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RICE UNIVERSITY

THE EXPRESSION AND REGULATION OF THE SINGLE CALMODULIN GENE OF DROSOPHILA MELANOGASTER

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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ABSTRACT

The Expression and Regulation of the Single Calmodulin Gene of
Drosophila melanogaster.

by

Bernard F. Andrus

Calcium and calmodulin-based regulation of cell activity is one of the
most widespread signaling mechanisms in eukaryotic organisms. Extensive
experimentation has uncovered many diverse roles for calcium signals,
many of which involve calmodulin. Calmodulin interacts with and
regulates a large and growing number of target proteins. For most of
these targets, interaction with calcium-bound calmodulin results in
activation of the target protein. In addition to regulating numerous
proteins directly, several calmodulin targets are also multifunctional
regulators such as kinases and phosphatases. Thus, Ca\(^{2+}\)-calmodulin-based
signaling is a powerful regulator of cell activities.

Consistent with its importance in cellular processes, calmodulins
from diverse eukaryotic species show remarkably high conservation at the
amino acid level, typically being greater than 90% identical. This strong
conservation and the discovery that Drosophila calmodulin is encoded by a
single gene make Drosophila melanogaster particularly well suited for the
study of the role of calmodulin in a multicellular organism.

The experiments presented here examine the expression of the single
calmodulin gene of Drosophila at the levels of mRNA and protein. *In situ* hybridizations to post-embryonic stages of Drosophila development reveal a complex pattern of calmodulin gene expression, as was suggested by previous examination of calmodulin transcript expression during embryogenesis. Some tissues such as the gut appear to have a greater need for calmodulin transcripts when they are highly active, while others, such as the musculature have higher calmodulin mRNA levels during their formation. The central nervous system produces high amounts of calmodulin mRNA during periods of inactivity that appear to be required for later function instead of immediate uses. Immunolocalization and immunoblotting experiments suggest the possibility of translational regulation of calmodulin protein levels in the central nervous system and indicate low turnover of calmodulin protein during most of embryogenesis. The expression and localization of calmodulin suggests roles for calmodulin in the regulation of neural function, mitosis and cell division cycles, cytoskeletal dynamics and cell shape changes, and secretion.
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<tr>
<td>AED</td>
<td>After Egg Deposition</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetraacetic Acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>[Ethylenebis(oxyethylenenitrilo)]tetraacetic Acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule Associated Protein</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule Organizing Complex</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>SPB</td>
<td>Spindle Pole Body</td>
</tr>
<tr>
<td>TDT</td>
<td>Tergal Depressor of the Trocanter</td>
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Chapter 1: Introduction

Calmodulin is one of the predominant messenger proteins in eukaryotic cells. The work presented here examines the expression of this multi-functional calcium signal transducer in the fruit fly *Drosophila melanogaster*. This chapter discusses the basic mechanisms of calmodulin-based signaling and the rationale for using Drosophila as a model organism. In addition, the major events of Drosophila development will be described here as a preview for later discussions.

A. The Universality of Calcium/Calmodulin Signaling

Calcium is a major second messenger in biological systems. For several reasons calcium is uniquely qualified to be a signaling molecule. Calcium is abundant in nature, typically found in millimolar concentrations in many environments. Calcium concentrations this high cause serious complications for living organisms including precipitation of macromolecules and interference with enzymatic reactions (Vogel, 1994). Calcium pumps are employed to lower intracellular calcium concentrations to approximately $10^{-7}$ molar in most cells. The existence of a sharp gradient between the external and internal concentrations of calcium make it uniquely suited to be an important messenger molecule (Vogel, 1994). Disruption of calcium signaling has been used extensively to determine roles for calcium in a great variety of cellular processes (Berridge, 1995; Takuwa et al., 1995).
Calmodulin is a major mediator of calcium signaling in eukaryotic organisms through its ability to regulate the activity of a wide variety of proteins in a calcium dependent manner (Manalan and Klee, 1984). Since calmodulin has been isolated from a large number of different species, it is considered a universal component of eukaryotic organisms (Cohen and Klee, 1988). The calmodulins from different species shows remarkable conservation such that vertebrate calmodulins have identical amino acid sequences even though they are encoded by three distinct genes (Nojima, 1989). In organisms as evolutionarily separated as plants, animals and fungi calmodulin sequence identity typically exceeds 85% (Smith et al., 1987).

Calmodulin was first identified as a heat stable component of brain extracts that was required for maximum activity of cyclic nucleotide phosphodiesterase (Klee and Vanaman, 1982). Since then, calmodulin has been shown to interact with a large and ever increasing variety of different proteins. Although most of them are activated by the calcium-bound form of calmodulin, targets that interact with the calcium-free form have been recently identified. Table I contains a partial list of known calmodulin targets and some of the cellular processes in which calmodulin is thought to function. Most of the proteins and processes listed in Table I have homologs in numerous species throughout the plant, animal and fungal kingdoms and are likely to be universal targets of calmodulin signaling.

Calmodulin is a small, acidic protein composed of 148 amino acids. Extensive conformational studies have demonstrated that calmodulin is composed of two terminal domains separated by a long central tether (Figure 1-1; Babu et al., 1985; Taylor 1991). Each terminal domain is
<table>
<thead>
<tr>
<th>Protein target</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic nucleotide phosphodiesterase</td>
<td>intracellular signal transduction</td>
</tr>
<tr>
<td>Plasma membrane Ca^{2+}-ATPase</td>
<td>intracellular signal transduction</td>
</tr>
<tr>
<td>Adenylate cyclase</td>
<td>intracellular signal transduction, neuronal plasticity, gene expression</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td>intracellular signal transduction, gene expression</td>
</tr>
<tr>
<td>Ion channels</td>
<td>intracellular signal transduction, neurotransmission</td>
</tr>
<tr>
<td>Ca^{2+}-calmodulin dependent kinases</td>
<td>intracellular signal transduction, neuronal plasticity, gene expression, cell cycle regulation, cell proliferation</td>
</tr>
<tr>
<td>calcineurin</td>
<td>intracellular signal transduction, gene expression, cell proliferation</td>
</tr>
<tr>
<td>phosphorylase kinase</td>
<td>intracellular signal transduction</td>
</tr>
<tr>
<td>receptor tyrosine kinases</td>
<td>signal transduction, cell proliferation, muscle contraction</td>
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<td>myosin light chain kinase</td>
<td>muscle contraction</td>
</tr>
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<td>caldesmon</td>
<td>muscle contraction</td>
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<tr>
<td>myosins</td>
<td>cellular movements and contractions</td>
</tr>
<tr>
<td>bHLH transcription factors</td>
<td>gene expression</td>
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<tr>
<td>nuclear DNA-binding proteins</td>
<td>DNA-matrix interaction, DNA synthesis</td>
</tr>
<tr>
<td>NAD kinase</td>
<td>cellular metabolism</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>cellular metabolism</td>
</tr>
<tr>
<td>Synapsin</td>
<td>vesicle trafficking, neurotransmission</td>
</tr>
<tr>
<td>Gap-43/ neuromodulin</td>
<td>cytoskeletal dynamics, neuronal pathfinding</td>
</tr>
<tr>
<td>adducin</td>
<td>cytoskeletal dynamics</td>
</tr>
<tr>
<td>spectrin</td>
<td>cytoskeletal dynamics, cell shape</td>
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<td>translation elongation factor 1α</td>
<td>cytoskeletal dynamics</td>
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<tr>
<td>microtubule associated proteins</td>
<td>cytoskeletal dynamics, mitosis, meiosis</td>
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composed of two EF-hand calcium-binding sites (Kretsinger and Nockolds, 1973) each composed of a calcium-binding loop flanked by alpha helices (Babu et al., 1985). Cooperative binding of calcium by the four binding sites in calmodulin induces a conformational change exposing hydrophobic residues in each domain that are believed to mediate target binding. The binding site for calmodulin on most proteins takes the form of a basic amphipathic helix (Meador et al., 1992; Ikura et al., 1992), to which Ca\textsuperscript{2+}-calmodulin binds inducing a conformational change in both calmodulin and the target protein. For targets with enzymatic functions the result is usually stimulation of the enzymatic reaction.

B. Calmodulin in *Drosophila melanogaster*

Several characteristics of the fruit fly, *Drosophila melanogaster*, make it a useful organism for studies of gene function and expression studies. Genetic analysis of Drosophila goes back nearly one hundred years (Flybase, 1994), making it one of the most well characterized organisms both genetically and developmentally. Its small size, rapid generation time, and the development of numerous genetic tools make a wide variety of experiments possible, including large scale random mutagenesis, generation of transgenic animals and inducible gene expression (Ashburner, 1989a). Furthermore, compared to vertebrate systems it has a simple and efficient genome, often possessing only a single gene for a given protein which in vertebrates is often represented by multiple copies (Perin et al., 1991; Tohtong et al., 1997).
Figure 1-1. Ribbon diagram representation of the crystal structure of Drosophila calmodulin. Calmodulin is composed of two domains that each have two calcium binding sites. The two domains are separated by a long stretch of amino acids that appear as an α-helix in the crystal structure. Adapted from Taylor et al., 1991.
Calmodulin is encoded by a single gene in Drosophila and differs in only three conservative amino acid substitutions from vertebrate calmodulin (Figure 1-2; Smith et al., 1987; Doyle et al., 1990). Extensive biochemical and biophysical studies of Drosophila calmodulin show that the protein adopts almost identical conformations and behaves very similarly to vertebrate calmodulins in calcium binding and target activation experiments (Maune et al., 1992a; Maune et al., 1992b; Gao et al., 1993).

Two transcripts of 1.7 and 1.9 kb are produced by the Drosophila calmodulin gene that differ only in the site of polyadenylation and do not affect the sequence of the protein product (Doyle et al., 1990). Although both transcripts can be detected at all stages, the relative abundance changes throughout development with the smaller transcript more abundant in ovaries and early embryos while the larger transcript is predominant in late embryos and pupae (Doyle et al., 1990; Kovalick and Beckingham, 1992).

A study of the calmodulin mRNA expression pattern during embryogenesis by in situ hybridization revealed a surprising pattern of transcript production and location (Kovalick and Beckingham, 1992). Maternally-contributed calmodulin mRNA is distributed uniformly in embryos from egg deposition through the beginning of gastrulation. During gastrulation these calmodulin transcripts are gradually degraded and have been eliminated from most tissues by maximal germ band extension. Zygotic production of calmodulin transcripts is seen only in cells of the central and peripheral nervous system (CNS and PNS; Kovalick and Beckingham, 1992).
Figure 1-2. The amino acid sequence of Drosophila calmodulin. Inside the circles are the single letter abbreviations representing the amino acid sequence of Drosophila calmodulin. The letters outside the circles represent the three amino acid differences between Drosophila and vertebrate calmodulins.
A calmodulin null mutant, \textit{Cam}^{n339}, has been generated in Drosophila by imprecise excision of a transposable element inserted just 5' of the calmodulin gene (Heiman et al., 1996). This mutation removes the first two exons of the gene and fails to produce zygotic calmodulin transcripts in homozygous embryos. Essentially no defects in embryogenesis are produced by the calmodulin null mutation and these embryos hatch as viable first instar larvae. Similar results have been observed with null mutations of other genes for which significant amounts of protein or mRNA are contributed by heterozygous mothers (Lee et al., 1993; Lane and Kalderon, 1993). CaM null larvae, however, show striking abnormalities in behavior, including sluggishness, frequent backward motion (interpreted as spontaneous avoidance behavior), and excessive head swinging motions (Heiman et al., 1996). These larvae die during the first instar stage and show greatly reduced levels of calmodulin protein on immunoblots (Heiman et al., 1996).

C. Overview of Drosophila Development

Figure 1-3 shows an overview of the Drosophila life cycle. During embryogenesis, which takes approximately 24 hours at 25°C, the unstructured egg transforms into a fully differentiated larva (Figure 1-4; Campos-Ortega and Hartenstein, 1985). Embryogenesis proceeds without growth but instead most proliferation occurs primarily through rapid, synchronous nuclear divisions in a common cytoplasm during the first two hours of development. The zygotic nuclei migrate to the extreme periphery of the embryo and cellularization occurs when invaginations of
Figure 1-3. The life cycle of *Drosophila melanogaster*.
Figure 1-4. The stages of Drosophila embryogenesis. This diagram shows the major morphological changes which have been used to divide embryogenesis into stages. The stippled area shows the central nervous system. From Wieschaus and Nüsslein-Volhard (1986).
the plasma membrane migrate between the nuclei, isolating them as single cells. Thus, an epithelium of approximately 6000 cells surrounding a large central yolk cell is produced (Foe et al., 1993). Over the next two and one half hours the global cellular migrations of gastrulation divide the embryo into three germ layers: ectoderm, mesoderm and endoderm (Costa et al., 1993). Organogenesis and terminal differentiation of larval tissues characterizes the remainder of embryogenesis (Hartenstein and Campos-Ortega, 1985).

The larval period is characterized by extensive growth when the animal increases its size by extensive foraging. Larval life is divided into three stages, termed instars, based on increases in size that correspond to periods between molting of the larval cuticle (Ashburner, 1989a). Growth of larval tissues is accomplished not by cell division, but by numerous rounds of endoreplication and cell expansion resulting in extensive polyploidy of the larval cells (Poodry, 1980).

The fly takes on its adult form (the imago) during the rapid rearrangements of pupal metamorphosis. During the larval instars, islands of cells that remained diploid and undifferentiated during larval life expand by cell division (Poodry, 1980). Pupuration occurs when the epidermis of the third instar larvae contracts and separates from its hardening cuticle. This is followed by massive rearrangement of the tissues inside the pupa as larval tissues undergo rapid histolysis and are replaced by imaginal tissues (Cohen, 1993). The cuticular structures of the adult fly, the appendages, body walls, and sensory organs, are formed from imaginal discs, which are a subset of the diploid cell clusters that form invaginations of the larval epidermis. Most internal organs such as the CNS, gut and musculature are
formed from islands of diploid imaginal cells scattered through each larval organ (Poodry, 1980; Hartenstein, 1993).

