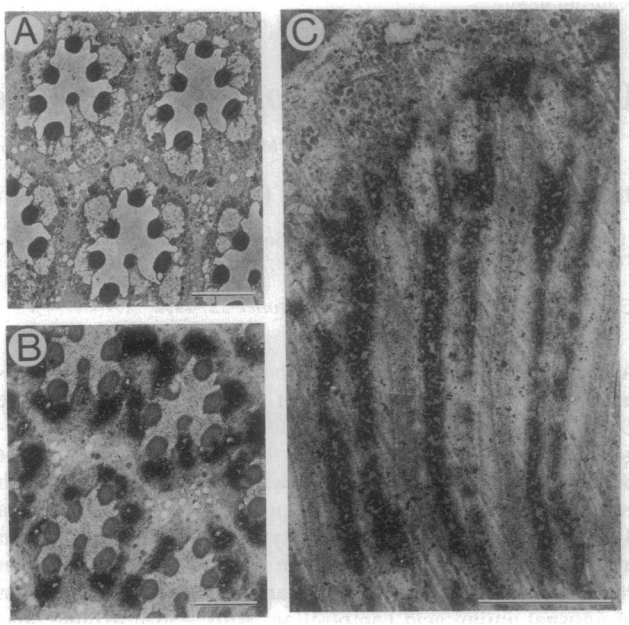

FIG. 2. Ultrastructural immunolocalization of calphotin *Drosophila* retina. (*A*) Section transverse to the axis of the photoreceptor cells; control without primary antibody. (*B*) Stained with mAb 23E9 and horseradish peroxidase-conjugated goat anti-mouse IgG. Immunolabel is localized to the photoreceptor cytoplasm peripheral to the rhabdomere. (*C*) Stained longitudinal section. Antigen extends along the length of the cell. (Bars = 10 μm.)

Calphotin cDNA. Screening the λ expression library with mAb 23E9 yielded a 1.6-kb cDNA clone with a long open reading frame. This cDNA was used to screen the Lambda ZAP II library, yielding a 2.3-kb clone with 740 bp more of 5′ sequence. An 800-bp fragment from the latter was used to probe a random-primed library, yielding an additional 550 bp upstream.

The complete cDNA contained a 166-bp 5′ untranslated region, a 2595-bp open reading frame encoding a protein of

