Structure and expression of the *Drosophila* calmodulin gene

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ABSTRACT

We have isolated and characterized cDNA and genomic clones representing the calmodulin gene of *Drosophila melanogaster*. As demonstrated by genomic blots and by reconstruction experiments, the calmodulin gene is represented once in the *Drosophila* genome. *In situ* hybridization of cloned probes to the polytene chromosomes of third instar larvae permitted the localization of the gene to region 49A on the left arm of the second chromosome. Two transcripts of 1.65 and 1.9 kb are produced from this gene. The accumulation of calmodulin message was measured at several stages of *Drosophila* development. The results of these experiments suggest developmental regulation of the gene. Three intervening sequences interrupt the protein coding nucleotides and two of these are located within calmodulin functional domains. The DNA sequence encoding the protein is presented; the derived amino acid sequence is compared to that of other species. The structural similarities of the *Drosophila* calmodulin gene to calmodulin genes of other species and to other calcium binding protein genes are discussed.

INTRODUCTION

The major calcium (Ca^{2+}) receptor in most eucaryotic cells is the small Ca^{2+} -binding protein calmodulin. This highly conserved protein is found throughout the plant and animal kingdoms, an observation which suggests a role for calmodulin in basic cellular processes. Indeed, data from a number of investigations, using a variety of techniques, support roles for calmodulin in such processes as secretion, mitosis, and cellular motility, while the results of biochemical studies demonstrate that calmodulin regulates many enzyme activities (1). In addition, structural studies on calmodulin and other Ca^{2+} -binding proteins have provided insights into the function of calmodulin at the molecular level (2-4).

A genetic approach has not been fully exploited in the study of calmodulin. Calmodulin mutations, especially conditional mutations, should complement structural and biochemical studies by allowing *in vivo* manipulation of the protein. *Drosophila melanogaster* is well suited for this

approach. Calmodulin has been purified from Drosophila melanogaster, and the available data indicate that the Drosophila protein is structurally and functionally similar to mammalian calmodulin (5). Furthermore, calmodulin-dependent cAMP phosphodiesterase and calmodulin-regulated protein kinase activity (6) are present in Drosophila. Analogous calmodulin-dependent enzyme activities have been described in other eucaryotes (7), indicating the usefulness of Drosophila as a model system for the study of calmodulin function. Finally, the vast array of genetic techniques available with Drosophila permit straightforward mutational analysis. This paper describes the isolation and characterization of the Drosophila calmodulin gene.

MATERIALS AND METHODS

Screening Libraries

A Drosophila genomic library, cloned into lambda charon 4 (8), was screened using the Benton-Davis procedure (9). Ten thousand plaques were plated per 15 cm plate. The prepared filters were prehybridized in 50% formamide, 5X SSC, 10 mM Hepes (pH 7.0), 1 mM EDTA, 5X Denhardt's (10) and 0.1% SDS at 37°C for at least 5 hours, then hybridized at 37°C overnight in the same solution to which was added 1×10^6 cpm of nick-translated probe per filter (8). After hybridization, filters were washed two times in 2X SSC, 0.1% SDS at room temperature for 15 minutes, then once at 50°C in 0.1 X SSC, 0.1% SDS for 30 minutes. The cDNA library in lambda gt10 was screened with the 500 bp EcoRI-PstI fragment (see Figure 1) by the same procedure except that the hybridization and prehybridization buffers were 50% formamide, 10 x Denhardt's (10), 50 mM Tris pH 7.5, 5 x SSC, 0.1% SDS, 100 μ g/ml E. coli DNA, 100 μ g/ml pBR322 DNA.

Phage Isolation, Plasmid Preparation, and DNA Purification

Purified phage were isolated from plate lysates by spinning through a CsCl step-gradient (11) or by adsorption to DE52 (12,13). Fragments prepared as described below were subcloned into pBR322 or pBR329. Small scale preparations of these plasmids were carried out by a rapid boiling procedure (14) or by alkaline lysis (15), while larger amounts were prepared by lysis (2 mg/ml lysozyme) followed by centrifugation in cesium chloride/ethidium bromide gradients (16). Phage DNA was isolated by phenol/chloroform extraction (11).

