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Original Article

Developmental expression of the *Drosophila melanogaster* calmodulin gene

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ABSTRACT The highly conserved, intracellular calcium binding protein calmodulin is present in all cells at all times. In addition to this constitutive level, the amount of calmodulin is highly regulated according to the tissue or stage of development. Since there are only a few genes or a single gene for this protein in most species, intricate regulatory elements may be necessary to effect its complex regulation. This report adds new information concerning the gene structure and outlines the developmental and spatial regulation of *Drosophila melanogaster* calmodulin transcripts. The gene contains five exons, including a 49 bp exon in the 5' untranslated region, and spans over 16 kb. Homologues to this small, 5' noncoding exon have not been found in other calmodulin genes. The combined level of the transcripts is developmentally regulated, and the relative amounts of the two transcript size classes (1.65 kb and 1.9 kb) are differentially regulated during development. Primer extension experiments and RNase protection mapping show that both size classes of *Drosophila* calmodulin transcripts initiate at the same site but undergo alternative termination within the final exon. The spatial distribution of calmodulin transcripts was examined by *in situ* hybridization to sections of adults and to developmentally staged whole mount embryos. Calmodulin transcripts are evenly distributed early in embryogenesis. In later stages of embryogenesis, higher levels accumulate in the developing nerve cord and other tissues. Elevated levels of calmodulin transcripts are seen quite distinctly in the adult neural tissues and in the photoreceptor region of the compound eye.

KEY WORDS: *calmodulin, calmodulin gene, development, in situ hybridization*

Introduction

Calmodulin is a highly conserved, acidic protein that functions as a universal regulator of intracellular calcium and modulator of cellular processes. Calmodulin is thought to have multiple general regulatory roles, probably due to its ability to interact with proteins involved in cell regulation (Lukas *et al.*, 1988). A series of structure-function studies using both a recombinant (Putkey *et al.*, 1986) and a cassette (Roberts *et al.*, 1985; Lukas *et al.*, 1988) approach to *in vitro* mutagenesis have demonstrated that different target proteins such as phosphodiesterase, myosin light chain kinase, the γ-subunit of phosphorylase kinase, and calmodulin-dependent protein-kinase II interact with different regions of the calmodulin molecule. Such studies, in combination with immunological (Hansen and Beavo, 1986), biochemical (Klevit and Vanaman, 1984; Ni and Klee, 1985), and genetic (Kink *et al.*, 1990) investigations, have revealed that calmodulin possesses multiple functional domains that interact separately with target enzymes (Putkey *et al.*, 1988). The interactions of calmodulin with multiple target proteins at non-overlapping binding regions may explain the extreme conservation of this protein among species, since a mutation might prevent one critical interaction. In yeast, calmodulin is an essential gene (Davis *et al.*, 1986; Takeda and Yamamoto, 1987). However, nonlethal mutants of *Paramecium* with altered swimming behavior have single amino acid changes in calmodulin (Schaefer *et al.*, 1987; Lucas *et al.*, 1989; Kink *et al.*, 1990), indicating that only one or a few calmodulin-target protein interactions are affected.

Although calmodulin is constitutively synthesized in all cells (Klee and Vanaman, 1982), the levels of calmodulin in different tissues vary several fold (Chafouleas *et al.*, 1979). In mammals, the

Abbreviations used in this paper: bp, base pairs; kb, kilobase pairs; MMLV, Moloney Murine Leukemia Virus; dpm, disintegrations per minute; cpm, counts per minute.

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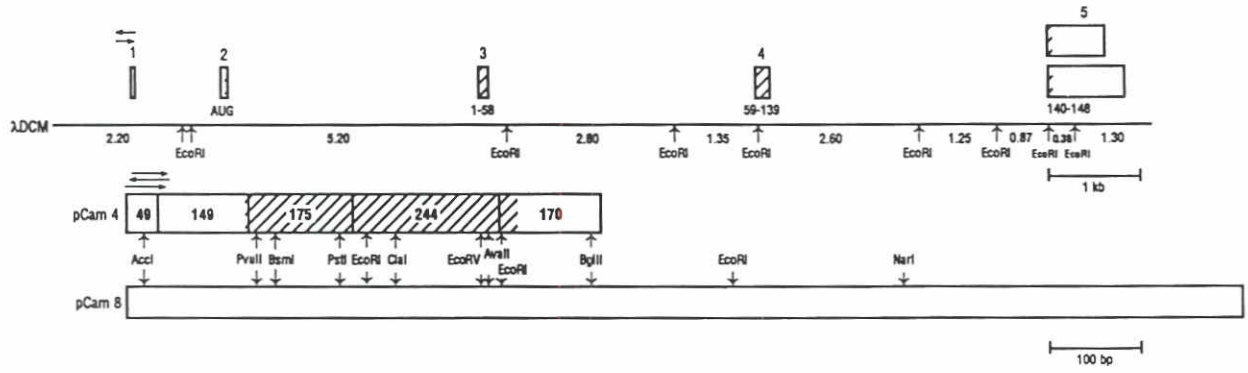

Fig. 1. Map of genomic and cDNA clones representing the *Drosophila* calmodulin gene. *The locations of the calmodulin exons are indicated by the boxes above the genomic clone, λDCM (Yamanaka* et al., *1987), and cross hatching indicates the locations of the protein coding sequence. The amino acid numbers encoded by each exon are given below each block. Maps of cDNA clones analyzed in detail (pCam4 and pCam8) are shown below the genomic clone. The coding regions as determined by sequence analysis are indicated by cross hatching. The exon lengths in bp are shown within each block representing an exon, and the vertical lines indicate the locations of the splice junctions. The sequencing strategy is symbolized by arrows above the maps. The locations of selected restriction enzyme sites are indicated. pCam8 is aligned with pCam4 by restriction mapping and Southern blot analysis.*

highest levels are found in brain tissue and in adult testes. High levels are also found in secretory and neurosecretory cells such as the islets of Langerhans of the pancreas and the electroplax organ of the electric eel (Klee and Vanaman, 1982). There is also evidence that the concentration of calmodulin in cells is differentially regulated during sea urchin (Floyd *et al.*, 1986) and *Xenopus* (Chien and Dawid, 1984) development. Cell-specific temporal regulation of calmodulin levels is seen during spermatogenesis (Feinberg *et al.*, 1985). Recent exciting evidence for the role of calmodulin in differentiation and regulation of specific cells comes from three transgenic lines of mice which overproduce calmodulin (Epstein *et al.*, 1989b) in pancreatic islet cells and become severely diabetic in the few days following birth.