D. Oogenesis in Drosophila

Oogenesis in Drosophila consists of continuous development of a cyst of germ cells as it passes in a posterior direction through the ovary. Figure 1-5 shows the morphology of the reproductive system of adult female flies. Each ovary is composed of about 16 independent ovarioles (Figure 1-5A), in each of which egg chambers are generated and matured (Spradling, 1993). Each egg chamber is composed of a cyst of 16 interconnected germ cells surrounded by a layer of somatic follicle cells. At the anterior end of each ovariole lies a germarium (Figure 1-5B), which contains the stem cells that give rise to the germ cell cyst and its surrounding follicle cells. The germline stem cells (2-3 per germarium) lie at the anterior end of the germarium in region 1. The stem cell divides asymmetrically to yield one daughter stem cell and one cystoblast. The cystoblast then undergoes four rounds of mitosis to yield 16 cystocytes (Mahowald and Klambysellis, 1980). Cytokinesis is incomplete during the cystoblast divisions resulting in cytoplasmic bridges called ring canals (Robinson et al., 1994). Due to the orientation of the cell divisions, two of the cystocytes will have four ring canals. One of these cells is determined to become the oocyte and takes up a posterior location within the cyst before exiting region 2b of the germarium (Spradling, 1993). Specific localization of certain mRNAs and proteins provides the earliest detectable evidence of oocyte determination (Spradling, 1993).
Figure 1-5. The Drosophila ovary. (A) A diagram of the anterior portion of one ovariole. Egg chambers originate at the anterior and migrate posteriorly as they develop. Inset - diagram of the reproductive tract. (B) A diagram of the gerarium. CB - cystoblast, CC - cystocyte, ES - sheath, FC- follicle cells, GSC - germline stem cell, NC - nurse cell, O - oocyte, TF - terminal filament. (C). A diagram of the fusome during cystoblast division. The fusome is a cytoskeletal structure that extends through the ring canals that connect the 16 germline cells each cyst. (A) and (B) are from King (1970), (C) is from Lin et al., (1994).
Regulation of germline stem cell and cystocyte division involves a membranous, cytoskeletal structure called the spectosome in stem cells and the fusome in cysts. Mutations in the genes *hu-li tai shao* (*hts*) and *bag-of-marbles* (*bam*), whose protein products are components of the fusome and spectosome, disrupt the germline stem cell and cystoblast divisions and can result in failure to properly determine the oocyte (McKearin and Ohlstein, 1995, Lin et al., 1995. The spectosome is located at the anterior of the germline stem cells and during mitosis extends toward the posterior pole. Cytokinesis cleaves the spectosome with the smaller portion segregating into the posterior daughter cell which is the cystoblast (McKearin and Ohlstein, 1995). During cystoblast divisions the fusome forms from the spectosome remnant and extends through each ring canal to form one pole of the cell division. When the cystoblast divisions are complete, the fusome is an extended branched structure (Figure 1-5C) that is thought to be the mechanism by which the oocyte is determined and accumulates localized products (Lin and Spradling, 1995).

The 15 remaining germ cells are termed nurse cells. They become highly polyploid and produce much of the material that fills the growing oocyte. Figure 1-6 shows the stages of egg chamber development. As egg chambers proceed down the ovariole, the cyst increases in size. During stages 1-6 the oocyte is similar in size to the other germ cells, and beginning in stage 7 the oocyte begins to expand due to the uptake of vitellogenins from the hemolymph (Mahowald and Klambysellis, 1980). During stage 11, nurse cells begin dumping their cytoplasmic contents into the oocyte and begin to rapidly degenerate as the oocyte expands (Spradling, 1993).
Figure 1-6. Stages of oogenesis. The images on the right are egg chambers viewed with Nomarski optics, those on the left are images obtained by fluorescent staining of the nuclei. From Spradling (1993).
Follicle cells play important roles in oogenesis. In early stages, they participate in intercellular signaling with the oocyte to determine the polarity of the both the oocyte and the identity of the follicle cells (Gonzalez-Reyes et al., 1995). During vitellogenesis, they contribute to the production of vitellogenins that are imported into the oocyte. Their final role in oogenesis involves laying down the protective coverings that will separate the embryo from its environment (Spradling, 1993). These are the inner vitelline membrane and the outer proteinaceous egg shell or chorion.

E. Studies Presented in this Manuscript

As outlined above, the high degree of conservation of calmodulin and the targets of calmodulin-based signaling point to the importance of understanding the functions of calmodulin. *Drosophila melanogaster* provides an elegant system for the study of calmodulin due to extensive genetic and developmental characterization, genomic simplicity, and experimental tractability.

The experiments to be presented here examine the expression of calmodulin mRNA and protein in *Drosophila melanogaster*. This work was undertaken to gain a better understanding of the dynamics of calmodulin expression and subcellular localization and how they relate to cellular and developmental processes. The expression pattern of calmodulin mRNA during post-embryonic development is presented in Chapter 3 followed by the production and characterization of anti-calmodulin antibodies in Chapter 4. The next two chapters discuss the use
of these antibodies for the immunolocalization of calmodulin during oogenesis and embryogenesis and quantitative immunoblot analysis of calmodulin levels in staged embryos. Finally, Chapter 7 summarizes and integrates the results of the previous chapters, with a discussion of potential roles of calmodulin during development.
Chapter 2: Materials and Methods

A. *In Situ* Hybridization

1. Preparation and sectioning of tissue

Fly strains yw\textsuperscript{67c23(2)} or Oregon R were used for *in situ* hybridization. Embryos, larvae and adults were prepared for cryosectioning by immersing animals in OTC embedding medium (Baxter) and freezing rapidly with dry ice or liquid nitrogen. Pupae were prepared similarly, but the pupal cases were opened to allow penetration of embedding medium. Adult heads were aligned in a Heisenberg collar (gift of Dr. R. Davis, Baylor College of Medicine) for the generation of frontal sections, while second and third instar larvae were oriented for both sagittal and horizontal sectioning. The size of embryos and first instar larvae precluded arrangement in specific orientations so randomly oriented sections were obtained. Larval and pupal stages were determined according to Ashburner (1989a). Blocks of frozen tissue were prepared at least one hour and not more than 24 hours before sectioning and were stored at -70°C wrapped in cellophane to prevent evaporation of the OTC. At least thirty minutes prior to use, the blocks were placed in the cryostat to equilibrate to 18°C.

Sections were prepared using a Jung 2800N cryostat. All sections were 7 μm thick except for frontal head sections, which were 10 μm in thickness. Sections were collected on gelatinized slides kept at room temperature. Gelatinized slides were prepared by briefly immersing well
rinsed slides in a solution of 0.5% gelatin and 0.05% chrome alum and then allowing the slides to air dry completely. The gelatin solution is prepared by dissolving the gelatin in warm water, allowing the solution to cool to room temperature and then dissolving the chrome alum (Hafen and Levine, 1986). Slides bearing tissue sections were placed on a 45°C hot plate for two to three seconds to assist in tissue adherence to the slide, then the OTC was allowed to evaporate for up to two hours at room temperature before fixation.

A 20% paraformaldehyde stock solution was made each day by heating a suspension of 20 grams paraformaldehyde (EM grade, Polysciences) in water to 80 degrees. Two to three drops of 5M NaOH were then added to assist dissolution of the paraformaldehyde. The solution was cooled and filtered through No. 1 Whatman filter paper. Slides were fixed 15 minutes in a freshly made solution of 4% paraformaldehyde in phosphate buffered saline (PBS; 160 mM NaCl, 7 mM Na2HPO4, 3 mM KH2PO4, pH 7.5). They were then rinsed three times for five minutes each in PBS. Slides were then passed through an ethanol series consisting of 30% ethanol in PBS, then 60%, 80%, 95% and finally 100% ethanol. Each wash was for 2 minutes except the 80% ethanol wash which lasted 5 minutes. The ethanol was evaporated and slides were stored at -70°C until used for hybridization.

2. Preparation of probes

Digoxigenin-labeled (DIG-labeled) DNA and RNA probes were generated using a 568 base pair EcoRI fragment from the 3'UTR of the
Drosophila calmodulin cDNA (δ2; Smith et al., 1987). Double-stranded DNA probes were prepared by randomly primed incorporation of digoxigenin-labeled (DIG-labeled) dUTP according to the protocol of Boehringer-Mannheim using 100 to 300 ng of gel-purified δ2 DNA. Probes were stored in hybridization buffer at -20°C.

Constructs were created by Alan Lu by inserting the δ2 fragment into the Bluescript KS+ vector in both orientations relative to the T7 polymerase promoter and were used to generate single-stranded sense and antisense RNA probes. The sense strand construct was named pAL2 and the antisense strand construct was named pAL3. Single stranded probes were generated by T7 RNA polymerase-driven in vitro transcription of these constructs with DIG-labeled UTP using a Boehringer-Mannheim protocol, with the exception that polymerization was performed at 30°C rather than 37°C, since transcription at 37°C produced transcripts significantly shorter than the expected size of around 600 bp.

RNA probes were reduced to approximately 200 bases by limited alkaline hydrolysis (Cox et al., 1984), to allow better penetration of the tissue. 50 μl of RNA probe at 16 mg/ml in water was mixed with 30 μl of 200 mM Na2CO3 and 20 μl of NaHCO3 and incubated at 60°C for 32.5 minutes. This time was designed to reduce the 700 base probes to an average length of 200 bases according to the following formula:

\[ t = \frac{(L_0 - L_f)}{(6.11 \cdot L_0 \cdot L_f)} \]

where \( t \) equals time in minutes, \( L_0 \) is the beginning length of the probe, and \( L_f \) is the desired final length of the probe (Cox et al., 1984). At the end of 32.5 minutes, probes were precipitated by addition of 5 μl 10% glacial acetic acid, 11 μl 3M sodium acetate pH 6.0, 1 μl 10 mg/ml tRNA, 1.2 μl
1M MgCl2, and 300 µl absolute ethanol. Probes were precipitated overnight then centrifuged at 14,000 g and vacuum dried. The pellet was taken up in 100 µl water treated with 1 µl RNAsin, an RNAse inhibitor, and stored at -70°C.

Before use, probes were diluted (1:1000 for RNA probes and 1:50 for DNA probes) into hybridization buffer [50% deionized formamide, 0.6 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1X Denhardt’s reagent (0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrrolidone), 0.5 mg/ml carrier RNA (Sigma, Type IV), 10% Dextran Sulfate, pH 7.5], denatured, and rapidly cooled in NaCl-ice water. DNA probes were denatured for 10 minutes at 95°C, RNA probes for 10 minutes at 80°C.

3. In situ hybridization to sectioned tissue

The method of Hafen and Levine (1986) was used for in situ hybridizations: Slides were rinsed in 0.2 N HCl for two minutes, then rinsed in SSC (150mM NaCl, 15mM sodium citrate, pH 7.0) then transferred to SSC at 70°C for 30 minutes. The slides were then rinsed in water at room temperature and fixed again for 15 minutes in 4% paraformaldehyde in PBS. This was followed by a wash in 3X PBS and dehydration through an ethanol series as before.

Hybridization was carried out by placing 20 µl of probe diluted in hybridization buffer on each slide and covering the slide with a coverslip. The slides were incubated for 12 hours at 37°C for DNA probes and 45°C for RNA probes, in a humid chamber. Coverslips were removed by immersion in warm hybridization wash solution (50% formamide, 0.6 M
NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5) at the same temperature as hybridization, and the slides were washed hybridization temperature with six to seven changes of hybridization wash solution over the next four to six hours.

Detection of the DIG-labeled probe was adapted from protocols supplied by Boehringer Mannheim. Slides were gradually equilibrated into by passing through a series containing hybridization wash buffer and genius buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) in ratios of 4:1, 2:1, 1:1, 1:2, and 1:4, all at room temperature for two minutes at each step. The tissue was then blocked for 30 minutes with genius buffer 1 plus 1% blocking reagent (Boehringer Mannheim) and 0.3% Triton X-100. The slides were then incubated under coverslips for one hour with anti-DIG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) at a 1:500 dilution in genius buffer 1 plus 0.3% Triton X-100. The slides were washed twice for 15 minutes each with genius buffer 1 and then for two minutes in genius buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5).

A colored precipitate is produced from the alkaline phosphatase conjugated to the anti-DIG antibody by incubation of the slides with the substrates NBT (4.5 µl/ml) and X-Phos (3.5 µl/ml) in buffer 3. The slides were incubated with this solution in a darkened humidified chamber for six to twelve hours until a colored product could be easily seen. The slides were then dehydrated and coverslips permanently mounted using Permount (Sigma). Hybridization patterns were shown to be specific by control hybridizations with a sense RNA probe, unless otherwise stated.
4. Histological stains

Some slides were treated to stain nuclei after in situ hybridization. Slides were treated with 1 N HCl at 60°C for 15 minutes, then stained for one hour in Feulgen reagent (0.5% basic fuchsin, 0.5% potassium metabisulfite in 0.1N HCl, charcoal filtered). Slides were rinsed twice with sulfite water (0.5% potassium metabisulfite in 0.05N HCl), then rinsed thoroughly with water and mounted as above. Standard hematoxylin and eosin staining was also performed on some sections to aid in tissue identification.

B. Generation of Anti-Calmodulin Antisera

1. Inoculation of rabbits

Purified recombinant Drosophila calmodulin was obtained from existing supplies in the laboratory that were prepared using standard purification protocols (Maune et al., 1989b). All protein preparations were sent to Cocalico Biologicals, Reamstown, PA, for mixture with Freund’s complete adjuvant and inoculation of New Zealand White rabbits. A pre-inoculation test bleed was taken from each animal and followed by an initial inoculation of 50 μg of antigen. This was followed by boosts of 10 μg each on days 14, 21 and 49 after the initial inoculation. Test bleeds yielding approximately 5ml of serum were taken on days 35 and 56. At the end of this schedule, all three serum samples from each rabbit were sent back for analysis.
2. Trypsin digestion of calmodulin

Purified calmodulin (265 μl at 6.8 mg/ml; 1.8 mg total) was digested by incubating with 36 μg trypsin (Sigma) in a buffer of 2 mM CaCl₂, 50 mM NH₄CO₃, and 50 mM NaCl for two hours at 30°C. The total reaction volume was 600 μl. The reaction was halted by adding 15 μg of TPCK-treated (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) trypsin inhibitor and shifting the reaction to -20°C.

3. Thrombin digestion of calmodulin

Thrombin digests were done in a 300 μl reaction consisting of 200 μl of 6.8mg/ml calmodulin plus 100 units (10μl of thrombin) in a 0.5 M Tris buffer at pH 8.0. The reaction was allowed to proceed for 16 hours at 37°C.

4. Performic acid oxidation of calmodulin

Performic acid oxidation was performed essentially as described by VanEldyk and Watterson (1981). Performic reagent was prepared by mixing 9.5ml formic acid with 0.5 ml 30% H₂O₂. This mixture was incubated for 2 hours at 25°C then cooled 30 minutes on ice. One milligram of calmodulin was lyophilized and dissolved into 0.5 ml formic acid to which 0.1 ml of methanol was added. This mixture was also cooled 30 minutes on ice. One milliliter of performic reagent was added to the
calmodulin solution which was incubated on ice for 150 minutes. The solution was then neutralized by adding an equal volume of 1M NH₄CO₃ and lyophilizing. The powdered protein was resuspended in 1 ml dH₂O and then dialyzed against four changes of PBS over 16 hours.

5. Denaturation of calmodulin

The detergent sodium-dodecyl-sulfate (SDS) was used to denature calmodulin by adding 10 µl of 10% SDS to a solution of 680 µg calmodulin in 200 µl of water. The solution was placed in a boiling water bath for five minutes, then cooled to -20°C.

6. Formation of calmodulin aggregates

Aluminum chloride was used to form aggregates of calmodulin by adding 8 µl of 250 mM AlCl₃ to a solution of 680 µg calmodulin in 200 µl of water. The solution developed a white precipitate immediately upon addition of the AlCl₃.