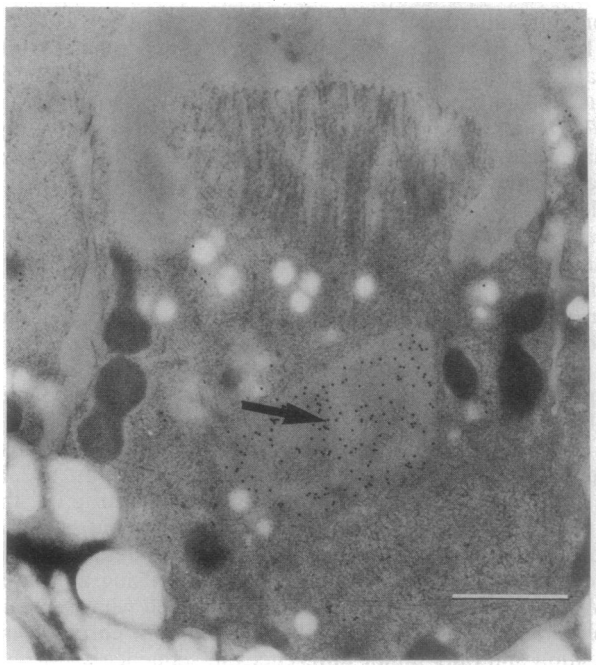

FIG. 3. Calphotin visualized by gold particles coupled to mAb 23E9. Specimen was prepared by rapid, high-pressure freezing and freeze substitution. Gold particles are localized to a circumscribed hypodense cytoplasmic region (arrow). (Bar = 1 μm.)

```
                                                                                                 GGCAAGTCGGTCGCT GTTTAAAACTATTCAACAAATTAAATTGTT 46
    GTCTTCTGACCATGAAAAGCTGGCCATGAA ACATTTTTGCAACTAAGCATTGGACAAGGC ACAATCCGCCCAAAAGGAAAAAACGGATTA ACTTTTAGTGAAAGTGGGCAAAGGATAGAC 166
    ATGGAACCAGGAACCATTCCATCGCCTGTT TCAGCACCAGTTGCTGCGCCTGTGACGCCA TCAGCAGTGGCTGCCCCTGTCCAAGTAGTA AGTCCTGCGGCAGTGGCTCCCGCACCAGCG 286
  1 M  E  P  G  T  I  P  S  P  V   S  A  P  V  A  A  P  V  T  P   S  A  V  A  A  P  V  Q  V  V   S  P  A  A  V  A  P  A  P  A
    GCACCGATTGCAGTCACTCCAGTTGCTCCA CCGCCCACCCTTGCAAGTGTGCAACCGGCA ACAGTTACTATTCCAGCTCCAGCTCCAATT GCTGCCGCATCCGTCACTCCTGTCGCATCT 406
 41 A  P  I  A  V  T  P  V  A  P   P  P  T  L  A  S  V  Q  P  A   T  V  T  I  P  A  P  A  P  I   A  A  A  S  V  T  P  V  A  S
    GTGGCGCCTCCAGTTGTTGCAGCTCCAACT CCGCCGGCAGCAAGTCCAGTTTCAACACCC GTTGCTGTTGCTCAGATTCCGGTCGCTGTT TCAGCACCAGTGGCACCTCCCGTTGCTGCA 526
 81 V  A  P  P  V  V  A  A  P  T   P  P  A  A  S  P  V  S  T  P   V  A  V  A  Q  I  P  V  A  V   S  A  P  V  A  P  P  V  A  A
    ACACCCACTCCTGTTGTCCAAATTCCTGTA GCTGCGCCTGTGATAGCGACACCACCCGTT GCTGCCTCTGCCCCCACTCCGGCTGCCGTG ACTCCCGTTATTTCTCCTGTGATCGCCTCA 646
121 T  P  T  P  V  V  Q  I  P  V   A  A  P  V  I  A  T  P  P  V   A  A  S  A  P  T  P  A  A  V   T  P  V  I  S  P  V  I  A  S
    CCACCTGTGGTTCCCGCGAACACCACGGTT CCCGTTGCAGCACCTGTAGCTGCTGTACCA GCAGCTGTTCCTGTTGTTGCCCCAGTTCTT GCGCCTGCCGTTGCTCCTGCAGTTGCCCCC 766
161 P  P  V  V  P  A  N  T  T  V   P  V  A  A  P  V  A  A  V  P   A  A  V  P  V  V  A  P  V  L   A  P  A  V  A  P  A  V  A  P
    GTCGTCGCCGAGACTCCAGCTCCACCACCT GTTGCTGAGATTCCGGTTGCAACCATACCG GAGTGCGTTGCACCTCTCATCCCCGAGGTT TCCGTCGTGGCAACGAAACCACTAGCTGCA 886
201 V  V  A  E  T  P  A  P  P  P   V  A  E  I  P  V  A  T  I  P   E  C  V  A  P  L  I  P  E  V   S  V  V  A  T  K  P  L  A  A