Some DNA fragments were isolated from agarose gels by electrophoresis onto, and elution from, DE 81 paper (16). Other DNA fragments were

electroeluted from agarose or acrylamide gel slices through an acrylamide plug into dialysis tubing (17). The 27-base oligonucleotide was synthesized on an Applied Biosystems DNA Synthesizer using phosphoramidite chemistry, and was purified by gel electrophoresis.

Labelling of Probes

Nick-translation was performed as previously described (18). To end label the oligonucleotide, a reaction mixture containing 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM DTT and 1 mM spermidine was used. Following the addition of 100 μ Ci of ³²P-ATP, 100 ng of oligonucleotide, and 5 units of T₄ polynucleotide kinase, the reaction was incubated at 37°C for 30 minutes. Unincorporated ³²P-ATP was removed by centrifuging the sample through a Sephadex G-25 spin-column (16). Labelled cRNA probes were synthesized in vitro using a T7 promoter system directed toward inserts subcloned into Gemini vectors (Promega Biotec) or pTZ vectors (PL Biochemicals). Single stranded probes were synthesized from the 500 bp EcoRI-PstI insert containing the coding region corresponding to amino acids 59-139.

DNA Sequence Determination

The DNA sequences of various subcloned fragments were determined by chemical modification (19) or by dideoxy chain termination methods (20,21). Computer analysis of sequence information was carried out with the use of the Bionet Computer Resource (Intelligenetics, Palo Alto).

RNA Isolation

Total RNA was isolated using guanidinium-isothiocyanate and centrifuging through a CsCl cushion (17,22). Poly(A) RNA was prepared using oligo-dT cellulose chromatography (P-L Biochemicals).

Preparation and Hybridization of Nucleic Acid Blots

Agarose gel electrophoresis, nucleic acid blotting, and dot blots were carried out as described (23). The labelled oligonucleotide probe was hybridized at 37°C in 6X SSC, 1 mM EDTA, 0.1% SDS, 5X Denhardt's (10) and 1 mg/ml heparin for at least 1 hour at a concentration of 10 ng oligonucleotide/ml hybridization solution. Filters were then washed twice at room temperature in 6X SSC, 0.1% SDS, then once in 6X SSC, 0.1% SDS at 37°C for 10 minutes. Southern (24) blots using nick-translated (18) probes were hybridized overnight at 37°C in 50% formamide, 5X SSC, 0.1% SDS, 1 mM EDTA, 1X Denhardt's, 100 μ g/ml denatured *E. coli* DNA containing 1 X 10⁶ cpm of labelled probe after at least 4 hours of prehybridization in the same buffer without probe. Experiments to look for the presence of homologous

sequences in the Drosophila genome were performed using cRNA probes complementary to the 500 bp EcoRI-PstI fragment. Both formamide concentration and temperature were varied. We tested for hybridization to additional bands on genomic blots under the following conditions: 50% formamide, 5X SSC, 0.1% SDS, 1 mM EDTA, 1X Denhardt's, 100 µg/ml denatured E. coli DNA, 200 µg/ml baker's yeast tRNA at 55° with washing in 0.2X SSC, 0.1% SDS at 65° (our normal stringency for these probes); 50% formamide at 42° with washing at 50°; 50% formamide at 37° with washing at 50°; 30% formamide at 37° with washing at 50°; and 0% formamide with washing at 50°.