C. Affinity Purification of Anti-Calmodulin Antibodies

Affinity purification of anti-calmodulin antibodies was based on the protocol of Dedman et al., (1978). One milliliter of CaM-agarose beads (bovine calmodulin immobilized on agarose beads, Sigma) was washed three times for ten minutes each with 5 ml of 10 mM Tris pH 7.5. Between each wash the beads were spun gently in a clinical centrifuge.
Three milliliters of antiserum was diluted into 5 ml of 10 mM Tris pH 7.5, and incubated with the washed CaM-agarose beads for one hour at room temperature. The beads were washed three times as above and then three times in 10 mM Tris, 300 mM NaCl, pH 7.5. Anti-calmodulin antibodies were eluted with two 1 ml washes of 3M NaSCN. The resulting aliquots were pooled. And assayed for the ability to bind calmodulin by either dot blot or conventional immunoblot.

D. Protein Gel Electrophoresis and Immunoblotting

1. Tricine gel electrophoresis of proteolytically-digested proteins

Tricine gels were prepared according to the procedure of Schägger and Von Jagow (1987). Stock acrylamide mixtures were made: 49.5%T, 3%C consists of 48% (w/v) acrylamide and 1.5% (w/v) bis-acrylamide; 49.5%T, 6%C consists of 46.5% (w/v) acrylamide and 3.0% (w/v) bis-acrylamide. 3X gel buffer consists of 3M Tris, 0.3% SDS, pH 8.45, anode buffer solution contains 0.2M Tris, pH 8.9, and cathode buffer solution contains 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25.

Resolving gels were prepared by combining the following:

10 ml 49.5%T,6%C
10 ml 3X Gel buffer
4 g glycerol
6 ml dH2O
100 µl 10% ammonium persulfate
10 µl TEMED
the solution was rapidly poured between two glass plates and overlaid with 
H$_2$O-saturated butanol. After the gel hardened for at least one hour, the 
overlay was washed off with water and a stacking gel was prepared as 
follows:

1.0 ml 49.5%T, 3%C
3.1 ml 3X gel buffer
8.4 ml dH$_2$O
100 µl 10% ammonium persulfate
10 µl TEMED

This was poured over the resolving gel and a comb inserted. The gel was 
allowed to harden an additional 30 minutes.

Protein samples were diluted with 2X sample buffer (0.8 g SDS, 2.4 
g glycerol, 0.5 ml 2 M Tris, 0.4 ml β-mercaptoethanol, 20 µl 10% Serva 
blue, pH 6.8) and placed in a boiling water bath for three minutes.
Samples were loaded on gels mounted in a vertical slab gel electrophoresis 
apparatus and a potential of 20 mV was applied to the gel and run for 
approximately four hours at 4°C. Proteins were visualized by staining the 
gels overnight in Coomassie staining solution (45% v/v methanol, 45% 
dH$_2$O, 10 % glacial acetic acid, 0.25% Coomassie Brilliant Blue R250), 
followed by destaining with destaining solution (45% v/v methanol, 45% 
dH$_2$O, 10 % glacial acetic acid; Maniatis et al., 1982).

2. Preparation of immunoblots

Gels for electrophoresis were prepared according to Maniatis 
(1981). For most experiments 12% resolving gels were used with 5%
stacking gels. Proteins extracts were boiled for three minutes and loaded into wells. The proteins were subjected to electrophoresis at 25 mV until the dye front was near the bottom of the gel.

Blotting of proteins was performed according to the procedure of Hulen et al. (1991). Gels were prepared by soaking five minutes in KP buffer (25 mM KH₂PO₄, 25 mM K₂HPO₄, pH 7.0). PVDF membrane (BioRad) was prepared by wetting with methanol then soaking for five minutes in KP buffer. The gel and membrane were then placed in a tank blotting apparatus filled with KP buffer and proteins were transfered to the membrane at 0.9 A for 17 hours at 4°C and an additional hour at 1.25 A.

In some experiments, dot blots were prepared instead of standard immunoblots. PVDF membrane was prepared as usual, purified calmodulin was dissolved in KP buffer. The membrane was placed in a dot blotting apparatus and the protein preparation added to the wells. The solution was drawn through the blot with a gentle vacuum. All subsequent processing was the same as for standard immunoblots.

After blotting, the membrane was placed in a solution of 0.2% glutaraldehyde in KP buffer for 45 minutes at room temperature to improve calmodulin retention on the membrane (Hulen et al., 1991). Blots were washed three times for five minutes in 5% nonfat dry milk in TTBS (TBS = 25 mM Tris, 135 mM NaCl, 2.7 mM KCl, pH 8.0; TTBS = 0.2% Triton X-100 in TBS), then soaked in the same solution for two hours at room temperature to reduce non-specific protein interactions. Blots were incubated with antibodies diluted in TTBS overnight at 4°C. Unless otherwise mentioned, antibodies were used at a dilution of 1:1000. Blots were washed at least three times for ten minutes with the milk solution,
then probed for two hours with secondary antibodies at room temperature. Anti-rabbit IgG conjugated to horseradish peroxidase (HRP) from Jackson Laboratories was used at dilutions ranging from 1:3000 to 1:5000 depending on the batch of antibody. Blots were rinsed in nonfat milk solution then in TTBS.

Antibody was detected by use of either colorimetric or chemiluminescent substrates of HRP. For colorimetric detection the blot was immersed in a solution of 600 μg/ml diaminobenzidine, 0.03% NiCl in 5mM Tris pH 7.6). H$_2$O$_2$ from 3% stock was added to 0.03% and the reaction was monitored visually. When the color was easily visible the reaction was stopped by washing with several quick changes of TBS and the blots were allowed to dry.

Chemiluminescent detection was performed according to the instructions provided with detection kits from either Amersham or Pierce. The blot was submerged in the substrate mixture for the indicated time period and then placed in a film cartridge. Blots were used to expose autoradiography film, typical exposure times ranged from a few seconds to five minutes.

3. Quantitation of bands from immunoblots

For quantitative immunoblotting, autoradiography films were quantitated using a Molecular Dynamics laser scanning densitometer. Volume integration was used to calculate the intensity of the band. For developmental immunoblots of Oregon R embryos, the volume of each band on three separate blots was averaged and the results were expressed as
a percentage of the average sum of the volumes in each blot. For experiments comparing homozygous and heterozygous Cam^{n339} embryos. At least two bands were quantitated for each time point and the results were expressed as a percentage of the highest value obtained (6-8 hour heterozygous embryos).

ANOVA (analysis of variance) calculations were used to determine if any significant differences exist in the data. The Oregon R embryo values in figure 6-1 showed no statistically significant differences (P < 0.01). Statistical differences were indicated for the data from Cam^{n339} embryos (P > 0.1). Two-tailed, unpaired Student t tests revealed that the difference is significant between heterozygous and homozygous embryos at 10-12 hours and 18-20 hours.

E. Preparation of Crude Protein Extracts from Drosophila Tissues

The adult and embryo protein extracts used for testing antibody specificity were from either yw^{67c23}(2) or Oregon R laboratory stocks. Approximately 100 mixed male and female adult flies or 150 µl of 0-18 hour embryos were collected in an microfuge tube and frozen on dry ice. Flies and embryos were ground extensively with a small pestle. 250 µl of 5 mM Tris, 1% Triton X-100, pH 7.5 was added to each tube and vortexed for about 20 seconds. The tubes were incubated on ice for one hour. The tubes were then pulse centrifuged in a bench top centrifuge and the supernatant decanted to a fresh tube containing 400 µl of 2X lysis/disruption buffer (20 mM Tris, 2 mM EDTA, 5% SDS, 10% β-mercaptoethanol, 10% glycerol, 0.02% Bromophenol Blue) and placed in a
boiling water bath for 3 minutes. Protein extracts were stored at -20°C.

Embryos used in quantitative blotting experiments were from the Oregon R strain, or collected from crosses of Cam<n>339/Cyo wg LacZ (Heiman et al., 1996; Kassis et al., 1992). Extracts were prepared as above except that embryos were collected for each two hour time period on yeasted grape plates. Twenty embryos were selected and proteins were extracted using 10 µl of extraction buffer (5 mM Tris, 1% Triton X-100, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 µM PMSF, 10 µM Leupeptin, pH 8.0) and 10 µl of 2X lysis/disruption buffer. Leupeptin and PMSF were added because initial experiments revealed some loss of calmodulin from the extracts.

Embryos from Cam<n>339/Cyo wg LacZ collections were stained for β-galactosidase activity before being collected. Embryos were dechorionated and placed in a biphasic mixture of heptane and standard β-galactosidase staining solution (Ashburner, 1989b) to which 1% Triton X-100 was added. The staining was monitored visually, typically taking 30 minutes and then was stopped by rinsing the embryos in PBS. The stained embryos were sorted into three classes based on staining pattern and morphology. Unstained embryos corresponded to Cam<n>339/Cam<n>339 homozygotes. Cam<n>339/Cyo wg LacZ embryos were stained in a segmentally repeated vertical stripe in the epidermis and had no defects in morphology. Cyo wg LacZ/Cyo wg LacZ embryos showed β-galactosidase staining but had profound morphological abnormalities and were discarded.
F. Immunolocalizations

1. Sources of antibodies

The generation of anti-calmodulin antibodies are discussed above and in Chapter 4. Polyclonal rabbit anti-α-spectrin antibodies (Ab 354, Lee et al., 1993) were obtained from Dr. J. Lee, University of California, San Diego, monoclonal anti-D4.1 antibodies (Fehon et al., 1994) were obtained from Dr. R. Fehon, Duke University, polyclonal rabbit anti-adducin antibodies (anti-ADD95; Zaccai and Lipshitz, 1996) were obtained from Dr. H. Lipshitz, The Hospital for Sick Children, Ontario, and monoclonal antibodies that bind CNS axons (BP102) were obtained from Dr. C. Goodman, University of California, Berkeley.

2. Embryo immunolocalizations

Embryonic immunolocalizations were carried out essentially as described by Wieschaus and Nüsslein-Volhard (1986). Embryos were dechorionated in bleach, and placed in a biphasic solution of heptane over fixative for 20 minutes with constant agitation. The fixative used for most experiments was 4% paraformaldehyde in PBS (see in situ hybridization protocol). To maintain microtubule structures in the embryos, the microtubule-stabilizing drug taxol was used at a concentration of 1 μg/ml in PBS for two minutes immediately before fixation (Baker et al., 1993). Vitelline membranes were removed by shaking embryos in a heptane/methanol wash. Embryos were equilibrated into BBX (0.1%
Triton X-100, 0.1% BSA in PBS), and washed several times. Primary antibodies were diluted in BBX and placed on embryos overnight at 4°C. RC20 antiserum was used at a 1:50 dilution, RC23 was used at 1:100. Anti-spectrin antibody was used at 1:500, anti-D4.1 was used at 1:250 and anti-ADD95 was used at a 1:1 dilution. Anti-tubulin antibodies were used at a 1:500 dilution and incubated for two hours at room temperature. Following primary antibody incubation embryos were washed four times for 10 minutes in BBX. Normal goat serum was added to 2% in the last two washes to reduce non-specific binding by secondary antibodies.

Detection of primary antibodies was accomplished using fluorescently labeled secondary antibodies. For rabbit primary antibodies, fluorescein conjugated goat anti-rabbit IgG (Vector) was used at a dilution of 1:300. For monoclonal antibodies, Cy3 conjugated donkey anti-mouse IgG (Jackson Laboratories) was used at a dilution of 1:500. Embryos were then washed with PBX and either mounted in Vectashield (Vector) or cycled back through for double labeling.

3. Ovary immunolocalizations

Ovaries were dissected from one to four day old Oregon R females into PBS. The ovarioles were teased apart at the anterior end and placed in paraformaldehyde fixative on ice for up to 30 minutes. Ovaries were immersed in fresh fixative and fixed for one hour at room temperature. They were then washed with several washes of BBX and treated the same as embryos. Antibodies were used at the same dilution as in embryos.
4. Larval CNS immunolocalizations

Larval offspring from the stock yw; Cam$^{339}$/ CyO y$^+$ were segregated based on whether they possessed pigmented mouth hooks indicating the presence of the CyO y$^+$ balancer chromosome (Heiman et al., 1996). Larvae were everted in PBS to expose internal organs, which were allowed to remain connected to the larval cuticle. These everted larvae were treated for immunolocalization in the same way as ovaries. The CNS was dissected away from the other larval organs just prior to mounting in Vectashield.

5. Phalloidin staining of actin

Actin was detected in tissues using rhodamine-labeled phalloidin (Wieschaus and Nüsslein-Volhard, 1986; Orsulic and Peifer, 1994). Ten microliters of rhodamine-phalloidin solution (Molecular Probes) was evaporated in vacuo and resuspended in 200 μl of PBS immediately prior to use. Embryos or ovaries prepared for immunolocalization were treated with this solution for 20 minutes at room temperature, then rinsed with PBX and mounted in Vectashield.

6. Confocal microscopy

Fluorescently labeled embryos and ovaries were observed by confocal microscopy using a Zeiss LSM410 laser scanning microscope equipped with an Argon/Krypton laser. Fluorescein was detected by
excitation of the probe with laser light at 488 nm wavelength and capturing the fluorescent emissions above 515 nm. Rhodamine and Cy3 were detected by excitation at 568 nm and collection of emissions above 590 nm. Images were collected digitally. For most experiments, tissues were prepared in parallel without the use of primary antibodies. These tissues were used to adjust the sensitivity of the detectors so that artifacts from autofluorescence and non-specific secondary antibody binding would be eliminated. Images were collected in the TIFF file format and analyzed using the software supplied with the confocal microscope.
Chapter 3: Calmodulin mRNA Distribution During Post-Embryonic Development

Calmodulin plays a major role in mediating intracellular responses to calcium and is generally viewed as a universal component of eukaryotic cells (Klee and Vanaman, 1982; Cohen and Klee, 1988). Despite this potentially ubiquitous presence, individual mammalian tissues vary in their levels of calmodulin, with organs such as the brain and testis containing very high calmodulin levels. In vertebrates, calmodulin is derived from three non-allelic genes that together produce at least five different transcripts all encoding the same protein (Nojima, 1989). In general, these genes appear to be coordinately expressed, with tissues containing high levels of protein also showing high levels of transcripts from all three genes (Weinman et al., 1991; Ikeshima et al., 1993; Gannon and McEwen, 1994). In the brain, this coordinate expression operates at the level of individual cells; all three genes are co-expressed in individual neurons (Ikeshima et al., 1993).

Previous studies established that the transcription of the single calmodulin gene of Drosophila melanogaster is dramatically regulated during embryogenesis (Kovalick and Beckingham, 1992). The newly-laid egg is supplied with maternally-derived calmodulin transcripts which disappear early in embryogenesis. Surprisingly, transcription of the gene is activated in only a single class of cells - the precursors of the peripheral and central nervous systems (CNS). Calmodulin transcription continues to be limited to the neural lineages until late in embryogenesis.

Given this unusual behavior during embryogenesis, it was of interest
to examine calmodulin expression at later stages. The question of when transcription in non-neural tissues is initiated was of particular interest. In addition, no complete analysis of calmodulin expression in the various body organs has been performed for any insect. This study allows the first comparison of tissue-specific variation in calmodulin expression between insects and vertebrates.