    GCTGAGCCAGTGGTCGTTGCTCCACCGGCT ACTGAAACCCCAGTCGTTGCTCCGGCTGCA GCTTCCCCCCATGTGTCGGTTGCACCAGCT GTGGAGACCGCAGTGGTCGCTCCTGTAAGT 1006
241 A  E  P  V  V  V  A  P  P  A   T  E  T  P  V  V  A  P  A  A   A  S  P  H  V  S  V  A  P  A   V  E  T  A  V  V  A  P  V  S
    GCTTCGACTGAGCCCCCGGTAGCTGCTGCG ACTCTAACAACTGCACCAGAAACACCAGCC CTTGCTCCAGTCGTTGCCGAGTCCCAAGTT GCTGCGAATACTGTTGTAGCTACACCTCCT 1126
281 A  S  T  E  P  P  V  A  A  A   T  L  T  T  A  P  E  T  P  A   L  A  P  V  V  A  E  S  Q  V   A  A  N  T  V  V  A  T  P  P
    ACTCCAGCCCCAGAGCCAGAAACCATTGCA CCACCTGTTGTTGCAGAAACCCCAGAAGTT GCTTCAGTTGCTGTTGCTGAGACAACACCA CCCGTTGTACCTCCTGTGGCCGCAGAATCA 1246
321 T  P  A  P  E  P  E  T  I  A   P  P  V  V  A  E  T  P  E  V   A  S  V  A  V  A  E  T  T  P   P  V  V  P  P  V  A  A  E  S
    ATACCAGCGCCAGTCGTTGCCACGACACCA GTTCCTGCAACACTAGCAGTAACGGATCCG GATGTCACTGCATCTGCTGTTCCCGAATTA CCTCCGGTGATCGCTCCATCACCCGTTCCT 1366
361 I  P  A  P  V  V  A  T  T  P   V  P  A  T  L  A  V  T  D  P   D  V  T  A  S  A  V  P  E  L   P  P  V  I  A  P  S  P  V  P
    AGTGCTGTTGCTGAAACTCCTGTCGACTTA GCGCCACCTGTTCTCCCTCCTGTGGCTGCC GAACCTGTACCAGCTGTAGTGGCTGAAGAA ACTCCTGAGACACCAGCACCAGCATCAGCA 1486
401 S  A  V  A  E  T  P  V  D  L   A  P  P  V  L  P  P  V  A  A   E  P  V  P  A  V  V  A  E  E   T  P  E  T  P  A  P  A  S  A
    CCAGTGACCATAGCAGCTTTAGATATACCA GAAGTTGCTCCTGTGATCGCCGCACCCTCT GATGCACCTGCTGAAGCTCCGTCTGCCGCT GCTCCCATCGTTTCTACTCCACCAACTACG 1606
441 P  V  T  I  A  A  L  D  I  P   E  V  A  P  V  I  A  A  P  S   D  A  P  A  E  A  P  S  A  A   A  P  I  V  S  T  P  P  T  T
    GCTTCTGTTCCCGAGACCACGGCACCACCT GCGGCAGTGCCTACGGAACCTATCGATGTT TCTGTACTATCAGAAGCTGCCATTGAAACG CCAGTTGCACCGCCTGTCGAAGTTACAACA 1726
481 A  S  V  P  E  T  T  A  P  P   A  A  V  P  T  E  P  I  D  V   S  V  L  S  E  A  A  I  E  T   P  V  A  P  P  V  E  V  T  T
    GAAGTTGCAGTTGCAGACGTTGCTCCACCT GAGGCAGCTGCGGATTTAATAATCGAGCCT GTGGAACCACCAGCTCCTATTCCGGATTTA TTAGAACAAACTACATCCGTGCCAGCTGTT 1846
521 E  V  A  V  A  D  V  A  P  P   E  A  A  A  D  L  I  I  E  P   V  E  P  P  A  P  I  P  D  L   L  E  Q  T  T  S  V  P  A  V