Regulation of calmodulin transcripts adds another level of complexity. Calmodulin transcripts are not coordinately regulated during the cell cycle (Lagace *et al.*, 1988). The low level of calmodulin in phosphorylase kinase-deficient skeletal muscle results from decreased calmodulin mRNA, apparently decreased in response to the lack of its target, phosphorylase kinase (Bender *et al.*, 1988). Primary cell culture experiments suggest that calmodulin transcript levels are differentially regulated during spermatogenesis (Slaughter *et al.*, 1989). These studies imply that there may be cell-specific and developmentally specific mechanisms of gene regulation. However, a great deal remains to be learned about the tissue-specific and temporal regulation of the calmodulin gene.

Some structural elements of calmodulin genes from different species, such as the position of an intron just after the ATG translation initiation signal, are conserved (Smith *et al.*, 1987; Yamanaka *et al.*, 1987; Means *et al.*, 1988; Koller *et al.*, 1990). This conservation may be due to positioning of complex regulatory elements in a gene that is expressed in all cells at all times, but at diverse levels. Analysis of the elements controlling gene expression has been complicated by the existence of more than one calmodulin gene in most species (Smith *et al.*, 1987; Fischer *et al.*, 1988). Chicken (Means *et al.*, 1988), however, like *Drosophila* (Yamanaka *et al.*, 1987; Doyle *et al.*, 1990) has one functional calmodulin gene. An extensive analysis of the chicken calmodulin gene promoter (Epstein *et al.*, 1989a) has revealed several regulatory regions. One

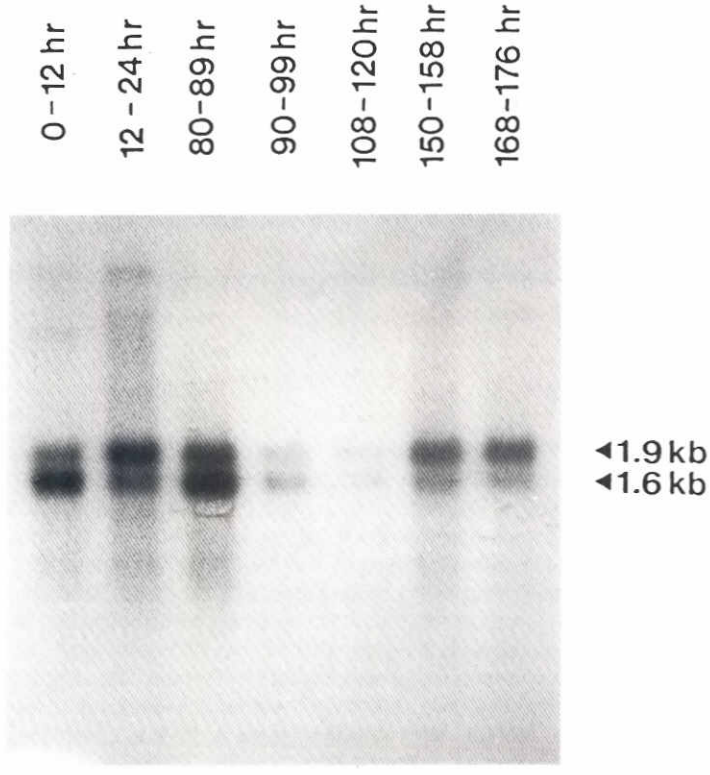

Fig. 2. Autoradiograph showing regulation of transcript accumulation during *Drosophila* development. *Poly A⁺ RNA was isolated from developmentally staged* Drosophila; *stages are shown at the top of each lane: 0-12 h, early embryos; 12-24 h, late embryos; 80-89 h, 90-99 h, and 108-120 h, early, mid, and late third instar larvae, respectively; 150-158 h, mid pupae; and 168-176 h, late pupae. The poly A⁺ RNA (1 μg per lane) was electrophoresed on a denaturing gel, blotted onto nylon membrane, hybridized with a probe specific for exon 1 as described in the text, and autoradiographed for 50 h. Sizes of the transcripts are shown and were determined by coelectrophoresis with RNA size markers.*