In vertebrates, tissue-specific calmodulin expression has largely been addressed through RNA Northern blot analysis (Weinman et al., 1991; Ikeshima et al., 1993; Gannon and McEwen, 1994). The small size of Drosophila precludes this approach and we have therefore mainly used in situ hybridization to sectioned animals to examine transcript abundance in various tissues and stages. Although this approach does not permit strict quantitative comparisons of transcript levels it has two significant advantages: first, given that all tissues present in a section are exposed to the probe, simultaneous assessment of transcript abundance in a large number of tissues is achieved and, second, the localization of the transcript within the individual cells of the various tissues is revealed. These studies have revealed interesting patterns of calmodulin transcript accumulation in several tissues.

A. Calmodulin Transcript Detection by In Situ Hybridization

1. General activation of calmodulin transcription in the late embryo

In previous studies (Kovalick and Beckingham, 1992), in situ hybridization to whole embryos was used to examine the embryonic
calmodulin mRNA expression pattern. However, this technique cannot be used to examine transcription after the larval cuticle becomes impermeable at about stage 15/16 of embryogenesis. Embryos were therefore sectioned for further analysis of transcription in the late embryo. In stage 17 embryos, transcription throughout the embryo was detected in a pattern that foreshadows that of the first instar larva (Figure 3-1A,B), that is, universal expression is seen with high levels in the gut and neural tissues. We conclude that generalized activation of calmodulin transcription occurs in the last stages of embryogenesis.

In order to give a more coherent description of calmodulin mRNA expression beyond embryogenesis, we describe below the individual hybridization patterns for the major tissues and organs throughout the life cycle. Unless otherwise indicated all hybridizations shown were performed using double stranded DNA probes, although most were also repeated using antisense RNA probes. Sense-strand RNA probes were used to verify hybridization specificity. Hybridization of the probes was detected by production of a blue or occasionally brown precipitate (see Chapter 2 for details).

2. The central nervous system

As described previously, the CNS shows exclusive and strong staining from mid- to late embryogenesis (Kovalick and Beckingham, 1992). Strikingly, from the end of embryogenesis onwards, the mRNA content of the CNS relative to other tissues decreases throughout the larval stages. In first instar larvae, hybridization in the CNS is significant but lower than that of
the larval gut (Figure 3-1B). During the second instar, calmodulin transcript levels are further reduced (Figure 3-1C), and by the third instar transcripts were undetectable in most CNS sections (data not shown). This absence of calmodulin transcription in the CNS at mid-third larval instar was confirmed by whole mount hybridizations to CNS preparations performed by Alan Lu in the Beckingham lab. However, with the initiation of pupation, calmodulin transcription appears to be activated in various neural populations. In sections of wandering third instar larvae, the innermost neural cells surrounding the neuropil of the CNS show weak calmodulin hybridization (Figure 3-1D). Presumably, this population of cells represent progenitors of the adult nervous system.

By the mid-pupal stages (P6/P7), the major elements of the developing adult nervous system (the supra- and sub-esophageal brain ganglia and the thoracic ganglion) were readily identifiable. All show moderately high levels of transcripts, comparable to those seen in other pupal tissues, from this point onwards throughout pupal life (Figures 3-2D, 3-3A, 3-4A). Transcript levels in the adult CNS are somewhat lower and, strikingly, are considerably lower than levels in the adult gut (Figure 3-3B). From the mid-pupal stages, regional differences in hybridization intensity throughout the CNS were detectable. Most noticeably, neurons in the brain cortex showed stronger hybridization than those in the optic lobes in both pupal and adult life (Figures 3-3A,B, 3-4A,B). In addition, cell-to-cell variation in signal intensity within the brain cortex was quite pronounced in P6 pupae (Figure 3-4A) and still detectable across fields of neurons in the adult central brain (Figure 3-3B). The more sensitive antisense RNA probe revealed that neuronal cell bodies in all regions of the
Figure 3-1. Calmodulin mRNA expression from late embryogenesis to the late third instar stages. Hybridization of the calmodulin probe to the following material is shown. A. Oblique horizontal section of a stage 17 embryo. B. Sagittal section of a first instar larva. C. Anterior region of a horizontal section of a second instar larva. D. Anterior region of a third instar larval sagittal section. Anterior is to the right. amg - anterior midgut, bl - brain lobes, gc - gastric caecum, mg - middle midgut, np - neuropil, op - outer proventricular wall, p - pharynx, sg - salivary gland, vc - ventral nerve cord. Scale bar = 100 μm.
Figure 3-2. Calmodulin mRNA expression in adult ovaries and pupal somatic tissues. Hybridization of the calmodulin probe to the following material is shown: A. Sagittal section of developing ovarian follicles in adult female. B. Horizontal section of developing thoracic musculature and gut in stage P9 pupa. C. Horizontal section through developing wing blade of a stage P9 pupa. D. Horizontal section showing brain, thoracic ganglion and thoracic musculature of a stage P10 pupa. For A, anterior is to the left and for B, C and D, to the top of the page. In A, nuclei are counterstained with fuchsin. cb - cell bodies of brain cortex, f - follicle cells, fd - follicle cells forming dorsal appendages of egg case, im - indirect flight muscle, j - jump muscle, mg - midgut, nc - nurse cell cytoplasm, nn - nurse cell nucleus, o - oocyte cytoplasm, pc - pupal case, sm - skeletal muscle, tg - thoracic ganglion, 4, 10, 12, stage of follicles indicated, other abbreviations as previously. Scale bar = 62.5 μm.
Figure 3-3. Comparison of calmodulin expression in P9 pupal and adult stages. Calmodulin hybridization to horizontal sections of the entire full-length of the animals are shown so that relative expression levels can be compared. Nuclei are counter-stained with fuchsin in both animals. A. Pupal section  B. Adult section. The pupal section is more ventral and includes the thoracic ganglion and little of the indirect flight muscles. In the more dorsal adult section the thoracic cavity is filled with sections of the indirect flight muscles but these show negligible hybridization.
lo - optic lobe, r - retina, other abbreviations as previously.
Scale bar = 100 μm
Figure 3-4. Calmodulin expression in the developing brain and eye.
A. Hybridization to oblique horizontal section of stage 6 pupal head.
B. Hybridization to an anterior frontal section of adult head.
C-G. Hybridization to sections of developing eye. C, D. Stage P9 pupa.
E. Stage P10 pupa. F. Stage P10 pupa. G. Adult. All eye sections are from horizontal sections of whole heads. In D, the section is tangential to the surface of the eye such that sections through the pseudocone and the apical regions of the cone cells and primary and secondary pigment cells are seen (asterisks). At the periphery of the eye, cup-shaped hybridization patterns to these cells are also seen (arrows). In E, hybridization forming a "collar" at the position of the bristle group of cells is seen (arrow). In C-F, arrowheads point to the strong line of hybridization at the position of the secretory region of the cone and pigment cells. B, antisense RNA probe; A, C-G, DNA probe. ad - antennal disc, cl - corneal lens, other abbreviations as previously. Scale bar = 50 μm.
brain and optic lobes showed some hybridization, while regional
differences in signal intensity were less dramatic.

3. The alimentary canal and accessory organs

By mid first larval instar, elements of the alimentary canal show the
highest levels of calmodulin transcripts seen throughout the entire
organism. In particular, hybridization to the proventriculus and the middle
midgut is very intense (Figure 3-1B). These two tissues show the highest
levels of calmodulin transcripts throughout the entire organism in all three
larval stages and again in the adult (see below). The pharyngeal epithelium
and salivary glands also show strong expression, with the gastric caecae and
anterior midgut showing somewhat lower levels and the Malpighian tubules
containing only moderate transcript levels. The fat body was poorly
preserved in these frozen sections but showed low to moderate
hybridization where examination was possible.

The proventriculus is formed by an infolding of the foregut into the
anterior midgut such that a three-walled structure is produced. Its function
is to generate the peritrophic membrane - a multi-layered, secreted lining
of the gut of unknown function. From morphological analyses, most layers
of the peritrophic membrane are thought to be secreted by cells of the
proventricular outer wall (King, 1988). Interestingly, at all three larval
stages, the intense calmodulin mRNA hybridization in the proventriculus is
confined to the outer wall, and is strongest in zone 4 (King, 1988) the
major secretory region of the organ (Figure 3-1B,C,D).

In pupal life, the gut is completely reorganized. All larval structures
except the Malpighian tubules degenerate and adult tissues are formed by
proliferation of imaginal cells located throughout the larval gut. The epithelium of the larval midgut is shed into the gut cavity and later excreted. Throughout the pupal stages, all elements of the alimentary canal examined showed low levels of calmodulin transcripts (Figures 3-2B, 3-3A) and the midgut epithelium appeared thin and undifferentiated. Even in late pupae, hybridization in the midgut and proventriculus was unremarkable and similar to that of most tissues of the organism. In marked contrast, in adult life, the epithelia of the alimentary canal again showed very intense hybridization and the proventriculus and midgut once more showed the highest levels of transcripts in the organism. As in larval life, intense hybridization in the proventriculus was limited to the outer wall. Figures 3-3A and 3-3B compare hybridization to sections through an entire adult and through a P9 stage pupa. These sections were hybridized simultaneously and processed in parallel, and therefore comparison of the signal strengths is appropriate. As can be seen, the difference in the signal for the pupal and adult gut is very striking.

4. The musculature

The major locomotor muscles of the larval stages lie flush along the inner surface of the larval body wall. These muscles are tubular, with a narrow core of cytoplasm running through a tube of myofibrils. Longitudinal sections are not optimal for examining hybridization to the cytoplasmic cores of these structures, since these regions will be absent in many sections. Thus, although muscle fibers were identified in sections through-out the larval stages, the absence of hybridization to these
structures must be interpreted cautiously.

In the adult abdomen, the body wall muscles also lie flush against the integument and again were difficult to examine in the sections used here. The major adult locomotor muscles are located in the thorax and include the indirect and direct flight muscles and the muscles for leg movement such as the large tergal depressor of the trocanter (TDT) or jump muscle. These muscles and many skeletal muscles of the head were readily visualized in our sections during both pupal and adult life. In early puparia, only large blocks of disorganized larval muscle, devoid of calmodulin staining and clearly undergoing autolysis, were detectable. In 24-48 hour (P6/P7) pupae, all the developing head and adult thoracic muscles showed significant calmodulin hybridization, comparable to that of other developing pupal tissues. This staining persists throughout pupal life. For the indirect flight muscles, which have a fibrillar structure, hybridization was rather diffuse and uniform throughout the muscles (Figures 3-2B,D, 3-3A). The direct flight muscles and other skeletal muscles like the TDT have a tubular structure such that in cross-section, the tightly packed myofibrils form an annulus around a central core of cytoplasm. All calmodulin transcripts were localized to the central cytoplasm in these muscles, producing a striking lattice-type pattern of hybridization to cross-sections of these muscles (Figure 3-2D).

Although the oldest pupae examined (stage P10) showed comparable levels of calmodulin transcripts in the muscles to those seen at earlier stages, a striking finding was that in one or two-day old adults, the hybridization to all types of thoracic muscles was very low (Figure 3-3B).
5. The ovary

The ovarian calmodulin hybridization pattern could easily be assessed from sectioned material. Hybridization levels were low in early follicles, but by stage 7 and the onset of vitellogenesis, strong hybridization was seen in the nurse cells with even more intense hybridization in the columnar follicle cells encasing the oocyte (Figure 3-2A). The follicle cells are actively engaged in the endocytosis of yolk during this period and subsequently secrete the components of the proteinacious egg shell or chorion. As shown in Figure 3-2A, even during advanced stages of choriogenesis, these cells contain high levels of calmodulin transcripts. In these later stages, the nurse cells undergo resorption, and lose their calmodulin transcripts (Figure 3-2A). Most of the transcripts of the oocyte proper are believed to be derived from its sister nurse cells, since examples of contribution of transcripts from follicle cells has been found and the oocyte nucleus is largely transcriptionally silent during oogenesis (Spradling, 1993). Throughout oogenesis, levels of transcripts in the oocyte cytoplasm are significantly below those in the nurse cells and the follicle cells (Figure 3-2A).

6. The imaginal discs and eye

During larval life, the imaginal discs undergo growth by cell division. Sections of second and third instar larval discs showed very low levels of hybridization to the calmodulin probe (data not shown). In cross-sections of the epithelial cells in everting wing discs at the mid-pupal stages
(P8/9) the calmodulin probe showed strong hybridization in a very punctate pattern (Figure 3-2C). During this period each of the wing epithelial cells is completing synthesis of a single hair and is also secreting cuticular components to cover the rest of its exterior face (Mitchell et al., 1983). This punctate localization might therefore relate to either of these specialized activities. However, exclusion of transcripts from the nuclei of these flattened cells could also contribute to this pattern.

By the mid-pupal stages, when the eye-antennal discs have everted and the developing eye is in its final location, calmodulin hybridization to the retina was strong (Figure 3-4C) but then decreased again to relatively low levels in the adult (Figures 3-4D,E,F,G). At all stages hybridization to the developing rhabdomeres was not uniform and in the adult was strongest at the mid-point of the photoreceptors, which corresponds to the region around the nucleus.

At mid-pupal stage when the secretion of the corneal lens has been initiated, a strong line of calmodulin mRNA hybridization is visible below the lens material (Figure 3-4C). In sections with more tangential views of the eye surface, this hybridization could be seen to form a "cup" and a "floor" around and beneath the developing lens and pseudocone, thus corresponding to the positions of the apical borders of the cone cells and primary and secondary pigment cells (Cagan and Ready, 1989). This line marks a region of intense exocytotic activity since these cells actively secrete the lens and pseudocone through the microvilli of their apical surfaces. In some sections, deep staining at the surface of the eye could also be seen as a "collar" at the base of each sensory bristle of the eye (Figure 3-4E). The four cells of each bristle group are known to form an
enwrapped structure of this kind (Cagan and Ready, 1989). Although the intense line of hybridization at the surface of the eye had disappeared by the adult stages (Figure 3-4G), when secretion of the lens and pseudocone are complete, interpretation of this hybridization pattern is complicated by our discovery that both sense and antisense RNA probes bind non-specifically to the periphery of the adult lens (Figure 3-4C). Thus, although this non-specific binding of RNA probes is distinctly different from the DNA hybridization seen during pupal stages, it nevertheless raises the possibility that, in contrast to RNA, DNA binds non-specifically to some precursor components of the lens and pseudocone to produce the strong staining seen at the surface of the eye.

B. Discussion and Conclusions

1. Developmental regulation of calmodulin transcript accumulation

We have identified striking changes in calmodulin mRNA accumulation throughout the Drosophila life cycle - both in terms of stage-specific and tissue-specific expression. Major changes in the calmodulin mRNA expression pattern occur at the end of embryogenesis and the onset of pupation. The general activation of calmodulin transcription seen in late embryogenesis might represent a systemic, hormonally-induced response. However, this period corresponds to the completion of organogenesis and thus the general onset of calmodulin transcript expression probably represents a consequence of terminal differentiation in all tissues. Similarly, most of the changes in calmodulin expression in pupal life
probably reflect the major reorganization of the organ systems within the animal instead of direct hormonal effects. The early activation of calmodulin transcription in neural precursors of the CNS at the onset of pupariation is, however, a candidate for a relatively direct hormonal effect. This activation corresponds in timing to the known burst of ecydsone secretion required to produce pupariation (Riddiford, 1993). In the adult stage there is evidence that juvenile hormone (JH) regulates the accumulation of calmodulin transcripts in the ovarian follicle cells of B. germanica (Iyengar and Kunkel, 1995).