RNA blots were prepared (16) by the electrophoresis of total RNA from embryos (0-24h) larvae (90-99h) or pupae (167-174h). Twenty micrograms total RNA was electrophoresed in a formaldehyde-agarose gel, using an RNA ladder (Bethesda Research Labs) as marker. Following electrophoresis, the marker lanes were stained in ethidium bromide, destained, and photographed. The sample lanes were transferred to ICN Biodyne nylon membrane using 20X SSC as the transfer buffer. Filters were baked at 80°C in a vacuum oven for one hour. RNA blots were prehybridized overnight in 50% formamide, 5 X SSC, 50 mM sodium phosphate pH 6.5, 250 µg/ml denatured E. coli DNA, 1 X Denhardts (10), 200 µg/ml baker's yeast tRNA. Hybridizations were carried out overnight at 55°C in 40% formamide, 4 x SSC, 40 mM sodium phosphate pH 6.5, 200 µg/ml denatured E. coli DNA, 160 µg/ml yeast tRNA, and 10% dextran sulfate containing 5 x 10⁶ cpm of labelled cRNA. The blots were rinsed four times in 2 x SSC, 0.1% SDS, then washed at 65°C in 0.1 x SSC, 0.1% SDS for at least 30 min with two changes of buffer.

In situ Hybridization Techniques

Tritiated cRNA complementary to the 5 kb PstI-PstI DNA fragment was hybridized in situ as described (17,25).

RESULTS

Isolation of the Drosophila calmodulin gene

The Drosophila calmodulin gene was isolated by screening a D. melanogaster genomic library (8) with an electric eel (E. electricus) cDNA clone (pCM 109, generously provided by A. Means; see 26) containing part of the calmodulin sequence. A screen of 40,000 plaques yielded a single lambda recombinant, designated λ DCM, containing an 18 kilobase pair (kb) insert. Restriction maps of the insert and several subclones are shown in Figure 1. As demonstrated by Southern blotting (24) experiments, the eel calmodulin

B

Figure 1. Physical map of the calmodulin gene recombinant lambda clone (A) and cDNA clone (B). Restriction endonuclease cleavage sites are indicated. The positions of amino acid residues, 1, 58, 59, 139, and 148 are shown. The DNA fragments which have been subcloned are indicated by name beneath each map. The orientation and extent of DNA sequencing runs are shown above each recombinant map. The direction of transcription is from left to right. Scale for diagram A is in kilobase pairs (kb); diagram B is presented in base pairs (bp).

cDNA hybridized to a 2.6 kb EcoRI-EcoRI fragment of the lambda clone. Subsequent analysis of this fragment identified a 500 base pair (bp) EcoRI-PstI fragment which cross hybridized with the eel calmodulin cDNA. This fragment was subcloned into pBR 322. In addition, the 2.6 kb EcoRI-EcoRI fragment which contains the 500 bp EcoRI-PstI fragment was subcloned. Subsequent nucleotide sequence analysis of the 500 bp EcoRI-PstI fragment confirmed that the insert contains part of the calmodulin sequence (see below).

Copy Number

To determine the arrangement of calmodulin sequences in the Drosophila genome, the labelled 500 bp EcoRI-PstI fragment was used to probe Southern (24) blots of genomic DNA. Figure 2 shows the autoradiogram resulting from hybridization of this labelled insert to Drosophila (Canton S) genomic DNA digested with various restriction enzymes. A single hybridization band at the position predicted from restriction endonuclease cleavage sites of the

Figure 2. *Drosophila* genomic DNA hybridization to calmodulin probe. Canton S strain DNA from a 24 hour embryo collection was cut with several restriction endonucleases: B (BamHI), E (EcoRI), H (HindIII), Pst (PstI), Pvu (PvuI), X (XbaI). 2.5 micrograms of cleaved genomic DNA were electrophoresed in each lane of an agarose gel, transferred to nitrocellulose, and probed with nick-translated (18) 500 bp EcoRI-PstI DNA. The resulting autoradiogram is shown with size markers (kb).

lambda recombinant is observed for each of the digested DNA samples except for the XbaI digested sample. In the XbaI sample lane, two labelled fragments are present, one at 2.6 kb and the other at 4 kb. The 4 kb XbaI fragment is probably not a partial digestion product because the predicted fragment size from the lambda recombinant is 4 kb. The presence of two bands may therefore indicate a restriction site polymorphism.