    GAAGCTGCAGAATCAACATCTTCACCAATA CCAGAGACCTCATTACCGCCGCCAAATGAA GCAGTTGCCTCTCCAGAAGTGGCTGTCGCT CCAATTACAGCTCCAGAGCCCATACCTGAA 1966
561 E  A  A  E  S  T  S  S  P  I   P  E  T  S  L  P  P  P  N  E   A  V  A  S  P  E  V  A  V  A   P  I  T  A  P  E  P  I  P  E
    CCGGAACCAAGCCTAGCGACCCCTACAGAA CCCATACCTGTGGAGGCACCTGTCGTAATC CAGGAAGCAGTGGATGCTGTAGAGGTGCCG GTGACAGAAACTTCAACATCGATTCCAGAA 2086
601 P  E  P  S  L  A  T  P  T  E   P  I  P  V  E  A  P  V  V  I   Q  E  A  V  D  A  V  E  V  P   V  T  E  T  S  T  S  I  P  E
    ACTACGGTTGAGTTTCCTGAAGCTGTAGCC GAAAAAGTCCTGGATCCTGCCATAACTGAA GCTCCTGTCACTACTCAGGAACCAGATGTT GCAAATATTAATGACGGTGCTCCAGCTACC 2206
641 T  T  V  E  F  P  E  A  V  A   E  K  V  L  D  P  A  I  T  E   A  P  V  T  T  Q  E  P  D  V   A  N  I  N  D  G  A  P  A  T
    GAAATCACCACACCCGCTGTTGAAATCGTT ACTGCTGCTGCAGAAGTATCTGATATTGCC ATACCGGTCATTGACCCTCCGGTTCCTCAA GAGATCGCAGTAGCTGAAATTCCAGAGACA 2326
681 E  I  T  T  P  A  V  E  I  V   T  A  A  A  E  V  S  D  I  A   I  P  V  I  D  P  P  V  P  Q   E  I  A  V  A  E  I  P  E  T
    GATACAAAGCCGGCGGAGGTCATTGTAGAA CAGAGCACCATCCCAATAGAGGCACCAGTC CCGGAGGTTTCAAAATACGCAGAACCAGTG ATTTCTGAAGCTCCAGCTGCCGAAGTACCA 2446
721 D  T  K  P  A  E  V  I  V  E   Q  S  T  I  P  I  E  A  P  V   P  E  V  S  K  Y  A  E  P  V   I  S  E  A  P  A  A  E  V  P
    ATTACGGCCGGGGACAACCCAGACAATACT AGTGTGGGAATATCTGAAGTTGTTCCCACC ATTGCAGAGAAACCAGTTGAAGAGGTTCCC ACTTCGGAAATCCCCGAACAATCATCGTCA 2566
761 I  T  A  G  D  N  P  D  N  T   S  V  G  I  S  E  V  V  P  T   I  A  E  K  P  V  E  E  V  P   T  S  E  I  P  E  Q  S  S  S
    CCCTCAGATTCCGTTCCCGTAGCAAAGATA ACTCCTTTGCTAAGAGATCTTCAGACCACC GATGTTTCGCTATTGGCTATTGCAGCCACC CTGGATGCCATTGGAGAAAAGCTAAAGGAT 2686
801 P  S  D  S  V  P  V  A  K  I   T  P  L  L  R  D  L  Q  T  T   D  V  S  L  L  A  I  A  A  T   L  D  A  I  G  E  K  L  K  D
    CAAAAGGCCAGAAATCAGCAGGTAATGGAC AGACTCTGCGAAATCGAGAAAATTCTTGGT CCTCCCAAATCCAATTAGCTCAAGTTTAAA GCCTGGATCATGTAAAGTAAAGCTACTAAT 2806
841 Q  K  A  R  N  Q  Q  V  M  D   R  L  C  E  I  E  K  I  L  G   P  P  K  S  N  *
    AAAGCGTTATTTCACCAATTTTAAAGTATA TAAAAAAAAAAA 2849
```