The developmental expression patterns for three major organ systems studied - the CNS, gut and adult muscles - are distinctive and unique. In the CNS, after intense expression during embryogenesis, transcript levels decrease through the larval stages to background, then are activated to levels comparable to other tissues during pupal life and maintained at moderate levels in the adult. In the gut, intense levels of hybridization are seen from the end of embryogenesis throughout larval life. Transcript accumulation is lower and comparable to other tissues in pupal life and very intense again in the adult. Although the larval muscles were not systematically studied, the behavior of the developing adult muscles contrasts with those of the both the CNS and the gut; during pupal life transcript levels are comparable to the other organs and then fall to very low levels in the adult. The extent to which calmodulin protein levels in these various tissues and stages will reflect these differences in mRNA expression remains to be determined. It seems likely that some discrepancies will exist, however, since differences are seen between calmodulin mRNA and protein levels during embryogenesis (discussed in
Chapters 5 and 6). In addition, the very low levels of mRNA in the larval CNS and adult muscles may reflect very low levels of calmodulin protein turnover. Similarly the very high levels in the gut may partially reflect a continuous need for calmodulin synthesis as a result of continuous shedding of intestinal cells. Studies of both Drosophila (Miller, 1950) and Manduca sexta (Bonfanti et al., 1992) suggest continuous gut regeneration in insects as in mammals.

In mammalian systems, down-regulation of calmodulin mRNA and protein levels during myogenesis has been linked to withdrawal from the cell cycle (Christenson and Means, 1993; Gruver et al., 1993). Since we have found that calmodulin mRNA levels are maintained high through to stage P10 of pupal life - well beyond cessation of mitosis at a point when myofibril formation is well advanced - it would appear that down-regulation of calmodulin mRNA levels in Drosophila muscles correlates with completion of muscle synthesis, not termination of cell cycling.

2. Differential tissue-specific calmodulin mRNA expression

The various tissue types within Drosophila show dramatic differences in levels of transcript accumulation. In addition, we have uncovered striking examples of differences in transcript abundance between the various cell types within particular tissues and organs.

Within the CNS, the marked cell-to-cell differences in calmodulin transcript levels in sectioned material from late third instar or early pupal life probably reflect developmental differences between the neural cells involved. Our data indicate that calmodulin transcription is activated in
cells that will generate new adult neurons, mirroring the activation of the
gene in neuroblast lineages during embryonic life. In the adult CNS, the
higher overall level of calmodulin expression in the central brain as
compared to the optic lobes probably reflects the greater complexity of
neural processing associated with this brain region. The cell-to-cell
variation seen within the adult central brain presumably also relates to
functional differences between neurons. The Drosophila brain is not
sufficiently well mapped to correlate cell location with function. For the
mammalian brain, however, differences in calmodulin mRNA levels have
been detected in identifiable neuronal subtypes within discrete brain
regions (Ni et al., 1992; Ikeshima et al., 1993) Cell-to-cell variations in
hybridization intensity seen in other tissues, such as the midgut, testes and
ovaries, can be more readily ascribed to functional differences, as outlined
below. It is not clear to what extent these differences in transcript levels
represent differences in transcription rates, since differential transcript
turnover could contribute significantly to overall mRNA levels.
Nevertheless, the cell-to-cell variations observed in the brain, alimentary
canal, and reproductive organs suggest that the Drosophila calmodulin gene
shows complex transcriptional regulation.

3. High levels of calmodulin transcripts in secretory and endocytotic
cell types

In general, the highest levels of calmodulin transcripts were found in
tissues or cells involved in secretory or endocytotic activity. The very
strong concentration of calmodulin transcripts in zone 4 of the
proventriculus correlates well with ultra-structural evidence of intense secretory activity towards synthesis of the peritrophic membrane (King, 1988). Excluding the possibility of non-specific hybridization to lens precursor materials, the very high levels of hybridization found within the cone cells and primary and secondary pigment cells of the developing retina also correlate with the intense secretory activity of these cells. These cell types, along with the middle midgut and ovarian follicle cells show the most intense calmodulin hybridization detected and represent the tissues/cells most actively involved in secretion or endocytosis. Many studies suggest a role for calmodulin in exocytosis in mammalian systems (for example, Chamberlain et al., 1995) and it has proved essential for exocytosis in Paramecium (Kerboeuf, 1993). A role for calmodulin in some endocytotic processes in also now emerging (Artlejo et al., 1996).

4. Comparison of the adult tissue distribution of calmodulin mRNA in Drosophila and other species

In adult Drosophila, the intestinal tract and the ovary show very strong expression of calmodulin transcripts, whereas expression in the CNS is at a relatively low level. There is detectable hybridization to Malpighian tubes but expression in the differentiated muscles is low. In situ hybridization of the calmodulin probe to whole mount preparations of Drosophila testes performed by Alan Lu in the Beckingham laboratory revealed high levels of calmodulin transcripts in this tissue as well. This pattern of expression shows some similarities to those found in adult mammals and other insects. In particular, the testis and gastro-intestinal
tract show high levels of calmodulin transcripts in mammalian systems (Ikeshima et al., 1993) and show high levels of calmodulin protein in the silk-worm *Bombyx mori* (Morishima, 1987).

The most surprising aspect of this tissue distribution is the lower level of calmodulin transcription seen in the adult Drosophila CNS as compared to mammalian systems. In mammals, the brain shows the greatest accumulation of calmodulin mRNAs and the highest concentration of calmodulin protein in the entire animal. Two factors may contribute to the lower levels of transcripts in the CNS in Drosophila. First, the requirements for calmodulin in the Drosophila brain may be lower, perhaps as a result of simpler neuronal functioning. Second, the rate of calmodulin turnover may be lower in Drosophila neural tissues such that lower levels of calmodulin transcription provide similar overall levels of protein. The observation that calmodulin protein is highly stable in the embryonic CNS (see Chapter 6) supports the latter hypothesis. In another insect species, *Leucophaea madera*, the CNS is one of the richest sources of calmodulin protein (Wright and Cook, 1985).

The much higher levels of calmodulin transcripts found in developing Drosophila ovarian follicles than in mammalian ovaries (Ikeshima et al., 1993) reflects the very different type of eggs formed in the two classes of organisms. The components of the large free-laid eggs of Drosophila must support the developing embryo throughout all of embryogenesis. In addition, the requirements in Drosophila for protective egg coverings (the vitelline membrane and egg shell) and uptake of yolk involve intense synthetic, endocytotic and secretory activities by the follicle cell layer surrounding the oocyte. The strong hybridization we see in these
follicle cells - particularly late in oogenesis during the vitellogenic and choriogenic phases - correlates well with findings for the cockroach *Blattella germanica*. Calmodulin transcript accumulation within the ovarian follicles cells of this species has been shown to be higher than in any other tissue of the organism examined (Iyengar and Kunkel, 1995). Interestingly, this laboratory has provided evidence that the high follicle cell calmodulin transcription in *B. germanica* provides calmodulin not only for follicle cell functions but also for transfer to the oocyte itself (Zhang and Kunkel, 1994). The lower levels of calmodulin transcripts we see in the nurse cell and oocyte cytoplasms, as compared to the follicle cells, could indicate that an equivalent process is ongoing in *Drosophila* follicles.

Further support for this model comes from germline clone experiments performed by Clare Bolduc in the Beckingham laboratory. These experiments were aimed at examining the development of germ-line cells homozygous for the calmodulin null mutation *Cam^n339* and thus incapable of synthesizing calmodulin protein. In egg chambers in which all of the germ cells are homozygous for the null mutation, undetectable levels of calmodulin protein are seen in the oocyte and nurse cells during early oogenesis. Surprisingly, however, by late oogenesis the germ line has accumulated calmodulin protein to levels equivalent to heterozygous egg chambers. The eggs produced from calmodulin null clones were normal in shape and morphology but had defects in the chorion which appeared very thin and variable. Since no evidence has been found for a direct role of the oocyte in chorion formation, this is presumably a result of the failure of calmodulin-depleted follicle cells to produce or secrete chorion components
properly. This implies that calmodulin is normally contributed to the oocyte from both follicle cells and nurse cells. However, in egg chambers in which the nurse cells are unable to produce calmodulin, the follicle cells transport a larger amount of calmodulin to the oocyte, thereby depleting calmodulin levels within the follicle cells and causing defects in chorion formation.

The low to negligible level of calmodulin mRNA detected in the muscles of adult Drosophila correlates well with findings for mammalian species. With the exception of the thyroid, calmodulin protein levels in skeletal muscle were the lowest in over twenty mammalian tissues examined (Klee and Vanaman, 1982), and transcript levels for all three mammalian calmodulin genes are low or barely detectable in fully differentiated mammalian muscle (Ikeshima et al., 1993).

5. Calmodulin expression in growing versus differentiated cells

Studies with cultured mammalian cells (Chafouleas et al., 1984; Rasmussen and Means, 1987), *Aspergillus nidulans* (Lu et al., 1993) and *Saccharomyces cerevisiae* (Anraku et al., 1991) have provided evidence that calmodulin plays a role in cell cycle progression. Further, Christenson and Means (1993) have demonstrated that in mammalian myoblasts and fibroblasts the transition from proliferation to quiescence is accompanied by a 2-4 fold down-regulation in transcript levels for all three calmodulin genes. It was of interest therefore to examine the calmodulin gene expression pattern during Drosophila development from the perspective of dividing versus differentiating cells.
Drosophila is unusual in that from the later embryonic stages until the onset of pupation the growth of most tissues is not accomplished by mitosis but through increase in cell size involving endoreplication of the nuclear DNA. It is unclear what roles calmodulin might have in this type of growth. The imaginal discs and elements of the CNS are exceptional and show normal cell division during the larval and pupal stages. Similarly, the reconstruction of the gut and muscles in pupal life is accompanied by normal cell division. Although the hybridization of the calmodulin probe to these mitotically active tissues is significant, it is clearly considerably lower than the levels associated with the fully differentiated state of many of the tissues, particularly the gut, ovary and testes. We conclude that the calmodulin transcript levels required for mitotic proliferation are considerably lower than those required to fulfill the differentiated functions of many tissues. This observation has relevance for a genetic analysis of calmodulin function in Drosophila.

C. Summary

In this study, we have determined that expression of the single calmodulin gene of Drosophila is expressed in highly regulated manner, resulting in wide variations in transcript levels within various tissues at different stages of development. Some of the cell and tissue specific differences can be ascribed to specific functions, such as secretion, while others, particularly cell-to-cell variations in the brain, are less easy to define. We have also determined that some tissues, such as muscle, have higher requirements for calmodulin transcription during cell division and
early development than in their fully differentiated forms, and the opposite is true of others, such as the alimentary canal. Finally, while most of our observations correlate well with those made in vertebrate systems, some aspects, particularly the levels of calmodulin transcripts in the brain and ovaries of Drosophila, differ significantly from their mammalian counterparts.
Chapter 4: Generation of Anti-Calmodulin Antisera

A. Overview of Strategy to Generate Anti-Calmodulin Antibodies

Antibodies that specifically bind a given protein are very valuable tools for analyzing many aspects of the protein's function. Antibodies are commonly used to quantitate, localize and isolate proteins and have been used to assay complex formation, disrupt protein function and a variety of other experiments. The potential to perform these types of experiments, and the failure of antibodies that bind calmodulin from other species to work with Drosophila calmodulin led to our attempt to produce antibodies against Drosophila calmodulin.

The general procedure for these attempts was to use various treatments of purified, recombinant Drosophila calmodulin to elicit an immune response against calmodulin in rabbits. Rabbits were chosen for several reasons. First, we desired to get polyclonal antibodies instead of monoclonal antibodies. Polyclonal sera have the advantage of often being able to recognize multiple epitopes on a protein. Post-translational modifications, conformational changes, complex formation, and other phenomena can sometimes interfere with the ability of an antibody to bind its target protein. A polyclonal serum is less likely to be affected by these events because they often contain a mixed population of antibodies that recognize different epitopes. Second, rabbit polyclonal sera are usually cheaper and faster to generate and often easier to work with due to a higher titer of antibody and the larger volume of serum. Third, a wide variety of secondary antibodies are commercially available for use with
rabbit antibodies in several relevant techniques.

Various preparations of recombinant Drosophila calmodulin were sent to a commercial firm (Cocalico Biologicals, Inc., Reamstown, PA) for combination with Freund's adjuvant and inoculation into rabbits. A pre-inoculation test bleed was taken from each animal and followed by an initial inoculation of 50 μg of antigen. This was followed by boosts of 10 μg each on days 14, 21 and 49 after the initial inoculation. Test bleeds yielding approximately 5ml of serum were taken on days 35 and 56. At the end of this schedule, all three serum samples from each rabbit were sent back for analysis. The animals that produced anti-calmodulin antibodies were then boosted twice again and larger production bleeds taken.

In order for an antiserum to be of maximum utility, it must be free from antibodies that bind other proteins from the organism under study. One of the best methods for eliminating undesirable antibodies from the serum is affinity purification. The commercial availability of agarose bead-immobilized bovine calmodulin (CaM-agarose) and the high identity (98%) between bovine and Drosophila calmodulin made affinity purification a quick and simple method for purifying anti-calmodulin antibodies. The antisera were mixed with CaM-agarose beads which were then washed extensively to remove the proteins that do not bind calmodulin. Anti-calmodulin antibodies were then eluted with 3M sodium thiocyanate, a chaotropic agent, and dialyzed extensively to remove the thiocyanate and get the antibodies into a storage buffer.

The antisera were screened for the presence of anti-calmodulin antibodies by immunoblotting and immunolocalization. Purified antisera
that were determined to have anti-calmodulin antibodies were also were
tested for the ability to cross react with other proteins in either crude
extracts of Drosophila protein or preparations of expressed proteins from
the calmodulin superfamily.

B. Preparation of Antigens for Inoculation

A total of eleven rabbits were injected with preparations of
Drosophila calmodulin in three separate projects. The first project
involved one animal, RC11, that was inoculated with untreated calmodulin.
While this project produced anti-calmodulin antibodies, subsequent projects
were undertaken using calmodulin protein treated in a variety of methods
to enhance the antigenicity of the protein. The second project consisted of
two rabbits inoculated with untreated calmodulin (RC13 and RC14), two
rabbits inoculated with trypsin-digested calmodulin (RC15 and RC16) and
two rabbits inoculated with thrombin-digested calmodulin (RC17 and
RC18). The third project involved two rabbits (RC20 and RC21)
inoculated with performic acid oxidized calmodulin, one rabbit (RC22)
inoculated with denatured calmodulin, and one rabbit (RC23) injected with
calmodulin treated with AlCl₃.