The results of the Southern (24) blotting experiments described above suggest the *Drosophila* calmodulin gene is a single copy sequence. Because Nojima et al. (27) have identified two bona fide calmodulin genes which encode identical proteins, but which cross-react only partially under normal conditions, this hypothesis was examined in greater detail. However, hybridization of similar genomic Southern blots at lower levels of stringency failed to reveal additional labelled restriction fragments (data not shown). To eliminate the possibility that more than one copy was present in the

Figure 3. In situ hybridization of cRNA complementary to 5 kb PstI-PstI DNA to salivary gland polytene chromosomes of Drosophila melanogaster. Tritiated cRNA was synthesized using the 5 kb PstI-PstI fragment as template (25) and hybridized to DNA of salivary gland chromosomes. The cytological locus 49A was autoradiographically labelled by this procedure, and that portion of chromosome 2 is shown here.

hybridizing band (either from very closely related genes or from tandemly repeated genes), a genomic reconstruction experiment (28) was performed. A known amount of genomic Drosophila DNA was digested with EcoRI and electrophoresed in parallel with amounts of the 2.6 kb EcoRI-EcoRI insert corresponding to a dosage of one to ten copies of the gene per haploid genome. This blot was transferred to nitrocellulose and probed with the labelled subcloned 500 bp EcoRI-PstI fragment. A comparison of the hybridization signals indicated that the labelled restriction fragment containing the calmodulin gene is present once per haploid genome (data not shown).

Chromosomal location

The cytogenetic locus of the calmodulin gene was determined by in situ hybridization of tritiated cRNA to polytene chromosomes from the salivary glands of third instar larvae (17, 25, 29). The probe was synthesized using the subcloned 5kb PstI-PstI fragment as template for cRNA synthesis (see Figure 1). In agreement with the results of the genomic reconstruction experiment, hybridization occurred at a single chromosomal site, in region 49A (30) on chromosome 2 (Figure 3).

Gene Expression

The expression of the calmodulin transcript during Drosophila development was examined by hybridization of labelled RNA complementary to the 500 bp EcoRI-PstI fragment. We have carried out a series of northern blot

Figure 4. Northern blot analysis. Equal amounts of total RNA from embryos (left lane), larvae (middle lane), and pupae (right lane) were electrophoresed on formaldehyde gels, blotted onto nylon membrane (16), and probed for calmodulin message using *in vitro* labelled RNA complementary to the 500 bp EcoRI-PstI fragment. Sizes of RNA ladder markers (kb) and exposed autoradiographic bands are indicated.

hybridizations probing RNAs isolated from 0-24 hour embryos, third instar larvae (90-99 hours), and pupae (150-159 hours) (Figure 4). All experiments demonstrated the existence of two calmodulin mRNAs of 1.65 and 1.9 kb in length. Both transcripts are present in each RNA population examined. The total amount of message was highest in the larval stage. The amount of the smaller transcript appears to be greater in embryos and larvae, but in pupae the transcripts have accumulated approximately equally.

Structure and Sequence

An investigation of the structure and sequence of the *Drosophila* calmodulin gene was carried out. Nucleotide sequence was obtained from the 500 bp EcoRI-PstI fragment and from the adjacent 200 bp HindIII-EcoRI fragment (see Figure 1). An analysis of these sequences revealed an open reading frame corresponding to amino acids 59-139 of bovine calmodulin (31,32). At both ends of this coding region, the reading frame is lost and nucleotides indicative of a possible splice junction are present.