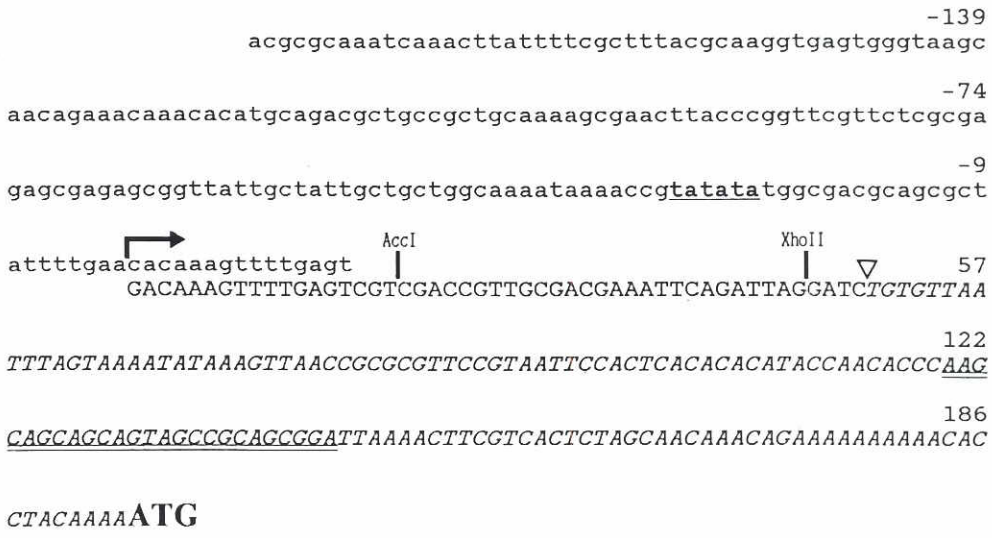

Fig. 3. DNA sequence of the 5' end of the *Drosophila* calmodulin gene. *Lower case letters denote the sequence of the genomic subclone pCam2.2 (2.2 kb EcoRI fragment of lDCM; see Fig. 1) in the region of the first exon, while capital letters denote the sequence of the 5' end of the cDNA clone pCam4 (see Fig. 1). The splice junction between exons 1 and 2 is marked with an open triangle. The italicized portion of the cDNA sequence is collinear with the genomic sequence (Smith* et al., *1987) and is part of the exon that ends after the translational start site (Bold ATG). The start site consistent with our primer extension experiments (Fig. 4) and with sequence from our cDNA clones is marked with a closed arrow. The sequence of the synthetic oligonucleotide used in primer extension experiments and as a probe for the developmental RNA blots is complementary to the doubly underlined portion. The AccI and XhoII sites used to produce a probe fragment specific for exon 1 are also indicated. The TATA consensus sequence is bold and underlined.*

of these has noticeable sequence similarity to the promoter region of one of the rat calmodulin genes (Nojima and Sokabe, 1987).

Drosophila calmodulin is the product of a single gene (Hanson-Painton *et al.*, 1987; Yamanaka *et al.*, 1987; Doyle *et al.*, 1990), located at cytological position 49A (Smith *et al.*, 1987; Yamanaka *et al.*, 1987) on the second chromosome. Two transcript size classes are produced, and the relative concentration of each of these size classes differs between embryo, larval, and pupal stages (Yamanaka *et al.*, 1987; Doyle *et al.*, 1990). This report completes the gene structure, further examines the temporal expression, and outlines the spatial distribution of transcripts of the calmodulin gene in *Drosophila melanogaster.*

Results

The structure of the *Drosophila* calmodulin genomic DNA and maps of the pertinent cDNA clones are shown in Fig. 1. This figure also summarizes the locations of subclones used as templates for the transcription of probes, the DNA sequencing strategy, and, as shown below, the complete structure of the gene. The *Drosophila* calmodulin gene produces two transcripts of 1.65 kb and 1.9 kb (Yamanaka *et al.*, 1987; Doyle *et al.*, 1990). During development, both the total level of all calmodulin transcripts and the relative amounts of the two size classes change (Fig. 2). The most obvious difference is an apparent down regulation of transcript levels during the third instar larval stage: early third instar larvae (80-89 h) have a large amount of calmodulin message which decreases drastically over the mid (90-99 h) and late (108-120 h) third instar. The relative amounts of the two transcripts are also differentially developmentally regulated. There is a striking difference in the relative amounts of calmodulin mRNA in each size class between the RNA from 0-12 h embryos and that from 12-24 h embryos, with the smaller size class preferred at the earlier time interval, while the larger transcript predominates in 12-24 h embryos. All three stages of third instar larvae contain a size distribution similar to that of 0-12 h embryo RNA in that there is a larger amount of the smaller transcript. In contrast, in mid (150-158 h) and late (168-176 h) pupal stages, the larger transcript is more abundant. The ratio between the two size classes remains constant throughout the subdivisions of the third instar larval stage. There is also no change in the size distribution of transcripts between the mid (150-158 h) and late (168-176 h) pupal stages.

Since there were differences in the relative abundance of the two transcript sizes during development, we isolated and analyzed several cDNA clones from different cDNA libraries. A cDNA library made from 0-12 h embryonic mRNA (Brown and Kafatos, 1988) yielded the most complete set of clones. Two clones isolated from this library, pCam4 and pCam8, are diagrammed in Fig. 1. The 5' end of the first clone isolated, pCam4, was sequenced (Fig. 3). When this sequence was compared with the published genomic sequence proposed as the start site of the gene (Smith *et al.*, 1987), an additional 49 bp of sequence was present at the 5' end of pCam4 which was not collinear with the genomic sequence. These data suggested that an additional exon was present on at least some of the calmodulin transcripts. Since our pCam4 cDNA was only 790 bp long, it did not represent a full-length transcript. We therefore isolated a second cDNA (pCam8 in Fig. 1) which corresponded to the length of the longer transcript. This cDNA was among those hybridizing to a labeled AccI-XhoII fragment from pCam4 (Fig. 3), a probe which is specific for the 49 bp exon (data not shown). By restriction mapping and Southern blot (Southern, 1975) analysis, pCam8 appears to represent the full length of the 1.9 kb transcript. Using the labeled AccI-XhoII (Fig. 3) probe and Southern blot analysis of our original lambda clone, λ DCM (Yamanaka *et al.*, 1987),