1. Purified calmodulin

Purified calmodulin protein was obtained from Poushali Mukherjea
from supplies she had generated for her biophysical and biochemical
studies of calmodulin, using procedures developed in the laboratory
(Maune et al., 1992). Briefly, a expression vector that contained the
coding sequence of Drosophila calmodulin was induced to produce
calmodulin in cultured bacteria. The bacteria were lysed and calmodulin
from the cells was purified by the ability of calmodulin to bind to phenyl-
Sepharose beads in the presence of calcium. The calmodulin was eluted
from the phenyl-Sepharose by addition of EDTA to chelate the calcium
(Maune et al., 1992). Three rabbits (RC11, RC13 and RC14) were
inoculated with this preparation of calmodulin without subsequent
modification.

2. Trypsin and thrombin digestion

The high degree of identity between Drosophila calmodulin and
vertebrate calmodulin caused concern that the fruit fly protein would be
unable to illicit an effective immune response, and that some kind of
modifications would be necessary to generate useful antisera. Although the
first animal inoculated with untreated Drosophila calmodulin did produce
antibodies to the protein, subsequent analysis (detailed below) indicated that
the serum is not highly polyclonal but rather contains antibodies that
recognize only one epitope that is located between amino acids 107 and
148.

One method chosen to avoid this problem was to proteolytically
cleave the protein in the hope that this would result in a more polyclonal
serum. Two proteases had been previously studied and the cleavage
patterns of these proteins had been well characterized. Although trypsin
can cleave C-terminal to all lysine and arginine residues, for the calcium-
bound form of calmodulin, only the two adjacent lysines in the central linker region (see Figure 1-2) of calcium-bound calmodulin are available to the enzyme (Walsh et al., 1977). Thus trypsin cleavage results in two fragments of calmodulin comprising amino acids 1 through 77 and 78 through 148. The other protease used was thrombin, which cleaves proteins on the C-terminal side of arginine residues. In the absence of calcium, thrombin can only cleave calmodulin after amino acid 106, yielding two fragments: one with amino acids 1 through 106 and the other with amino acids 107 through 148 (Wall, et al., 1981).

Both enzymes result in fragments in which the epitope recognized by the RC11 serum is separated from the amino-terminal end of the protein. In the case of trypsin digestion it was hoped that this would result in sera that recognized epitopes in both halves of the protein. The thrombin digestion was designed to eliminate the most antigenic region of the protein thereby increasing the likelihood that antibodies would be formed against the N-terminal or central regions of the protein.

Figure 4-1 shows the results of digestion of calmodulin with trypsin and thrombin. Proteins were digested as described in Materials and Methods and resolved by PAGE to resolve the proteolytic fragments. Scanning densitometry indicated that the digests were approximately 75% complete. The trypsin digest was used to inoculate rabbits RC15 and RC16, while the thrombin digest was used to inoculate rabbits RC17 and RC18.
Figure 4-1. Proteolytic Digestion of Calmodulin. Purified recombinant calmodulin was digested with either thrombin or trypsin and resolved by electrophoresis. Both digests were estimated to be approximately 75% complete.
3. Performic acid oxidation

Another method used to increase the antigenicity of calmodulin was performic acid oxidation. Performic acid is thought to oxidize methionines to methionyl sulfones. VanEldyk and Watterson (1981) used this procedure to treat mammalian calmodulin for use in generating antibodies and showed that it resulted in enhanced ability to generate an immune response to calmodulin. Drosophila calmodulin has nine methionine residues scattered throughout the protein (see Figure 1-2).

The performic acid oxidation is performed by dissolving lyophilized calmodulin in formic acid with 20% methanol. The solution is incubated with an excess of performic acid, a mixture of 1.5% H$_2$O$_2$ in formic acid, then neutralized. This preparation was used to inoculate two rabbits, RC20 and RC21.

4. Denaturation

Denaturation of calmodulin with SDS was also performed to improve the chances of getting antibodies that recognize calmodulin. Denaturation with heat or chemicals such as urea or detergents were considered. Ultimately, we chose to denature calmodulin by boiling for five minutes in 0.5% sodium dodecyl sulfate (SDS). This protein was used to inoculate rabbit RC22.
5. Aggregation

Proteins will often generate a stronger immune response when presented as aggregates (Harlow and Lane, 1988). While there are numerous methods for aggregating proteins, we chose to use aluminum to induce aggregation of calmodulin. AlCl₃ was added to purified calmodulin to a final concentration of 10 mM. A white precipitate formed immediately in the protein solution, and was vortexed gently to form a suspension. This protein preparation was used to inoculate rabbit RC23.

C. Characterization of Antisera.

1. RC11 antiserum detects calmodulin

a. Characterization by immunoblotting

RC11 antiserum was generated by inoculation of rabbits with purified recombinant Drosophila calmodulin. Extensive characterization of the serum was conducted to determine the utility of the serum for studies of calmodulin. Immunoblots to recombinant and endogenous proteins were used to measure the affinity and specificity of the serum.

i. RC11 test bleeds recognize native calmodulin

The whole serum was tested to determine if it contained antibodies capable of binding calmodulin by immunoblot (Figure 4-2A). Purified
Figure 4-2. Characterization of RC11 antiserum by immunoblot. (A) Blots of 50 and 200 ng of non-denatured recombinant CaM with RC11 test bleeds. Control antiserum is rabbit anti-bovine CaM (gift of Dr. W Radding). RC11.1 denotes first test bleed, RC11.2 denotes second test bleed. (B) Native blots of wild-type or B1234Q CaM probed with preimmune serum (lane 1), unpurified RC11.2 (lane 2), or affinity purified RC11.2 (lanes 3, 4 and 5). (C) Immunoblots against denatured CaM with purified RC11 serum. Lane 1 - purified CaM, lane 2 - extract from 0-2 hr Oregon R embryos, lane 3 - extract from male and female adult Oregon R flies. (D) Immunoblots of purified recombinant CaM, Androcam, and centrin (50 ng each) with purified RC11 serum. Left two lanes show Coomassie staining of undigested and thrombin digested CaM. Right two lanes show RC11 detection of undigested CaM and the C-terminal thrombin fragment of CaM.
recombinant calmodulin was subjected to non-denaturing polyacrylamide gel electrophoresis, transferred to membrane and probed using several dilutions (1:100, 1:500, and 1:1000) of the test bleeds from the inoculated rabbit, as well as positive and negative controls. Non-denaturing conditions were used in this experiment to eliminate the possibility of denaturation as a source of negative results. As seen in Figure 4-2A, all dilutions of the first or second test bleed were able to detect 50 ng of calmodulin, while the pre-immune serum and negative control could not detect 200 ng of calmodulin. This confirmed that the animal produced an immune response to the injected protein resulting in anti-calmodulin antibodies.

ii. Affinity purified RC11 detects both native and denatured calmodulin

The anti-calmodulin antibodies were then affinity purified from the RC11 serum. Immunoblots against non-denatured recombinant calmodulin (Figure 4-2B) shows that affinity purified RC11 antiserum can detect native calmodulin. Figure 4-2C (lane 1) shows that the affinity purified RC11 serum also can detect purified calmodulin on immunoblots prepared under denaturing conditions.

iii. RC11 can recognize mutant calmodulin

In addition to the ability to recognize wild-type calmodulin protein, we were interested in whether the antiserum would detect mutant
calmodulin as well. The mutant chosen was a site-directed, multiple mutant the first calcium coordinating residue in each calcium binding site had been mutated to a glutamine residue, denoted B1234Q (Mukherjea et al., 1996). This mutant calmodulin is unable to bind calcium, and spectroscopic and target-binding and activation studies have suggest that this calmodulin mutant adopts an unusual conformation in comparison with wild-type protein. Figure 4-2B (lane 5) shows that RC11 antiserum is able to detect the mutant protein, indicating that the antibodies in the serum are not sensitive to mutations that cause small changes in the conformation of the protein.

iv. RC11 detects an epitope in the C-terminus of calmodulin

One of the concerns about using a highly conserved protein in an inoculation is that the protein may not have many antigenic sites. The result could be generation of antibodies that are limited a single epitope, which could reduce the utility of the antiserum for experiments such as immunolocalization. To determine the distribution of epitopes recognized by RC11 antibodies, blots of proteolytically cleaved calmodulin were probed with RC11 serum. This was done in collaboration with Poushali Mukherjea, who was investigating the conformation of calmodulin with different concentrations of calcium. She digested purified calmodulin with thrombin under conditions which would result in two fragments, one fragment contained the first 106 amino acids of calmodulin while the second fragment contained amino acids 107 - 148. She then resolved the fragments by PAGE and transferred them to a PVDF membrane. I probed
the membrane with purified RC11 serum. Figure 4-2E shows that the serum recognizes only two bands, the higher band corresponds to undigested calmodulin while the lower band is the 107 - 148 fragment. This indicates that the epitopes recognized by the RC11 antibodies are all located in the C-terminal 41 amino acids of calmodulin. Two of the three amino acid differences (see Figure 1-2) between vertebrate and Drosophila calmodulin occur with in the last ten amino acids of the protein, which may explain why the antibodies only recognize this part of the protein. The possibility remains, however, that the proteolysis of the protein may have disrupted other epitopes of the protein. The observation that RC11 antibodies only bind to the C-terminal tail indicates that the serum is not highly polyclonal and may explain the problems encountered in using this antiserum for immunolocalizations (discussed below).

v. RC11 affinity for calmodulin is stronger than for other related proteins

To investigate the specificity of the RC11 antibodies, the serum was tested for the ability to detect closely related members of the EF-hand calcium-binding protein family. Fig. 4-2D show the results of immunoblots to purified calmodulin, Androcam, and centrin. Androcam is a calmodulin related protein in Drosophila that is similar in size (149 amino acids versus 148 for calmodulin) and organization to calmodulin and is 66% identical in amino acid sequence (Fyrberg et al., 1994). Human centrin has four EF-hand calcium binding sites as do both calmodulin and Androcam, but is only 50% identical to calmodulin and is larger by an
extra 24 amino acids. 150 ng of each protein was immunoblotted and probed with RC11 serum (Figure 4-2D). RC11 is capable of binding Androcam, but the signal is approximately four fold stronger in binding to calmodulin. RC11 was unable to detect human centrin.

Although Androcam is the most closely related to calmodulin identified thus far in Drosophila, other proteins with similarity to calmodulin have been identified in Drosophila. Three genes encoding troponin-C isoforms were identified in the same screen that identified Androcam (Fyrberg et al., 1994). These isoforms are less similar to Drosophila calmodulin, sharing only 40 - 42% identity. These proteins were not available for assay by immunoblot, but it seems unlikely that they would crossreact with the RC11 serum. E63-1 is a gene that produces a protein of 193 amino acids with two pairs of EF-hand motifs separated by a central helix, that shares 40% identity with calmodulin (Andres and Thummel, 1995). Based on the differences in sequence and the fact that no higher molecular weight bands are seen in immunoblots to Drosophila protein extracts (Fig 4-2C, lanes 2 and 3), it is unlikely that RC11 cross reacts with E63-1. Although the cross reaction observed with Androcam is significant, experiments performed by Alan Lu have demonstrated that Androcam is only expressed in the testes of male pupae and adult flies, and that no Androcam transcripts or protein are detectable in embryos nor in any stage of development in female flies. In addition, he has determined that calmodulin is present in at least three fold excess in the testes of adult flies. These data suggest that cross reactivity with Androcam and other EF-hand motif proteins should have little effect on most experiments performed with RC11 serum.
vi. RC11 detects only calmodulin in extracts from embryos and adults

It is important that the purified antibodies not cross react with other Drosophila proteins. To examine the specificity of RC11 serum, immunoblots were prepared using crude protein extracts made from wild-type embryos and adult flies. Figure 4-2C shows that a band of the same approximate size of recombinant calmodulin is detected by RC11 without any other bands being seen on the gel. This film was overexposed to detect any weak crossreactivity that might be present. Further proof that the bands detected by RC11 in tissue extracts represent calmodulin was obtained by use of the antibody on crude extracts from calmodulin mutants. Robert Heiman used this antibody to perform immunoblots to proteins from larvae homozygous for a calmodulin null allele Cam$^{n339}$ (Heiman et al., 1996). These animals showed greatly reduced levels of the protein detected by RC11 than heterozygous animals. The slightly faster mobility of calmodulin in tissue extracts will be discussed below.

b. Characterization by immunolocalization

Numerous attempts were made to immunolocalize calmodulin in Drosophila embryos using the RC11 serum. *In situ* hybridizations performed previously revealed zygotic calmodulin transcripts only in the CNS and PNS of the embryo (Kovalick and Beckingham, 1992), so these tissues were expected to show high levels of calmodulin protein. The first
few attempts were undertaken using standard procedures for staining embryos with the one exception that fixation of the embryos included 0.2% glutaraldehyde along with the standard 4% paraformaldehyde. The glutaraldehyde was used on the advice of other researchers who reported that calmodulin is not easily fixed with formaldehyde, and that glutaraldehyde would improve the fixation of calmodulin. We also chose to detect the antibodies using fluorescently-labeled secondary antibodies so that we could use confocal microscopy. Initial attempts showed high background levels of fluorescence across the visual spectrum, whether using anti-calmodulin antibodies or control antibodies (anti-Kruppel and anti-twist). Omission of glutaraldehyde from the fixation eliminated the non-specific fluorescence, but no specific signal was seen in embryos with RC11 antibodies, even though control antibodies showed specific staining.

Many modifications were attempted to try to get the RC11 antibodies to show specific detection of calmodulin. First, several fixatives were tried under the assumption that the calmodulin might not be getting sufficiently fixed in the tissue or that fixation might be altering the availability of the protein for antibody binding. The fixations attempted included: 4% paraformaldehyde with and without 0.2% glutaraldehyde and for times varying from 15 to 30 minutes, methanol fixation from 15 minutes to 5 hours, Zenker’s fixation, periodic acid/lysine/paraformaldehyde fixation, and 2% HgCl2 with paraformaldehyde. None of these different fixations gave any specific staining.

Standard fixation uses a buffer containing 1.33 mM EGTA which chelates calcium and other cations. Since removal of calcium might affect the ability to fix calmodulin in the tissue, an immunolocalization attempt
was also performed with calcium added to 5mM in excess of the EDTA concentration. This treatment also failed to give specific staining.

The next modification tried with embryos was designed to expose masked epitopes. The finding that only the C-terminus of calmodulin is detected by RC11 serum raised the possibility that the epitopes recognized by the antisera might have been blocked from the antibody by fixation, binding of other proteins or by adopting an unusual conformation. Attempts to eliminate this as a possibility involved: i) treating the embryos with Proteinase K, to remove any masking proteins, and ii) by denaturing with SDS. Several dilutions of proteinase K from 20 μg/ml to 50 μg/ml were used on embryos for five minutes then washed and probed with RC11 antiserum. Other embryos were treated with concentrations of SDS from 0.01% to 1% for five minutes before being washed and probed. Only the 1% SDS trial gave any promising results, with some nuclear staining in amnioserosa cells of germ band extended embryos. Subsequent attempts to optimize the SDS treatment failed to give any positive results; treatments of 1% SDS for less than 5 minutes failed to show specific staining while longer time periods resulted in failure to maintain tissue morphology.