Because the eel calmodulin cDNA does not extend any further in the 5' direction, an oligonucleotide probe (5'-TTGATAAGGATGGCAATGGCACCATC-3') was

Figure 5. DNA sequence of Drosophila calmodulin gene coding region and derived amino acid sequence. Residues at which the amino acids of bovine (31,32) and Drosophila calmodulin differ are underlined. Positions of intron-exon splice junctions are marked with arrows.

used to identify the 5' protein-coding exon of the Drosophila gene. This 27 base oligonucleotide corresponds to amino acids 19-27 of bovine calmodulin. In blotting experiments, the probe hybridized to the 5.4 kb EcoRI fragment of λ DCM (see Figure 1). Sequence analysis of this fragment identified an open reading frame corresponding to amino acids 1-58 of the bovine calmodulin sequence. Both sides of this coding region are also flanked by consensus splice junction sequences. Thus, the initiating methionine is separated from the first amino acid (alanine) of the calmodulin sequence by an intron. The identical situation occurs with the chicken calmodulin gene (33,34).

A similar approach to identify the 3'-end of the gene, using oligonucleotides as probes, proved unsuccessful. Therefore, several calmodulin cDNA clones were isolated from a cDNA library that was synthesized using as template polyA RNA isolated from the heads of adult flies (0-24 hours after eclosion) of the Oregon R P2 strain (a gift of B. Yedvobnick and S. Artavanis-Tsakonas). Sequence analysis of one of these cDNA clones provided the sequence encoding amino acids 140-148 of Drosophila calmodulin. A subcloned probe derived from the cDNA clone (115 bp EcoRI-EcoRI) encoding these last nine residues plus 80 base pairs of the 3'-untranslated region failed to hybridize to the original lambda recombinant, indicating that the 3'-end of the calmodulin gene is not present in λ DCM.

The completed nucleotide sequence of the Drosophila calmodulin coding region is summarized in Figure 5. This sequence encodes a 148 amino acid protein which is very similar to bovine calmodulin. The derived Drosophila calmodulin sequence differs from bovine calmodulin (31,32) at three

positions; these include phenylalanine rather than tyrosine at residue 99, threonine rather than glutamine at residue 143, and serine rather than alanine at residue 147.

DISCUSSION

We conclude that Drosophila calmodulin is encoded by a single gene that spans over 10 kb of genomic DNA and contains a minimum of three introns. One of the introns separates the initiating methionine from the rest of the protein, while the other two are inserted into the calmodulin coding region at positions corresponding to amino acids 58 and 139 (31,32). The intron at position 58 is approximately 4 kb in length. The sizes of the other two introns are unknown, although the intron at position 139 extends at least 5.9 kb.

Like Drosophila, Dictyostelium possesses a single calmodulin gene (35), as do the genomes of yeast (36) and sea urchin (37). Multiple calmodulin genes have been reported in other eucaryotes, however. Trypanosome calmodulin is encoded by three tandemly repeated genes, all of which code for the identical protein (38). In X. laevis, two genes are present (39); again, both encode the same calmodulin sequence. Two calmodulin genes also occur in the chicken, but in this case, the genes are different. One encodes a protein that is identical to bovine calmodulin, while the other is a processed pseudogene (40) encoding a protein with 19 amino acid substitutions (41). Rat contains four related calmodulin genes (27); two are bona fide genes, and two are processed pseudogenes derived from the bona fide genes. Whatever the reason for the occurrence of multiple calmodulin genes in these eucaryotes, the presence of a single calmodulin gene in Drosophila is convenient for the isolation of mutants. Analysis of calmodulin mutants, coupled with studies on the properties of mutant calmodulin proteins which have been expressed in bacteria (41,43), should provide insights to the molecular and functional properties of calmodulin.

The expression of the calmodulin gene shows a variable pattern of accumulation during Drosophila development. The accumulation of calmodulin messages is highest in larvae and lowest in pupae. These levels may be a reflection of the role of calmodulin in the regulation of cellular contractile events. The ratios between the two transcripts also change among the stages. These results are, of course, the total of the average accumulation of calmodulin gene transcripts throughout the whole organism. We plan in situ hybridization studies to permit the determination of

calmodulin message accumulation in each specific tissue during Drosophila development.