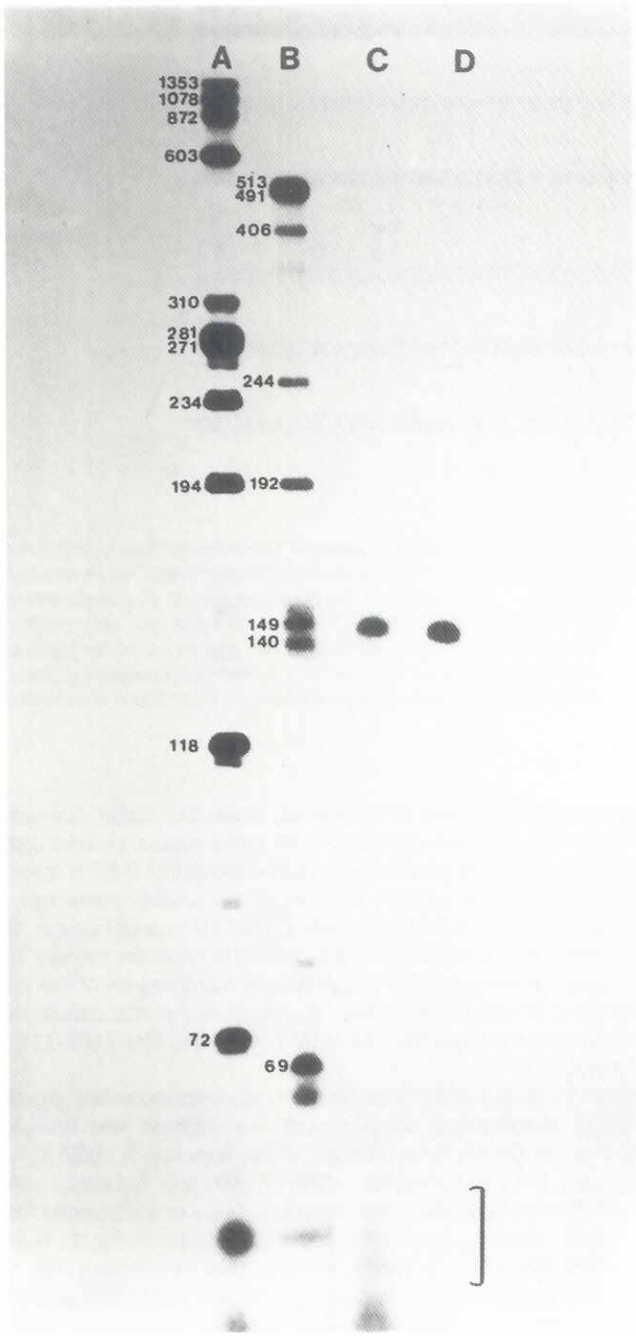

Fig. 4. Autoradiogram of primer extensions of *Drosophila* poly A⁺ mRNA. *An oligonucleotide chosen to hybridize to a region of exon 2 (See Figs. 1 and 3) was labeled with γ-^{32}P ATP, annealed to 4 μg of poly A⁺ RNA and extended with MMLV reverse transcriptase as described in the text.* **(Lane A)** *HaeIII fragments of phiX 174;* **(Lane B)** *HpaII fragments of pTZ19;* **(Lane C)** *extension of poly A⁺ mRNA isolated from 0-12 h embryos;* **(Lane D)** *extension of poly A⁺ RNA isolated from 12-24 h embryos. Numbers indicate lengths of molecular weight standards (bp).*

the 49 bp exon was localized within a 2.2 kb EcoRI fragment, approximately 650 bp upstream from the exon containing the translational start site (Fig. 1). The genomic sequence in the region of this exon is shown in Fig. 3. No CAAT consensus sequences were found in the -75 bp region, but a TATA consensus sequence was found beginning at -29 bp. The initial G in the cDNA sequence at the start of transcription was clearly present in multiple sequencing runs. Because of the discovery of this additional exon, we wished to determine whether alternative 5' initiation was involved in the generation of the two transcript size classes. We therefore carried out primer extension experiments to determine whether these two transcripts shared a common initiation site. We used a 24 bp primer which is complementary to a DNA sequence starting 76 bp upstream from the ATG initiation codon (Fig. 3). This primer was chosen to detect use of the previously proposed transcriptional start site (Smith *et al.*, 1987) as well as any other start site upstream. The results of primer extension of embryonic poly A⁺ RNA are shown in Fig. 4. The presence of a single extension product indicates that there is a single start site for all transcripts. The length of this product places the transcriptional initiation site at the 5' end of exon 1 (Figs. 1 and 3). The bracket indicates the expected location of an extended product from the start site previously proposed (Smith *et al.*, 1987).

Since there appeared to be only one transcriptional start site, we carried out RNase protection experiments to determine whether the two transcript size classes were a result of heterogeneity at the 3' end of calmodulin transcripts. A cRNA probe was generated by T7 RNA polymerase using a 1.3 kb fragment containing the 3' portions of exon 5 (Fig. 1) as template. RNase protection of this probe with 2 μg of poly A⁺ RNA yielded two fragments of 590 bp and 950 bp (Fig. 5). The lengths of these fragments are consistent with the hypothesis that the two calmodulin transcripts are created by alternative 3' termination and processing within the 1.3 kb EcoRI fragment. This hypothesis was further examined by hybridizing the cRNA transcriptional probe from the same 1.3 kb EcoRI fragment to RNA blots. The 1.3 kb probe hybridized to both size classes of calmodulin transcript at all developmental stages examined (data not shown). In addition, RNase protection experiments using either the 0.38 kb genomic EcoRI fragment or the 0.38 kb EcoRI fragment of pCam8 yielded protected fragments (data not shown) consistent with the alternative 3' processing sites described above.

To determine the tissue distribution of *Drosophila* calmodulin transcripts, *in situ* hybridizations were performed using whole mounts of developing embryos (Fig. 6). During early stages of embryogenesis, calmodulin transcripts are evenly distributed. Fig. 6A shows a stage-6 embryo at cellular blastoderm, showing a uniform level of labeling. As development proceeds, calmodulin transcripts accumulate at higher levels in specific tissues, but are present in all cells. Fig. 6B illustrates the segmental labeling of the nerve cord and labeling of the supraesophageal ganglion in a stage-14 embryo. In addition, the region of the developing midgut is labeled. A stage-15 embryo (Fig. 6C) shows sharper anterior localization of signal, associated with the developing anterior sensory organs. A higher level of calmodulin transcripts can also be seen in the brain. By stage 16 (Fig. 6D) the labeling in the brain and nerve cord is dramatic. In a stage-17 embryo seen from above (Fig. 6E), the lobes of the brain can be seen extending laterally from the position of the central nerve cord. A stage-16 control embryo hybridized with unlabeled probe is shown in Fig. 6F. Nonspecific binding of the detection reagents to the developing trachea can be observed; otherwise, the embryo is unstained.