FIG. 4. Calphotin cDNA nucleotide and deduced amino acid sequence numbered 5′ to 3′. Potential polyadenylylation signal is double underlined. Leucines in a putative leucine zipper motif are boxed. Amino acid differences of our sequence from that of Ballinger *et al.* (10) are as follows: aa-37, P to V; aa-43, I to T; aa-64, I to V; aa-76, T to A; aa-126–127, VQ to AP; aa-154, I to V; aa-160, S to T; aa-534, A to E; aa-699, I to T; aa-703, V to L; aa-721, D to E. Their sequence also contains a 7-aa insert at position 40 and a proline inserted at position 100.

865 amino acids (85 kDa), and an 87-bp 3′ untranslated region (Fig. 4). A consensus polyadenylylation site was 30 bp from the 3′ end. An ATG codon at position 167 was a possible translation start site; upstream, all three reading frames contained stop signals. The sequence flanking the transitional start site AGACAUG was close to the consensus sequence of translational start sites in *Drosophila* (C/A)AA(A/C)AUG (22). The predicted protein was acidic (pI 3.4), containing much alanine, valine, and proline (175, 146, and 178 residues, respectively). The C-terminal segment contained a leucine zipper-like motif (23). Beginning with Leu-817 and ending with Leu-859, leucine recurred every 7th residue. However, the sequence lacked the flanking basic residues that are typical of DNA-binding proteins (23).

Searches of the GenBank data base revealed no significant sequence similarity to other proteins. In particular, calphotin did not contain any Ca^{2+}-binding EF-hand motifs (24) (as determined by Prosite in Genetics Computer Group), nor did it show any relationship to the Ca^{2+}-binding annexin family. The sequence is also very different from that of drosocalbin (K. Zinsmaier, personal communication).

Chromosomal Localization of the Calphotin Gene. Hybridization *in situ* to salivary gland polytene chromosomes from third-instar larvae (25) showed a single hybridization site at band 86E/F–87A/B on chromosome 3, the same as that identified by Ballinger and Benzer (11) for a cDNA derived from a different antibody, 72H5, leading us to compare the two sequences. Indeed, the two cDNA isolates proved to be essentially identical [see the accompanying paper (10)].

Calphotin mRNA Expression. The *in situ* hybridization pattern (Fig. 5) was closely similar to the immunocytochemical staining of Fig. 1, but no signal was evident in the photoreceptor axons or optic lobes. This may be due to much lower abundance in these structures or lack of migration of the mRNA into them. No hybridization was seen in sections of the brain or the body. Northern blots of head poly(A)$^{+}$ mRNA were probed with ^{32}P-labeled calphotin cDNA. A single message of 3 kb was identified (Fig. 6*A*). The sizes of the cloned cDNA and the mRNA were nearly equal, indicating that the clone was close to full length. The mRNA was found in wild-type head but not body and was greatly reduced in heads of eyeless mutant (*eya*) flies. Although *eya* flies have intact ocelli, their combined mass is small compared to the compound eyes; the contribution of ocellar RNA to the blot should have been minimal.

Expression of Calphotin Protein. Western blot comparison of *eya* heads with wild type showed the protein to be restricted to the eyes, not in the brain (Fig. 6*B*). The mass of 200 kDa, as compared to the standards, was considerably

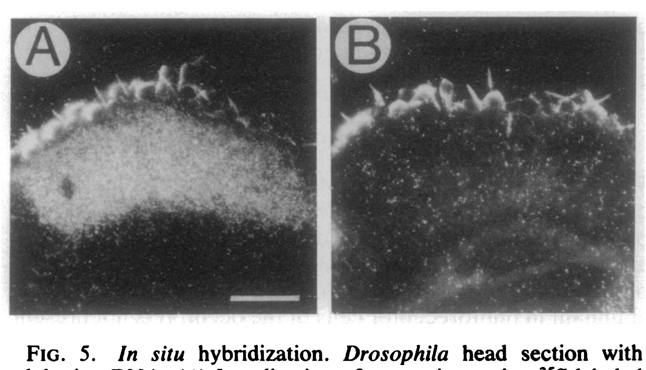

FIG. 5. *In situ* hybridization. *Drosophila* head section with calphotin cDNA. (*A*) Localization of transcripts using ^{35}S-labeled cDNA. (*B*) Control treated with RNase before hybridization. (Bar = 50 μm.)