Another concern that was addressed was the possibility that calmodulin was being leached out in the heptane or methanol washes that are standard in the embryo staining procedure. Elimination and reduction of these steps in the embryo protocol was attempted without success. Dissected tissues were then attempted because these tissues do not require washing with heptane or methanol in order to be permeabilized. Ovaries were stained with RC11 at several dilutions from 1:50 to 1:1000 and detected with both fluorescent and histochemical methods without specific
staining being detected. Ovaries were tried with varied fix times in paraformaldehyde and were also fixed with 2% HgCl₂ without success. Finally, dissection and fixation in PBS solutions with and without 5mM EGTA were tried without resulting in specific staining.

Finally, embryos and ovaries were tried with standard treatment but incubated with RC11 antibodies for various times at different temperatures. Embryos were incubated at 4°C for 24 and 36 hours, at 25°C for four or 16 hours, and at 37°C for 2 hours again without observable staining. Ovaries were incubated for the shorter time period at each temperature and again failed to stain.

c. Conclusions on RC11

The experiments detailed above demonstrate that RC11 serum contains antibodies directed against Drosophila calmodulin. Although these antibodies are able to detect wild-type and a mutant calmodulin in both native and denatured conformations as well as calmodulin from tissue extracts when used on immunoblots, the serum is unable to detect calmodulin by immunolocalization in fixed Drosophila tissue. This may be explained by the fact that only the C-terminus of the protein is detected by the antibody, which could be masked in some way when fixed in tissues. Although it is not useful for immunolocalizations, RC11 antiserum was used for subsequent immunoblot analyses (see Chapter 6).
2. RC13 and RC14 antisera, but not RC15 through RC18, have anticalmodulin antibodies

My second attempt to generate anti-calmodulin antibodies involved inoculating six rabbits, RC13 through RC18. RC13 and RC14 were inoculated with purified calmodulin, RC15 and RC16 were injected with trypsin digested calmodulin, and RC17 and RC18 were injected with thrombin digested calmodulin.

Since the first attempt to generate anti-calmodulin antibodies had produced a serum that could detect calmodulin on immunoblots but did not work for immunolocalization in Drosophila tissue, highest priority in examining these new preparations was to find antisera that could be used for immunolocalization. Special attention was paid to the central and peripheral nervous systems since previous studies in the laboratory had established that calmodulin expression is specifically activated in these tissues (Kovalick and Beckingham, 1992). The sera were used to probe 0 - 16 hour embryos collected from flies that were heterozygous for the calmodulin null mutation, Camn339. These embryos were used because 25% of the embryos will be homozygous for the null mutation and would be expected to have lower levels of calmodulin in the CNS and PNS.

a. RC13 and RC14

The RC13 and RC14 sera showed high levels of probably nonspecific fluorescence in the tracheal system and in the lumen of the salivary glands, but no specific localizations were detected (Figure 4-3). The RC13 and
Figure 4-3. Immunolocalizations performed with RC13 and RC14 antisera on embryos. Embryos were stained with 1:100 dilutions of undiluted RC13 (A) and RC14 (B) antisera and detected by FITC-conjugated goat anti-rabbit IgG antibodies. The images are projections of 16 images taken at 3 μm intervals through the embryos. (A) Lateral view of an embryo stained with RC13. Anterior is up in both figures. Scale bar = 50 μm. (B) Dorsal view of an embryo stained with RC14. Scale bar = 100 μm.
RC14 sera were checked for the ability to detect calmodulin on immunoblots of purified calmodulin, embryo protein extracts and adult protein extracts (Fig. 4-4A). Both sera detected calmodulin in all three lanes, but also several other bands were detected by both antisera. These antisera were affinity purified and the resulting antibodies were able to detect calmodulin on dot blots (Fig 4-4B).

The strength of detection of calmodulin by unpurified RC13 and RC14 antisera is approximately equal suggesting that the sera have similar titers of anti-calmodulin antibodies. Affinity purified RC13 serum, however, can detect much smaller amounts of calmodulin at equivalent dilutions than RC14 which has roughly the same titer as affinity purified RC11. This difference may be a result of different affinities for the bovine calmodulin used in affinity purification.

Immunolocalizations were repeated with the purified sera with nearly identical results to the unpurified sera. Because no specific patterns of staining were seen and no differences were detected within the population of embryos no further immunolocalization attempts were made with these sera.

b. RC15 and RC18

RC15 and RC18 showed patterns very similar to RC13 and RC14 but at much lower intensity. Subsequent affinity purification and probing of dot blots with these sera indicated that they possessed no anti-calmodulin antibodies. No further experiments were performed with these sera.
Figure 4-4. Characterization of antisera RC13 and RC14 by immunoblot analysis. A. Immunoblots to embryo and adult extracts and recombinant CaM using unpurified RC13 and RC14. Embryo extracts are from 0-18 hr embryos. Adult extracts are from mixed male and female adults. B. Dot blots with recombinant calmodulin. 100 and 7.5 ng of purified CaM were applied to each strip and probed with affinity purified RC13 and RC14. Purified RC11 was used as a control in both experiments.
c. RC16

RC16 showed the most interesting localization pattern in embryos from the heterozygous mutant parents. In most of the later stage embryos the connectives and commissures, of the CNS stained brightly (Fig 4-5A), while in some the staining was less intense. The connectives and commissures are axon bundles that run anterior to posterior and across the midline, respectively, within the central nervous system of the embryo. Preliminary quantitation of the embryos showed that roughly 25% of the embryos had the less intense staining. It seemed possible therefore that these were the calmodulin null embryos, with the residual staining in the CNS representing perdurance of maternally supplied calmodulin. In addition to the CNS staining, the chordotonal organs of the PNS showed staining in the scolopale cell, a support cell of that sensory organ.

An interesting finding was seen with very early syncytial blastoderm embryos. In the anterior, a long coiled structure was detected (Fig. 4-5B). Consultation with T. Karr (University of Illinois) established that this is the sperm tail, which is completely internalized by the egg in Drosophila (Karr, 1991).

Several experiments were undertaken to determine whether the staining seen with RC16 serum was due to detection of calmodulin. RC16 serum was used to probe blots of purified calmodulin and extracts from embryos and adult flies (Fig 4-6A). The serum failed to detect purified calmodulin and detected very little in the extracts and nothing close to the size of calmodulin. Affinity purified RC16 also failed to detect calmodulin on dot blots as well (Fig 4-6B). Furthermore, preincubation of RC16
Figure 4-5. Immunolocalizations of embryos with RC16 antiserum. 0-16 hour collections from Cam^{n339} /CyO embryos were probed with unpurified RC16 serum and detected with FITC-conjugated anti-rabbit IgG. (A) Stage 14 embryo with staining in the connectives and commissures of the central nervous system. Connectives run anterior to posterior while connectives cross the midline twice per segment. (B) Early embryo showing sperm tail staining by RC16 antiserum. Anterior is to the left in both images which are projections of 13 (A) or 20 (B) images taken at 2.5 μm intervals. Scale bar = 50 μm.
antiserum with an excess of purified calmodulin prior to immunolocalization fails to eliminate the CNS staining pattern.

In order to determine whether the embryos showing reduced levels of RC16 staining were indeed the calmodulin null homozygous embryos, immunolocalization was repeated using embryos from parents heterozygous for the Cam$^{n339}$ mutation over a CyO balancer chromosome carrying a construct expressing β-galactosidase in the wingless transcription pattern (CyO wg LacZ; Kassis et al., 1992). This construct can be used to identify the calmodulin null embryos since they will be the only embryos produced that do not show the wingless β-galactosidase expression pattern. The embryos were stained simultaneously with: i) RC16 antiserum detected with fluorescein conjugated anti-rabbit IgG secondary antibodies, and ii) a mouse generated anti-β-gal antibody detected with Cy3 conjugated anti-mouse IgG secondary antibody. Stripes of red fluorescence identified all embryos carrying the balancer chromosome (Cam$^{n339}$/CyO wg LacZ and CyO wg LacZ/CyO wg LacZ embryos), while homozygous null embryos (Cam$^{n339}$/Cam$^{n339}$) had no red fluorescence. The Cam$^{n339}$/CyO wg LacZ embryos can be distinguished from the CyO wg LacZ/CyO wg LacZ homozygous embryos because the latter also display morphological abnormalities during embryogenesis. The embryos were scored for their genotype and the intensity of the CNS staining with RC16. The results showed no correlation between homozygosity of Cam$^{n339}$ and low levels of RC16 staining in the CNS (Table II). These results prove that the localization pattern seen with RC16 is not due to the detection of calmodulin.
Figure 4-6. Characterization of RC16 antiserum by immunoblot analysis. (A) Immunoblots to embryo and adult extracts and recombinant CaM using unpurified RC16. Embryo extracts are from 0-18 hr embryos. Adult extracts are from mixed male and female adults. (B) Dot blots with recombinant calmodulin. 100 and 7.5 ng of purified CaM were applied to each strip and probed with affinity purified RC16. Purified RC11 was used as a control in both experiments.
Table II. Staining of the CNS axons by RC16 is independent of the calmodulin null allele Cam\textsuperscript{n339} in embryos.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CyO wg LacZ</th>
<th>Cam\textsuperscript{n339}</th>
<th>Cam\textsuperscript{339}</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos counted (^a)</td>
<td>21 (16.0%)</td>
<td>64 (48.8%)</td>
<td>35 (26.7%)</td>
<td>131</td>
</tr>
<tr>
<td>Strong CNS staining (^b)</td>
<td>17 (81.0%)</td>
<td>47 (73.4%)</td>
<td>28 (80.0%)</td>
<td>92</td>
</tr>
<tr>
<td>Weak CNS staining (^b)</td>
<td>4 (19.0%)</td>
<td>17 (26.6%)</td>
<td>7 (20.0%)</td>
<td>28</td>
</tr>
</tbody>
</table>

Results of two experiments were combined. The first experiment consisted of 37 embryos of which 1 was undeveloped and could not be genotyped, the second experiment consisted of 94 embryos of which 10 were undeveloped. \(^a\) Numbers in parentheses reflect the percentage of embryos of each genotype out of the total number of embryos. \(^b\) Numbers in parentheses reflect the percentage of embryos counted out of the total number of embryos of that genotype.

d. RC17

The RC17 antiserum also gave an interesting localization pattern that is apparently unrelated to calmodulin. This serum stains the pole cells from very soon after their formation at the posterior pole of the embryo. The staining is cytoplasmic and appears to be concentrated in two or three areas within the cell (Fig 4–7). Immunoblot analysis showed however that this serum does not recognize calmodulin either (Fig. 4–8). Pole cell staining is a frequently observed artifact encountered when generating antisera to Drosophila proteins (K. Aurora, personal communication).
Figure 4-7. Immunolocalization with RC17 antiserum. O-16 hour collections from Camn339/Cyo embryos were probed with unpurified RC17 serum and detected with FITC-conjugated anti-rabbit IgG. An embryo during germ-band extension (stage 8) showing fluorescence in the germ cells. Anterior is down. This image is a projection of 16 images taken at 3 μm intervals. Scale bar = 50 μm.
Figure 4-8. Characterization of RC17 antiserum by immunoblot analysis. A. Immunoblots to embryo and adult extracts and recombinant CaM using unpurified RC17. Embryo extracts are from 0-18 hr embryos. Adult extracts are from mixed male and female adults. B. Dot blots with recombinant calmodulin. 100 and 7.5 ng of purified CaM were applied to each strip and probed with affinity purified RC17. Purified RC11 was used as a control in both experiments.
3. RC20 through RC23

The third project to generate antisera against calmodulin involved three rabbits, two inoculated with performic acid oxidized calmodulin (RC20 and RC21), one inoculated with SDS-denatured calmodulin (RC22) and the fourth inoculated with AlCl₃-treated calmodulin (RC23). By the time that these antisera were received for testing, characterization of the calmodulin null allele and immunoblot analysis suggested a change in the screening procedure. Characterization of the Cam¹³³⁹ mutation showed that it does not cause lethality during embryogenesis and that embryonic development is normal in homozygous animals (Heiman et al., 1996). Furthermore, immunoblots performed on wild-type and mutant embryos (see Chapter 6) indicated that maternal supplies of calmodulin perdure throughout embryogenesis and that mutant animals have calmodulin protein levels equivalent to those of wild-type embryos until just prior to hatching as first instar larvae. Thus, in contrast to our previous assumptions, we could not expect to see differences in immunolocalization of calmodulin in mutant versus wild-type individuals during embryogenesis. We could, however, expect differences in calmodulin expression in the CNS and perhaps other tissues of first instar larvae since this is the stage in which the phenotypes are manifested in the mutant animals.

Antisera from RC20 through RC23 rabbits were affinity purified and used to probe dot blots of purified calmodulin (Fig 4-9A). RC20 and RC21 antisera were able to detect 100 ng of calmodulin and RC23 was able to detect 7.5 ng. Since RC22 serum was unable to detect calmodulin in this assay, no further characterization was conducted with this serum.
a. RC20 and RC23 detect calmodulin in fixed tissues

Affinity purified antibodies from sera RC20, RC21 and RC23 were used to probe dissected CNS and alimentary canal tissues from homozygous Camn339 first instar larvae and their heterozygous siblings. As can be seen in Figure 4-10 for RC20 staining of the CNS, the intensity of fluorescence was much lower in the homozygous organs when compared to the heterozygous ones. RC23 antiserum also displayed lower levels of fluorescence in the CNS of homozygous larvae, while fluorescence levels were very low for both sets of larval organs treated with RC21.

b. Characterization of RC20 and RC23 by immunoblotting

RC20 and RC23 sera were then tested for specificity by immunoblot analysis. Immunoblots with RC20 and RC23 (Fig. 4-9B) against purified calmodulin, embryo extracts and adult extracts show that both sera detect a band the same approximate size as recombinant calmodulin. RC23 also detects several smaller molecular weight bands. Since smaller bands are found both in extracts and in purified calmodulin lanes it is likely that these band represent degradation products of calmodulin. It was also determined that at equivalent dilutions, RC23 serum results in three fold higher intensity of detection of calmodulin, suggesting approximately three-fold higher titer or affinity than RC20 and RC11.

RC20 and RC23 were also tested for the ability to detect centrin and Androcam by immunoblot (Fig 4-9C). Neither antiserum detected human
Figure 4-9. Characterization of RC20 through RC23 antisera by immunoblot analysis. (A) Dot blots of recombinant calmodulin probed with affinity purified sera. 100 and 7.5 ng of purified CaM were applied to each strip and probed with affinity purified RC20 through RC23. Purified RC11 was used as a control. (B) Immunoblots to embryo and adult extracts and recombinant CaM using purified RC20 and RC23. Embryo extracts are from 0-2.5 hr embryos. Adult extracts are from mixed male and female adults. (C) Immunoblots of purified recombinant calmodulin, Androcam, and centrin (50 ng each) with purified RC20 and RC23.
Figure 4-10. RC20 antiserum detects calmodulin in fixed Drosophila tissues. RC20 immunolocalization to the CNS of Cam^{n399} heterozygous (A) and heterozygous (B) 1st instar larvae. Images are projections of 16 confocal images taken at 1.5 μm intervals. Both mutant and heterozygous tissues and images were treated identically. Anterior is to the left. The bright fluorescence on the left side of panel B is non-specific adherence of the secondary antibodies to a cuticular structure lining the gut.
centrin. Quantitation revealed that detection of calmodulin by RC20 has at least a 35 fold greater affinity for calmodulin than for Androcam, while RC23 has approximately two fold greater affinity for calmodulin. As discussed for RC11, the difference in affinity and the restricted pattern of Androcam expression indicate that RC20 can be used reliably for all immunolocalizations while RC23 must be used with greater caution to avoid detection of Androcam. In all tissues examined by both RC20 and RC23, no significant differences in localization pattern have been identified, although RC23 fluorescence is usually more intense.