Transcript distribution was further examined by *in situ* hybridization to sections of sn^w adults 1 to 2 h post eclosion. White-eyed

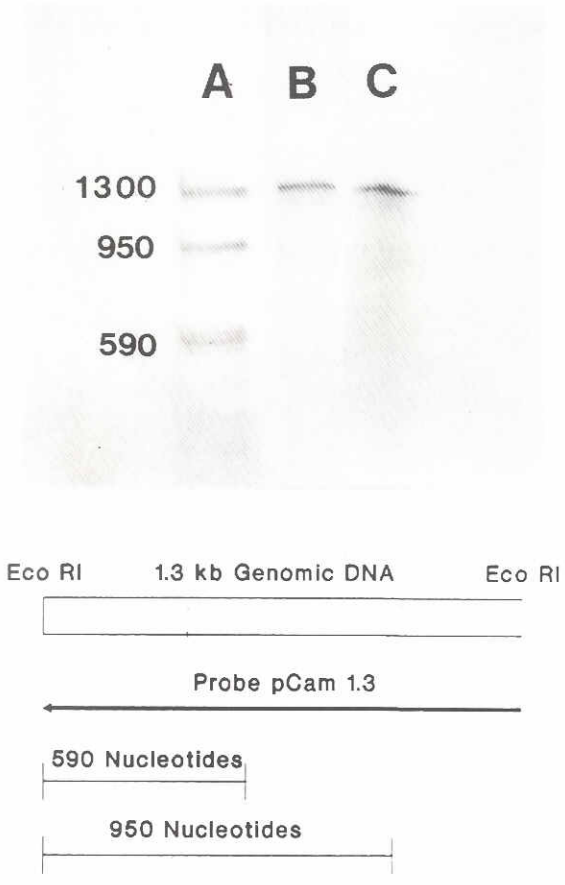

Fig. 5. Mapping of the 3' end of the gene by RNase protection experiments. *A labeled cRNA produced from the 1.3 kb EcoRI fragment was hybridized to 2 μg of poly A⁺ RNA as described in Materials and Methods. Digestions of unhybridized RNA were performed with RNase T1 and autoradiographic exposure was for 60 h at -70°C with an intensifying screen. Other RNase protection details are described in the text.* **(Lane A)** *cRNA probe protected by larval poly A⁺ RNA.* **(Lane B)** *cRNA probe carried through the experiment in the presence of 10 μg of tRNA.* **(Lane C)** *cRNA probe carried through the experiment in the presence of 10 μg tRNA, but without RNase T1 and with only 6 h of autoradiography. Lengths of the original probe and the protected cRNA fragments are given in bp, calculated by using labeled phiX174 fragments as molecular weight markers.*

mutants were used because the absence of eye color enhances the visualization of the autoradiographic grains. The results are shown in Fig. 7. The highest concentration of transcripts, revealed by the deposition of silver grains, follows a neural and photoreceptor distribution. Accumulation is very high in the photoreceptor cell layer (retina) of the compound eye (Fig. 7A) and is more concentrated at the base of the retina. High levels of transcript are also quite evident in the cell body-rich cortical rind surrounding the lamina, medulla, and lobula (Fig. 7A), as well as in the cortical rind surrounding the subesophageal ganglion. The nerve tissue throughout the organism also exhibits high levels of accumulation of the transcript (data not shown). The similar section shown in 7B was hybridized with a sense probe to estimate the level of nonspecific binding.

Discussion

The structure of the *Drosophila* calmodulin gene differs from those described in other species (Epstein *et al.*, 1987; Zimmer *et al.*, 1988; LèJohn, 1989; Nojima and Sokabe, 1989; Koller *et al.*, 1990) in that there is an additional small exon (Exon 1, Fig. 1) present in the 5' noncoding region. All transcripts apparently initiate at a single point at the 5' end of this exon (Fig. 4); the primer used was selected because it would have extended to either a proposed start site in exon 2 (Smith *et al.*, 1987) or to any site upstream. Surprisingly, the exon 2 start site was not used, even though appropriate signals appear to be present. Extensions of either 0-12 h or 12-24 h embryonic mRNA produced fragments of the same length, even though the relative populations of transcript lengths differ significantly in these two developmental time periods. Therefore, transcript length is apparently independent of 5' initiation. Primer extension experiments using pupal poly A⁺ RNA as a template (data not shown) gave similar results, suggesting that the same start site is used in other stages. Primer extension experiments reported by Doyle *et al.* (1990) also indicate a single start site at a 51 bp exon in a similar location. However, the oligonucleotide used for extension was not specified, making a direct comparison of the two results difficult.

The genomic sequence adjacent to exon 1 contains a TATA box 29 bp upstream from the transcriptional start site defined by the primer extension, although no CAAT consensus sequences were apparent in the -70 to -80 bp region. The G present at the start of transcription of the cDNA clone is apparently not an artifact of cloning, since the cloning procedure (Brown and Kafatos, 1988) should have introduced a C at this position. This difference may represent a population polymorphism, particularly since the cDNA library is derived from a different strain of flies than that from which the genomic library was produced. The sequence surrounding the TATA box is quite similar to that of one of the rat calmodulin genes (Nojima and Sokabe, 1987). The region conserved between the chicken and one of the rat calmodulin gene promoters (Epstein *et al.*, 1989a) was not evident. The sequence obtained is similar to that previously reported (Doyle *et al.*, 1990), including the G residue at the 5' end of the cDNA; however, the length of the first exon predicted from our data is 49 rather than 50 base pairs.

The difference in length between the two transcripts is apparently due to alternate termination within exon 5, since RNase protection using the 1.3 kb genomic fragment as template (Fig. 5) produced protected fragments of 950 and 590 bases. These lengths fully account for the mRNA lengths of 1.65 kb and 1.9 kb previously observed (Yamanaka *et al.*, 1987). RNase protection experiments have the ability to reveal whether transcription termination occurs within contiguous segments of DNA. Such information is important because considerable microvariation in use of termination and polyadenylation sites can occur (Platt, 1986) and thus may not be detected by sequence analysis of a limited number of cDNA clones. However, the positions identified by our RNase protection experiments do correspond to putative polyadenylation sites revealed by DNA sequence analysis (Doyle *et al.*, *1990*).