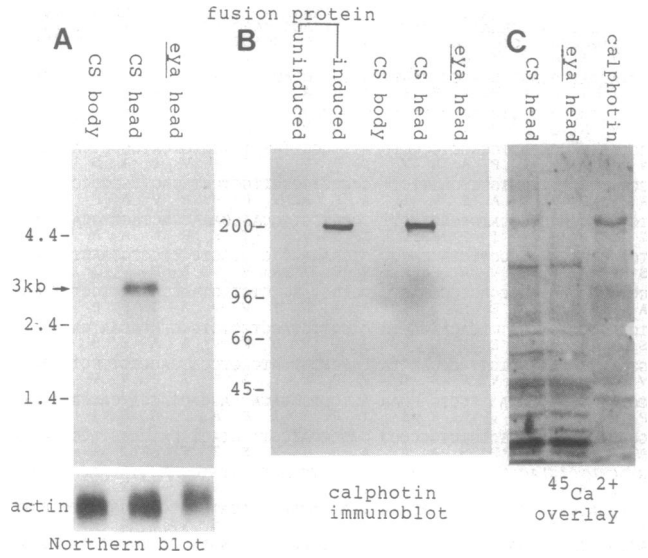

FIG. 6. (*A*) Northern blot of *Drosophila* poly(A)$^+$ mRNA probed with ^{32}P-labeled calphotin cDNA. (*Lower*) β-Actin probe confirms that RNA is intact. Scale on left is in kb. A single 3-kb transcript is identified in the head but not body of wild type. It is absent in the head of *eya* mutant. (*B*) Western blot, stained with mAb 23E9 of *Drosophila* proteins from wild-type and *eya* mutant head and body homogenates and fusion protein. Markers on left are in kDa. Fusion protein was induced in *E. coli* by 0.3 mM isopropyl β-D-thiogalactoside. Epitope of mAb 23E9 is in the C-terminal half of the protein (data not shown). It differs from the epitope of mAb 72H5, which is in the 44 aa of the N-terminal end (10). (*C*) Autoradiograph of a $^{45}CaCl_2$ overlay of a Western blot. $^{45}Ca^{2+}$ is bound to purified calphotin as well as to a protein of the same size in the Canton S (CS) head homogenate. Conversly, no $^{45}Ca^{2+}$ binding is seen in the *eya* mutant homogenate at the corresponding size.

FIG. 7. Visualization of calcium in compound eye photoreceptor cells by the oxalate-antimonate method. There is a high abundance of precipitate in the same regions where calphotin occurs. Arrows indicate these regions in cells R2 and R7. (Bar = 1 μm.)

larger than the 85 kDa estimated from the cDNA. However, a fusion protein made from the cDNA that should encode a polypeptide of 100 kDa also ran anomalously at a rate corresponding to 200 kDa. Although dimerization of the protein—e.g., via the leucine zipper—cannot be excluded, that would seem improbable after boiling in 3.5% SDS/0.7 M 2-mercaptoethanol for 5 min.

Calphotin Protein Binds Calcium. In $^{45}Ca^{2+}$ overlay experiments (21), Western blots of fly head homogenates showed several Ca^{2+}-binding proteins, whereas affinity-purified calphotin protein showed a single $^{45}Ca^{2+}$-binding band (Fig. 6*C*). Fly bodies or *eya* mutant heads lacked that band. Using Ponceau S staining to estimate protein, and liquid scintillation counting of $^{45}Ca^{2+}$ bound, calphotin was found to bind on the blot ≈0.3 mol of Ca^{2+} per mol of protein. That value is comparable to those observed for other calcium-binding proteins using $^{45}Ca^{2+}$ overlay (26).

In addition, preliminary histological evidence using the oxalate-pyroantinomate procedure (15) indicates a high concentration of calcium in the photoreceptor cell region where calphotin occurs (Fig. 7).

DISCUSSION

We describe a protein, calphotin, contained in a hypodense cytoplasmic compartment of the *Drosophila* compound eye photoreceptor cell, that extends along the length of the cell and into the axon. It does not appear to be associated with other intracellular organelles or reticular structures. It is also abundant in photoreceptor cells of the ocelli. It is not present in accessory (nonphotoreceptor) cells of the retina, the brain, or the body. Baumann and Walz (8) have described cisternae in the photoreceptor cells of the honey bee retina that sequester calcium. Although the morphology differs from *Drosophila*, it is possible that similar functions are served by these structures in the two organisms.

The same gene for calphotin is described in the accompanying paper by Ballinger *et al.* (10). The nucleotide sequences are nearly identical; both clones were derived from Canton S strains but from different cDNA libraries with possible polymorphisms. The sequences differ in 12 amino acids; their sequence also has a 21-bp insertion at amino acid position 40 and a 3-bp insertion at position 100. Neither insertion changes the character of the protein.