Transcripts of two sizes (1.65 and 1.9 kb) are produced by the Drosophila calmodulin gene. These two transcript sizes are surprisingly similar to the sizes of the major calmodulin transcripts of chicken (44), which were reported to be 1.6 and 1.9 kb in length. However, the smaller chicken calmodulin transcript is much more abundant than the larger chicken calmodulin transcript. In chicken RNA populations, however, the relative proportions of the two transcripts appear to remain constant in various tissues and stages of development. Three transcripts of 1.4, 1.7 and 4.0 kb are observed in the rat (27). The 1.4 kb transcript appears to be brain-specific and is derived from one gene, while the 1.7 and 4.0 kb transcripts are derived from the other gene and are expressed at similar levels in brain, liver and muscle. Multiple calmodulin transcripts have also been observed in the electric eel and in rat (45). Because the Drosophila calmodulin gene appears to be represented only once per haploid genome, we conclude that the two Drosophila transcripts are the result either of production of two different primary transcripts or of differential processing of a single primary transcript.

One aspect of this study which has proven particularly interesting is the conservation of calmodulin intron positions. The locations of the three Drosophila intervening sequences are in exactly the same positions relative to the calmodulin protein coding sequence as three of the six chicken introns (33,34). Intervening sequences are also found in these positions in the rat (46). The location of these conserved introns is surprising, particularly because two of the insertion points are within calcium-binding domains. Calmodulin contains four calcium-binding sites, each made up of a 12 amino acid calcium-binding loop flanked by two helices (4). The Drosophila introns interrupt calcium-binding subdomains II and IV. The intervening sequences of Drosophila calmodulin thus do not mark the boundaries between different functional domains of the protein. It may be, as hypothesized by Babu *et al.* (4), that these introns separate regions that are on the surface of the molecule. If the comparison is extended to other members of the EF-hand calcium binding superfamily for which gene structures are known, the similarities are remarkable. These include sea urchin Spec 1 genes (47) and myosin light chain genes (48,49). Even parvalbumin, which has lost one of its calcium binding domains entirely, has a similar gene structure (50). Since these introns do not lie at the boundaries of calcium binding domains,

Hardin *et al.* postulated that the present gene structure of this superfamily evolved after the four calcium binding domains had arisen from gene duplications (47). Information on the exact positions of calmodulin gene introns in other organisms has not yet been reported, but at least in chicken (33,34), rat (46), and Drosophila, the structural characteristics of this gene family have been conserved.

The Drosophila amino acid sequence derived from the nucleotide sequence illustrates the highly conserved nature of this protein. Compared to the sequence of bovine brain calmodulin (31,32) there are only three amino acid substitutions, occurring at positions 99 (Tyr to Phe), 143 (Glu to Thr), and 147 (Ala to Ser). The identical amino acid substitutions have been described in the scallop (51). Amino acids 143 and 147 reside in the last helix outside the calcium binding segment (4). Although position 99 lies within one of the calcium binding segments, the substitution of phenylalanine for tyrosine is conservative. In addition, phenylalanine is found at this position in other invertebrates (52). Because calmodulin interacts with so many different proteins and occupies such a central role in the structural and metabolic regulation of the eucaryotic cell, it is reasonable to suppose that most of the protein is involved in critical functional roles. Different reactive domains on the calmodulin molecule might be utilized during the many interactions of calmodulin (1, 53-56). Calmodulins altered by genetic engineering and expressed in bacteria interact differentially with myosin light chain kinase and calcineurin or calmodulin-dependent multiprotein kinase (41).

The small size, multiple interactions, and conserved nature of calmodulins imply that even minor structural alterations would not be tolerated. However, some of the genetically engineered calmodulins mentioned above (41) retained significant activity, leading these authors to suggest that some mutations could occur which would not necessarily be lethal but which would alter the ability of calmodulin to interact with particular sets of calmodulin-dependent proteins. The introduction of genetically engineered forms of this Drosophila calmodulin gene into mutant embryos will provide the means to analyze the myriad of functional interactions of calmodulin.

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