The overall accumulation of the transcripts is developmentally regulated (Fig. 2). Accumulation decreases dramatically during the third instar larval stage; this decrease has been seen for other genes and may be related to a reduction in overall transcription in preparation for pupation (Vigoreaux and Tobin, 1987). The differential distribution of the two transcript size classes between the early

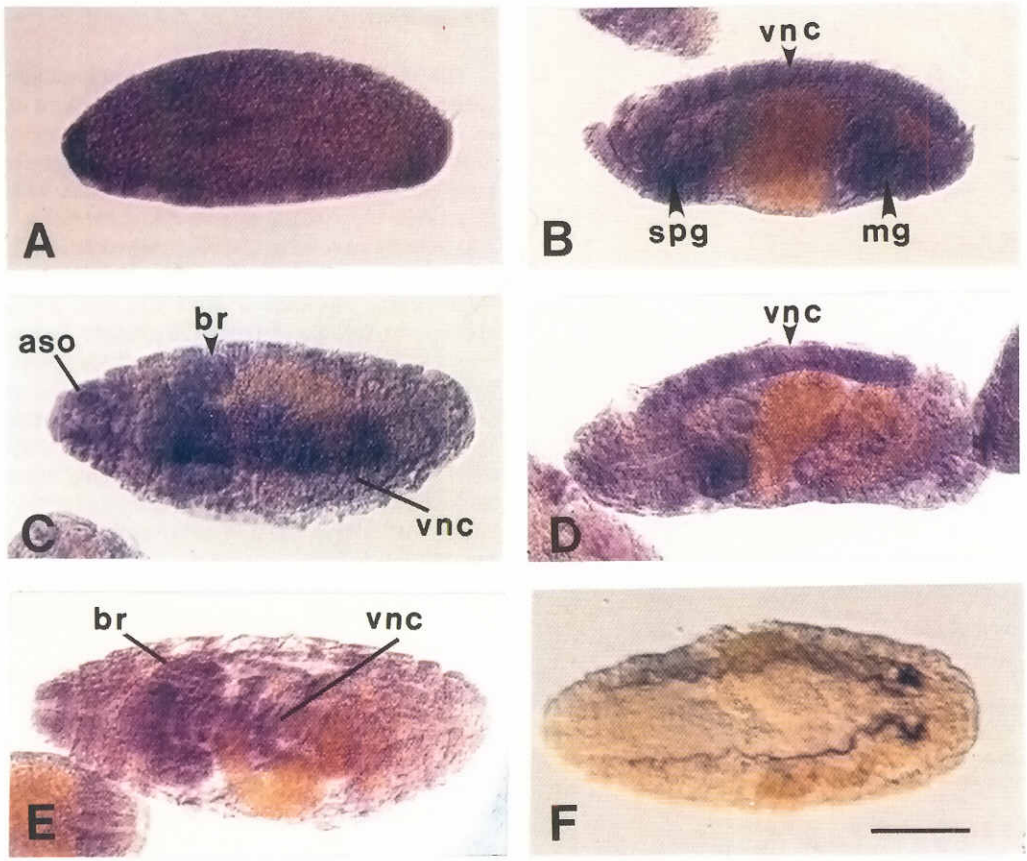

Fig. 6. *In situ* hybridization of digoxigenin-labeled calmodulin probe (pCam4, Fig. 1) to *Drosophila* whole mounts. (A) *Stage-6 embryo showing homogeneous distribution of transcript;* **(B)** *lateral view of stage-14 embryo with transcript accumulation in the ventral nerve cord, supraesophageal ganglion and developing gut;* **(C)** *ventral view of stage-15 embryo, showing higher concentration of calmodulin transcripts in the ventral nerve cord, developing brain, and anterior sensory organs;* **(D)** *lateral view of stage-16 embryo illustrating the distribution in the developing neural tissue;* **(E)** *dorsal view of stage-17 embryo with striking accumulation in the ventral nerve cord and brain;* **(F)** *stage-16 control embryo hybridized with unlabeled probe. vnc: ventral nerve cord; spg: supraesophageal ganglion; mg: midgut; br: brain; aso: anterior sensory organ. For all embryos, anterior is to the left. Scale bar indicates 0.1mm.*

and late embryonic stages (Fig. 2) is striking. It may be that the smaller transcript represents persistent maternal message and the longer transcript is produced by the embryo when transcriptional activity begins at approximately 2 hours. This phenomenon was observed for calmodulin transcripts in sea urchin embryos (Floyd *et al.*, 1986). In the third instar larvae and the pupae, the shorter transcript again accumulates preferentially. There was no detectable differential regulation of the transcript size classes during the later stages we examined. Previous studies using both total (Yamanaka *et al.*, 1987) and poly A^+ RNA (Doyle *et al.*, 1990) suggested that transcript sizes differed among embryonic, larval, and pupal stages. However, these blots were of RNA collected over broader sampling times and thus did not detect the mid-embryonic switch seen in the present study.

The patterns of calmodulin transcript distribution in embryos detected by *in situ* hybridization of calmodulin probes to whole mounts (Fig. 6) forecast the neurological distribution in adult tissues discussed below. In early embryogenesis, calmodulin transcripts are evenly distributed, as are other maternal messages (Burn *et al.*, 1989). While the accumulation of calmodulin transcripts continues to be observed in all embryonic tissues, increased levels in neurological tissue are consistently apparent. As development proceeds, localization of calmodulin transcripts becomes prominent in ventral nerve cord, brain, and sensory organs. These results are in keeping with the important role of calmodulin in neurological tissue.