The 85-kDa encoded protein is unusual: >50% is alanine, proline, or valine. The apparent size on SDS/PAGE for both native and fusion proteins was about twice the predicted size. This may be attributable to the amino acid composition and/or phosphorylation, as has been found with other proteins (28). Indeed, several putative phosphorylation sites (Prosite data bank) are present in calphotin.

The protein sequence suggests three domains. The first 200 N-terminal amino acids are mostly hydrophobic, with only one negatively charged residue; alanine, proline, and valine account for 78% (28%, 26%, and 24%, respectively). The next segment of 600 also is high in alanine, proline, and valine (19%, 21%, and 16%) but contains many negatively charged amino acids (9% glutamic acid plus aspartic acid), with only a few basic residues. The C-terminal 80 amino acids include equal proportions of basic and acidic residues. At positions 813–860, there are no prolines at all.

Within the latter segment is a putative leucine zipper. Leucines occur every 7th residue at positions 817–859, except for an asparagine at the L5 position. A similar asparagine substitution is found in the yeast transcription factor yAP-1; that protein, nonetheless, forms functional heterodimers (29). The number of repeated leucines is greater than in most other leucine zipper motifs (23), equaling that

found in *Drosophila* paramyosin (30). According to Chou–Fasman analysis (19), this region should be capable of forming an α-helix, as there are no prolines to disrupt the structure. Calphotin has no EF-hand motifs, nor is it capable of binding phospholipids in the presence of Ca^{2+} as annexins do (data not shown). It does contain negatively charged residues, separated by short helical hydrophobic sequences. Such structures are capable of binding Ca^{2+} (31).

Transcription factors, the most common class of proteins with the leucine zipper motif, typically have two flanking basic regions separated by alanine spacers. Calphotin lacks such basic regions and is not found in the nucleus. Nevertheless, a zipper sequence might serve in forming dimers, which could provide a reversible mechanism for binding Ca^{2+}. Although the sequence shows little overall homology to others in the data base, there are proteins that have regions rich in alanine, proline, and/or negatively charged amino acids. These include the transcription factor CTF/NF-1 (32), the APEG protein of *Xenopus laevis* (33), and acidic, proline-rich proteins in saliva that serve as weak calcium binders (34). In muscle sarcoplasmic reticulum, an intracellular membrane system responsible for regulating Ca^{2+}, several acidic proteins bind calcium and interact with the luminal face of the junctional membrane. Calsequestrin binds Ca^{2+} with high capacity and moderate affinity (35). Interestingly, in the presence of Ca^{2+}, calsequestrin is able to dimerize *in vitro* (36). Another protein, calreticulin, has a high-affinity Ca^{2+}-binding site in the N-terminal third of the protein. In its C-terminal segment, it has a proline-rich region and an acidic region, which may provide low-affinity, high-capacity Ca^{2+} binding (27).

Calphotin, as detected by mAb 23E9, is present in a circumscribed compartment of the cytoplasm of the photoreceptor cell, which apparently also binds calcium. It is tempting to speculate that this structure functions as a calcium store. Such an organelle would be expected to play an important role in development of the photoreceptor cell in phototransduction, in transport needed to regenerate the rhabdomeric membranes, and in maintenance of the integrity of the cell against degeneration.

We thank Dennis Ballinger for exchange of results and manuscripts, Kent McDonald and Mary Morphew for quick-freeze specimen preparation, Robert Buchanan for help in chromosomal localization, and Rosalind Young for excellent technical assistance. J.H.M. was supported by a postdoctoral fellowship from the National Institutes of Health. This work was supported in part by Grants AGO5142 and MH39145 (C.A.M.) and EYO9278 (S.B.) from the National Institutes of Health and Grant 8908154 (S.B.) from the National Science Foundation.