In adults, the tissue distribution of calmodulin mRNA accumulation follows a neural and photoreceptor distribution (Fig. 7A, B). Accumulation is quite high in the cell bodies surrounding the lamina, the medulla, and the lobula, as well as the cell bodies of the subesophageal ganglion, the mass of neurons providing communication from the head organs to the rest of the neurons extending into the thorax. In the compound eye, the striations in the grain distribution follow the length of the ommatidia, the structure containing the eight specialized photoreceptor cells (Campos-Ortega, 1980). The observed pattern suggests that different cells of the ommatidia accrue differential amounts of calmodulin transcripts. Calmodulin may be present at high levels in some of these

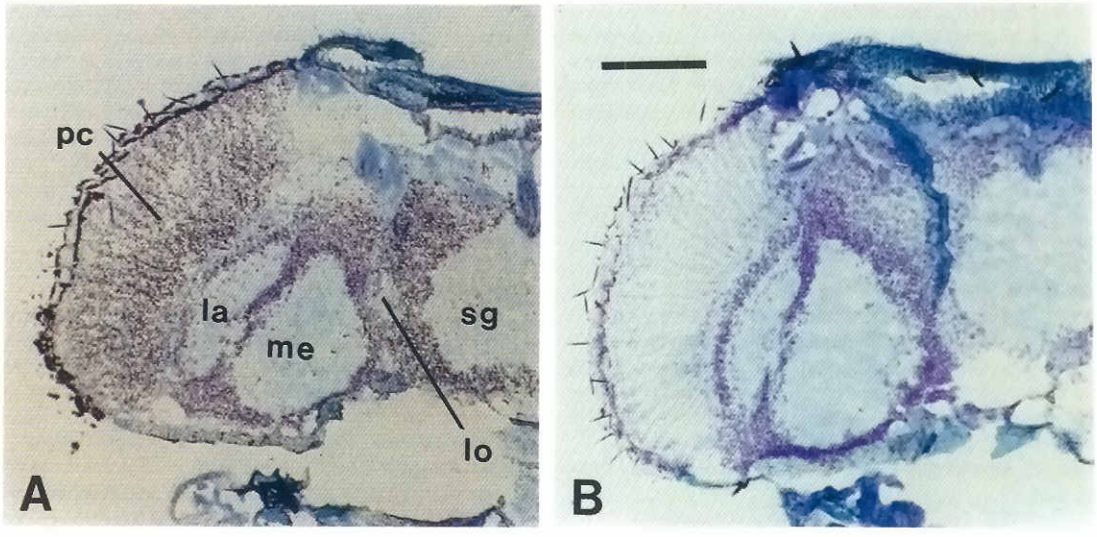

Fig. 7. Distribution of calmodulin mRNA in adult *Drosophila* heads as determined by *in situ* hybridization. *Eight micron frozen sections were hybridized with tritium-labeled sense and antisense cRNA probes made from pCam4 as described in Materials and Methods and exposed for 5 days. The sense probe provides a control to determine non-specific binding of probe to the sections.* **(A)** *Section hybridized with an antisense probe. The distribution of silver grains represents the accumulation of calmodulin transcripts.* **(B)** *Section hybridized with a sense cRNA probe. pc: photoreceptor cells; la: lamina; me: medulla; lo: lobula; sg: subesophageal ganglion. Scale bar indicates 0.1 mm.*

cells in order to participate in the visual transduction signals, although signal transduction in the invertebrate compound eye is thought to be mediated by hydrolysis of phosphoinositol triphosphate via phospholipase C (Yamada *et al*, 1990). The ommatidial distribution of calmodulin transcripts differs from that of phospholipase C *(norpA)* transcripts in that calmodulin transcripts are concentrated near the base of the retina, while *norpA* transcripts are concentrated at the outer regions (Bloomquist *et al.*, 1988). Whether or not the levels of calmodulin transcript are truly regulated at the level of the individual photoreceptor cell awaits *in situ* hybridization studies at higher resolution.

The *Drosophila* calmodulin gene is different from those of other species in that there is a small, 49 bp exon in the 5' noncoding region. The presence of this exon does not associate with a particular transcript length, since both transcript size classes possess this exon. The two transcripts produced from the gene are regulated differently during the developmental stages examined. This differential regulation may be a result of changing requirements in efficiency of translation or in message half life that allow the organism to effect the changes in calmodulin protein levels necessary for each developmental stage. *Drosophila* is a good model for the study of such a hypothesis, since it has well-defined developmental stages and our studies show that the relative amounts of transcript size classes change during development. The tissue distribution of transcript accumulation follows a pattern that suggests that calmodulin may participate in signal transduction. The highest levels of transcript are found in neural tissue and in the photoreceptor region of the compound eye, although it is not clear whether all of the cells in the retina have high concentrations of calmodulin transcript. Whether or not each transcript size class is differentially spatially accumulated is the subject of future investigations.

Materials and Methods

Isolation of recombinant clones

A cDNA library from 12-24 h embryos was a gift of Nicholas Brown (Brown and Kafatos, 1988). The library was screened at high density (Hanahan and Meselson, 1980) with a nick-translated (Rigby *et al.*, 1977) 500 bp EcoRI-PstI genomic DNA fragment (Yamanaka *et al.*, 1987) containing the DNA sequence encoding amino acids 67-139 of *Drosophila* calmodulin. Positive clones were rescreened with a nick-translated genomic EcoRI 5.2 kb fragment, containing the coding sequence for amino acids 1-58, until purified positive colonies were obtained. Subsequently, clones were screened with probes specific for individual exons labeled by filling in 3' recessed ends with Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982) or by nick translation (Rigby *et al.*, 1977). Plasmid DNAs were prepared by the method of Birnboim and Doly (1979).

DNA sequence analysis

DNA fragments were subcloned into m13 phage vectors (Messing, 1983) and sequenced by the chain termination method (Sanger and Coulson, 1975; Sanger *et al.*, 1977) using the Promega TaqI kit or the Sequenase (USB) kit. Sequences were compiled and analyzed using the Intelligenetics programs available through Bionet (Kristofferson, 1987) and the University of Oklahoma Genetic Computer Group programs (Devereux *et al.*, 1984; Pearson and Lipman, 1988) available on the University of Oklahoma computer.

RNA isolation and analysis

The developmentally staged RNA samples were obtained by timed collections and 25°C incubations of the progeny of Canton S strain of *Drosophila melanogaster*, using the developmental recommendations of Roberts (1986). Flies (1-3 days post eclosion) were allowed to lay on cornmeal/molasses/agar medium (Sang, 1978) in a population cage. Embryos (0-12 h) were collected after 12 h; identical collections were incubated at 25°C for 12 h and represented 12-24 h embryos. For the other

timed collections, embryos were collected for 8, 9, or 12 h. The larvae were incubated at 25°C and harvested at 80, 90, and 108 h after the period of egg deposition, while the pupae were collected at 150 and 168 h. Total RNA was isolated according to the method of Chirgwin *et al.* (1979). Poly A^+ RNA was selected by oligo (dT) cellulose chromatography (Type 7, PL Biochemicals) and analyzed by electrophoresis in gels containing formaldehyde (Maniatis *et al.*, 1982). Each gel lane was loaded with 1 μg of poly A^+ RNA and RNA lengths were determined by staining a parallel lane with RNA size markers (Gibco BRL). The gels were blotted onto nitrocellulose (Schleicher and Schuell) and baked for 2 h at 80°C in a vacuum oven or blotted onto nylon (Amersham) membrane and irradiated on a UV transilluminator (Fotodyne) for 7 min. For analysis of expression over time, the blots were probed with an oligonucleotide ($1.5x10^8$ cpm/μg) labeled at the 5' end with γ-^{32}P ATP (Maxam and Gilbert, 1980). Autoradiography was at -70°C using Kodak XAR-5 film with an intensifying screen (Dupont Cronex). The oligonucleotide was chosen to hybridize with a 24 base region 76 bp upstream from the exon containing the ATG start of translation, based on sequencing of cDNA clones. RNA blots were also hybridized with cRNA probes generated by T7 transcription from T3/T7 (Boehringer Mannheim) subclones of the λDCM genomic clone (Fig. 1).

Primer extensions

Primer extensions of poly A^+ RNA from 0-12 h and 12-24 h embryos were carried out according to Akhurst *et al.* (1987). The hybridization temperature was 70°C and the specifically hybridized oligonucleotide (described above) was extended with MMLV reverse transcriptase (Pharmacia) at 42°C. The resulting reaction products were electrophoresed on a 6% polyacrylamide, 8 M urea gel (Maxam and Gilbert, 1980). The gel was dried on a slab gel dryer (BioRad) and autoradiographed as above. Molecular weight standards included were: HaeIII fragments of phiX174 labeled by kinase exchange (Berkner and Folk, 1977) with γ-^{32}P ATP (Amersham), and HpaII fragments of pTZ18 (PL Biochemicals) labeled by fill-in with Klenow fragment of DNA Polymerase I (Maniatis *et al*, 1982).

RNase protection experiments

The 3' end of the gene was mapped by RNase protection assays according to modifications of the methods of Melton *et al.* (1984) using reagents supplied by Ambion, Inc. (Austin, TX). The 1.3 kb and the 0.38 kb EcoRI genomic fragments and the corresponding EcoRI fragments of pCam8 (Fig. 1) were cloned into T3/T7 vectors (Boehringer Mannheim). Transcriptions were carried out with T7 RNA polymerase (McAllister *et al.*, 1981) in the presence of α-^{32}P UTP (New England Nuclear) to generate a radioactive cRNA probe ($1.0x10^6$ dpm/μg). Probes ($1.0x10^5$ cpm) were hybridized to 2 μg of poly A^+ RNA by heating to 90°C in 20 μl of 80% deionized formamide, 40 mM PIPES pH 6.4, 400 mM sodium acetate pH 6.4, 1 mM EDTA, then immersing in a 42°C water bath for 4 h. Control samples consisted of a parallel treatment with 10 μg yeast tRNA. Single-stranded RNA was digested with 20 units of RNase T1 or 20 units each of RNase A and T1 for 30 min at 37°C. Digestions were then terminated by a 15 min, 37°C incubation with 10 μl of 20% SDS and 10 μl of proteinase K/yeast RNA mixture (supplied by Ambion). Digests were extracted with phenol/$CHCl_3$/isoamyl alcohol (25/24/1), and precipitated with 2.5 volumes of 95% ethanol. The precipitates were redissolved in 8 μl of loading buffer (Sanger and Coulson, 1975), heated for 4 min to 90°C, and loaded onto a 6% polyacrylamide, 8 M urea sequencing gel (Maxam and Gilbert, 1980). The gel was transferred to Whatman 3mm paper, wrapped in Saran wrap and autoradiographed.

In situ *hybridizations*

For the embryo collections, Canton S adults were allowed to lay on agar medium for 24 h. The embryos were washed off the surface of the medium into a series of USA standard testing sieves. The upper sieves removed the adult flies and debris and the number 60 sieve contained the embryos, which were then dechorionated by placing them in 50% Clorox for 3 min. They were collected onto silk bolting cloth, washed extensively with water, and air dried briefly. Whole mount embryos were prepared and probed with digoxigenin-labeled pCam4 (see Fig. 1) according to Tautz and Pfeifle (1989), using the Genius Kit (Boehringer Mannheim). Embryonic stages were determined according to Campos Ortega and Hartenstein (1985). The embryos were photographed using Nomarski interference optics on an Olympus Vanox AH2 microscope. For the adult *in situ* hybridizations, sense and antisense tritiated cRNA probes were synthesized also using pCam4. Adult w sn^w flies (strain provided by W. Gehring) were embedded in OCT (Miles Inc.) and sectioned at 8 microns. The sections were post fixed and hybridized with 400,000 cpm of probe according to the procedure of Hafen and Levine (1986) with modifications (Courchesne-Smith and Tobin, 1989). The hybridization probe ($6.3x10^6$ dpm/μg) was generated from the same recombinant as for the embryo hybridizations. Sections were postfixed, exposed for 5 days, developed, and stained with Geimsa.

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