1. Baimbridge, K. G., Celia, M. R. & Rogers, J. H. (1992) *Trends Neurosci.* **15**, 303–308.
2. Johnston, P. A., Perin, M. S., Reynolds, G. A., Wasserman, S. A. & Sudhof, T. C. (1990) *J. Biol. Chem.* **265**, 11382–11388.
3. Kelly, L. E. (1990) *Biochem. J.* **271**, 661–666.
4. Ranganathan, R., Harris, W. A. & Zucker, C. S. (1991) *Trends Neurosci.* **14**, 486–493.
5. Minke, B. & Selinger, Z. (1992) in *Progress in Retinal Research*, eds. Osborne, N. N. & Chader, G. J. (Pergammon, New York), pp. 94–124.
6. Montell, C. & Rubin, G. M. (1989) *Neuron* **2**, 1313–1323.
7. Walz, B. (1982) *J. Ultrastruct. Res.* **81**, 240–248.
8. Baumann, O. & Walz, B. (1989) *Cell Tissue Res.* **255**, 511–522.
9. Volpe, P., Krause, K. H., Hashimoto, S., Pozzan, F., Meldolesi, T. & Lew, D. P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1091–1095.
10. Ballinger, D. G., Xue, N. & Harshman, K. D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1536–1540.
11. Ballinger, D. G. & Benzer, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9402–9406.
12. Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. & Shotwell, S. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7929–7933.
13. Sved, J. (1986) *Drosoph. Inf. Ser.* **73**, 169.
14. McDonald, K. & Morphew, M. (1992) *Microsc. Res. Tech.* **24**, in press.
15. Peute, J., van Linder, A. T. M., Zanderbergen, M. A. & de-Bruijn, W. C. (1990) *Histochemistry* **94**, 601–607.
16. Sambrook, J., Fritch, E. F. & Maniatis, T. (1988) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Hafen, E. & Levine, M. (1986) in *Drosophila: A Practical Approach*, ed. Roberts, D. B. (IRL, Oxford), pp. 139–157.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
20. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
21. Maruyama, K., Mikawa, T. & Ebashi, S. (1984) *J. Biochem. (Tokyo)* **95**, 511–519.
22. Cavener, D. R. (1987) *Nucleic Acids Res.* **15**, 1353–1361.
23. Busch, S. J. & Sassome-Corsi, P. (1990) *Trends Genet.* **6**, 36–40.
24. Kretsinger, R. H. (1975) in *Calcium Transport in Contraction and Secretion*, eds. Carafoli, E., Clementi, F., Drabikowski, W. & Margreth, A. (North-Holland, Amsterdam), pp. 467–478.
25. Pardue, M. L. (1986) in *Drosophila: A Practical Approach*, ed. Roberts, D. B. (IRL, Oxford), pp. 111–137.
26. Garrigos, M., Deschamps, S., Veil, A., Lund, S., Champeil, P., Moller, J. V. & le Maire, M. (1991) *Anal. Biochem.* **194**, 82–88.
27. Fleigel, L., Burns, K., MacLennan, D. H., Reithmeier, R. A. F. & Michalak, M. (1989) *J. Biol. Chem.* **264**, 21522–21528.
28. Kaufmann, E., Geister, N. & Weber, K. (1984) *FEBS Lett.* **170**, 81–84.
29. Moye-Rowley, W. S., Harshman, K. D. & Parker, C. S. (1989) *Genes Dev.* **3**, 283–292.
30. Vinos, J., Maroto, M., Garesse, R., Marco, R. & Cervera, M. (1992) *Mol. Gen. Genet.* **231**, 385–394.
31. Sambrook, J. F. (1990) *Cell* **61**, 197–199.
32. Mermod, N., O'Neill, E. A., Kelly, T. J. & Tijian, R. (1989) *Cell* **58**, 741–753.
33. Gmachl, M., Berger, H., Thalhammer, J. & Kreil, G. (1990) *FEBS Lett.* **260**, 145–148.
34. Bennick, A. (1982) *Mol. Cell. Biochem.* **45**, 83–99.
35. Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. R., Reithmeier, R. A. F. & MacLennan, D. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1167–1171.
36. Mitchell, R. D., Simmerman, H. K. B. & Jones, L. R. (1988) *J. Biol. Chem.* **263**, 1376–1381.