D. Comparisons of Immunoblot Detection of Calmodulin by Different Antisera.

A mobility difference is often seen between purified recombinant calmodulin and calmodulin in crude protein extracts from Drosophila. In particular, RC11, RC13 and RC14 antisera detect calmodulin bands from adult and embryonic extracts that show faster mobility than recombinant calmodulin (Fig. 4-3C). These antisera also detect calmodulin in extracts as a double band. The mobility differences seen with these antisera may be due to differences in calcium content in the protein preps as loaded on the gel. One of the unique characteristics of members of the calmodulin family is the presence of a calcium-dependent mobility shift on SDS PAGE gels (Klee et al., 1979). Calcium-bound calmodulin has faster mobility than calcium free calmodulin. Although no efforts were made to adjust the calcium levels in the crude protein extracts, calcium was removed from the purified recombinant protein during purification, making it likely that the
altered mobility of calmodulin from crude extract is due to the presence of calcium. This may also explain the frequent observation of doublets of calmodulin from the crude extracts.

Differences are seen between the bands detected by RC11, RC20 and RC23 sera even in experiments in which the same extracts are probed with all three antisera (Figure 4-11). The calmodulin detected by RC11 in embryo extracts generally has faster mobility than recombinant calmodulin and there is significant variability in the mobility from sample to sample. The same extracts probed with RC20 and RC23 show less variability in the mobility of the bands detected and these are very similar in mobility to the purified protein. Also, RC20 has not been observed to detect doublets bands of calmodulin as have the other sera. While it is likely that the mobility differences are due to the amount of calcium present in the extracts, these antisera appear to have different affinities for the calcium-bound and calcium-free isoforms of calmodulin.

Post-translational modifications of calmodulin could be responsible for some of the differences in the pattern of bands detected by the different sera. First, the lysine at position 115 is trimethylated in several species (Newton et al., 1984; Yazawa et al., 1980; Seamon and Moore, 1980) and dimethyllysine has been reported in a some species (Schaefer et al., 1987; Molla et al., 1981). While amino acid sequencing of Drosophila calmodulin did not detect methylated amino acids (Gorlach et al., 1985), the trimethyllysine residue has been found in other Dipteran insects (Dudoignon et al., 1983) and suggests that methylation of the protein may be important in insects. Also, phosphorylated calmodulin has been isolated from mammalian tissues (Plancke and Lazarides, 1983; Joyal and Sacks,
Figure 4-11. Developmental immunoblots stained with RC11, RC20 and RC23 antisera. Crude extracts staged embryos, larvae and adults were loaded in equal amounts on three gels and used to prepare immunoblots. All three were treated identically except for probing with RC11 (A), RC20 (B) or RC23 (C). Lanes 1 and 12 = 100 ng recombinant CaM, lane 2 = 0-4 hr embryos, lane 3 = 4-8 hr embryos, lane 4 = 8-12 hr embryos, lane 5 = 12-16 hr embryos, lane 6 = 16-20 hr embryos, lane 7 = 20-24 hr embryos, lane 8 = 1st instar larvae, lane 9 = 3rd instar larvae, lane 10 = male adults, lane 11 = female adults.
1994) and *in vitro* phosphorylation of calmodulin affects the kinetics of activation of several calmodulin targets. (Sacks et al., 1992). One would expect, however, that modifications such as these would result in slower mobility of the modified protein rather than the faster mobility seen in immunoblots with RC11, RC13 and RC14. More experimentation will be required to determine whether mobility differences seen are due to post-translational modifications, the extent of calcium-saturation, or artifacts of the electrophoresis of the protein samples.

E. Conclusions

In summary, eleven rabbits were inoculated with preparations of calmodulin. Of these, six appear to have mounted some immune response to the protein. All attempts with both unmodified calmodulin and performic acid oxidized calmodulin resulted in successful production antibodies as did the single trial of aggregated calmodulin, while proteolytic digestion and denaturation were unsuccessful. Of the five antisera that contained anti-calmodulin antibodies, only two, RC20 and RC23, appear to contain antibodies that can detect calmodulin in immunolocalization. It should be noted that RC13 and RC14 were abandoned after no differences were seen in staining the offspring of Cam$n^{339}$ heterozygous crosses and were not tried on CNS or gut tissue from first instar larvae. These sera might contain antibodies that detect calmodulin in fixed tissue. RC13 has been used by collaborators to immunoprecipitate calmodulin and for detection of Drosophila calmodulin in mammalian cells (J. Joyal and D. Sacks, personal communication).
The antisera RC11, RC20 and RC23 were deemed to be the most useful for subsequent studies of calmodulin expression and localization. Affinity purified RC11 antiserum has been the serum of choice for immunoblotting of calmodulin, while RC20 and RC23 have been used for immunolocalizations.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Preparation</th>
<th>Immunoblot</th>
<th>Crossreaction</th>
<th>Immunolocalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC11</td>
<td>unmodified</td>
<td>+</td>
<td>++ (Androcam)</td>
<td>-</td>
</tr>
<tr>
<td>RC13</td>
<td>unmodified</td>
<td>++</td>
<td>NA</td>
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<td>RC16</td>
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¹ Not tested against larval tissues, may be positive (see discussion above). ² Affinity purified; non-purified serum detected calmodulin as strongly as RC13. ³ Not affinity purified. ⁴ See chapter 5 for localization patterns.
Chapter 5: Immunolocalization of Calmodulin

Immunolocalization studies can be a powerful tool for examining the function of a protein in the tissues of an organism. Observation of the spatial distribution and relative expression level of a protein at the tissue, cellular and sub-cellular levels can provide strong clues as to cellular functions of the protein.

The complex pattern of calmodulin transcripts observed by *in situ* hybridization (discussed in Chapter 3) raises a number of interesting questions about the role of calmodulin in Drosophila development. Some of these questions were addressed by immunolocalizations to look at the expression and localization of calmodulin protein in Drosophila tissues.

One of the questions of interest is whether calmodulin is required in all cells, as is commonly assumed (Klee and Vanaman, 1984). *In situ* hybridizations revealed that most tissues of the embryo lack detectable levels of calmodulin transcripts for most of embryogenesis (Kovalick and Beckingham, 1992). In addition, *in situ* hybridizations performed on larvae, pupae and adults revealed that numerous tissues are devoid of calmodulin mRNA at various stages (see Chapter 3). While it is possible that calmodulin is not essential in all cells at all times, a more plausible explanation is that calmodulin protein is very stable in these tissues and perdures between periods of calmodulin transcript production.

Another question concerns whether the amount of calmodulin protein is determined directly by the amount of calmodulin mRNA. Although examples to the contrary have been found, it is generally assumed that timing and amount of protein produced in Drosophila generally
correlate with transcription of the corresponding gene. However, some tissues and cells may have higher turnover rates for certain proteins or transcripts which could lead to differences in the number of transcripts present in a cell without having an effect on protein levels. Evidence exists for tissue-specific differences in calmodulin protein levels from mammalian systems (Klee and Vanaman, 1982). In particular, high levels of calmodulin have been identified in mammalian brain (Smoake, et al., 1974), which correlates well with the presence of abundant calmodulin transcripts in the embryonic nervous systems of Drosophila. This suggests that high levels of calmodulin protein could be expected in this tissue and in the CNS during embryogenesis and at later stages of development. Comparing the calmodulin immunolocalization pattern with the in situ hybridization pattern may reveal details about the regulation of calmodulin expression post-transcriptionally.

Finally, comparison of calmodulin levels between different tissues and developmental stages could also lead to inferences about the types of processes for which calmodulin is particularly important. The position of calmodulin within a cell may indicate the location of important signaling mechanisms and represents a first step in identifying upstream events or downstream targets of calmodulin signaling.

The immunolocalization of calmodulin was examined in two developmental systems: embryogenesis and oogenesis. Both systems have been extensively studied in Drosophila and involve a number of interesting cell biological processes such as cell-to-cell signaling (through a variety of pathways), cytoskeletal dynamics, mitosis, meiosis, endoreplication, and the dynamics of stem cell divisions.
For these experiments, we chose indirect immunofluorescent labeling of calmodulin in fixed tissue with detection by confocal microscopy. The embryos and developing oocytes of Drosophila are large structures and a process of interest may be buried over 100 μm deep in the tissue making resolution poor using conventional fluorescent microscopy. This is especially true when the protein of interest is expressed at high levels in the cytoplasm of all cells, as expected for calmodulin. Confocal microscopy allows high resolution imaging of objects in thick tissues by passing the fluorescent emission through a pinhole, thereby preventing out-of-focus light from degrading the resolution of the image from the focal plane. Images are collected electronically by use of a photomultiplier tube and converted into image files.

Two affinity purified anti-calmodulin antisera were used to detect calmodulin protein in immunolocalization: RC20 and RC23. These antisera are specific to calmodulin as determined by immunolocalization to mutant and wild-type larval tissue and by immunoblot analysis. The generation and characterization of these antisera are described in detail in Chapter 4. In all experiments, control samples were treated identically to the experimental samples except that the anti-calmodulin antibodies were omitted. These samples were used to set the sensitivity of the detector such that background caused by autofluorescence of the sample or by non-specific binding of secondary antibodies was minimized.

The results of immunolocalization of calmodulin during oogenesis and embryogenesis will be discussed in the next two sections. These will be followed by discussions of what localizations of calmodulin tell us about its role in cell functions and development.
A. Calmodulin Localization in the Ovary

1. Calmodulin is transiently localized to the spectrosome/fusome in the germarium

Figures 1-5 and 1-6 provide diagrams of the Drosophila ovary and the stages of oogenesis, respectively. Oogenesis begins in the germarium at the anterior tip of the ovariole, which contains the germline stem cells and is the site of egg chamber formation. Immunolocalization of calmodulin in the germarium is shown in Figure 5-1. Some germline stem cells, at most one per germarium, exhibit concentration of calmodulin above cytoplasmic levels in discrete structures lying at the anterior end of the cell (Figure 5-1A). Based on the asymmetry of the staining and its subcellular distribution this localization of calmodulin appears to reflect association with the spectrosome (Lin and Spradling, 1995; McKearin and Ohlstein, 1995). The spectrosome is a germline stem cell-specific organelle consisting of cytoskeletal proteins and membranes that has been implicated in regulating the asymmetric division of these stem cells (Lin and Spradling, 1995; McKearin and Ohlstein, 1995). Staining of the centrosomes, which are closely associated with the spectrosome (McKearin and Ohlstein, 1995), probably contributes to the localization of calmodulin in these cells (see below).

Drosophila homologs of the membrane cytoskeletal proteins α-spectrin and adducin have been identified as components of the spectrosome (Lin and Spradling, 1995). Mammalian adducins and α-spectrins bind
Figure 5-1. Immunolocalization of calmodulin in the gerarium. (A) and (B) show two confocal sections through a gerarium stained with anti-calmodulin antiserum RC23. (C) Confocal section of a gerarium stained with anti-α-spectrin antibody. Arrowheads mark the spectroyme in germline stem cells, arrows point to the fusomes in germline cysts located in region 2a of the gerarium. Anterior is to the upper left. Scale bar = 20 μm.
calmodulin (Bennett and Gilligan, 1993) making these likely targets for calmodulin localization at the spectrosome. Anti-α-spectrin staining of the spectrosome in germline stem cells is similar to anti-calmodulin staining except that the spectrosome can be detected in all of the germline stem cells in the germarium (Figure 5-1C). Therefore, while α-spectrin localization to the spectrosome is constant, calmodulin appears to associate only transiently with this organelle, since the anti-calmodulin antibodies only detect the spectrosome in at most one germline stem cell per germarium. Calmodulin localization to the spectrosome may be in coordination with stem cell division. Attempts to localize Hts protein, the Drosophila adducin homolog, to the spectrosome were unsuccessful, probably because the antibody used detects a different isoform (ADD-95; Zaccai and Lipshitz, 1996) than that used to show localization to the spectrosome (ADD-140; Lin and Spradling, 1995).

After a stem cell division, one cell, the cystoblast, undergoes four rounds of cell division with incomplete cytokinesis to form a cyst of 16 germ cells interconnected by cytoplasmic bridges called ring canals (King, 1970). Although the localization of calmodulin in these cells is not highly specific, calmodulin appears to be enriched near the fusome (Figure 5-1B), which extends through the ring canals connecting the cells of a cyst. The fusome is derived from the spectrosome and is similar in composition and structure. It serves as one pole in each cystocyte cell division then elongates to extend through the ring canal into the distal daughter cell as the new ring canal forms (McKearin and Ohlstein, 1995). Although the fusome remains intact through regions 2a and 2b of the germarium, calmodulin localization near the fusome is only seen in region 2a, which
contains cysts that are still in the process of cell division (Spradling, 1993).

By the time a germ cell cyst reaches region 3 of the germarium, it has been surrounded by somatic epithelial cells, called follicle cells, to form a stage 1 egg chamber (see Figure 1-5C). Cysts at this stage have slightly higher levels of calmodulin protein than in region 2, but no specific localization pattern was observed in the germ cells (Figure 5-1).

2. Calmodulin is localized to the cortical cytoskeleton and centrosomes in follicle cells

Follicle cells originate in region 2 of the germarium, where they migrate to form an epithelial layer completely surrounding each cyst of germ cells (Margolis and Spradling, 1995). From the earliest point at which these cells can be identified, they have high levels of calmodulin protein with significant concentration near the plasma membrane (Figure 5-2A). This pattern of localization persists throughout oogenesis. Staining of spectrin (Figure 5-2B; Deng et al., 1995), actin (Figure 5-1C; Riparbelli and Callaini, 1995), and three isoforms of Hts protein (Lin et al., 1994; Zaccai and Lipshitz, 1996) in follicle cells reveals a pattern similar to that seen by anti-calmodulin antibodies suggesting that calmodulin is localized to actin/spectrin networks in the sub-cortical cytoskeleton of these cells.

Beginning at stage 9 of oogenesis, the follicle cells at the posterior end of the egg chamber begin to take on a more cuboidal shape while those at the anterior flatten to become squamous. Thus, by stage 10 the majority of the follicle cells have migrated to cover the oocyte with a few flattened cells left covering the nurse cells (King, 1970). An interesting change in
Figure 5-2. Co-localization of calmodulin with the cortical cytoskeleton in follicle cells. Confocal images of anti-calmodulin (A) and anti-α-spectrin staining (B) in early stage egg chambers. Egg chamber in (A) is at stage 4, egg chamber in (B) is at stage 2. (C) shows the localization of calmodulin to the intersections of every three follicle cells in a stage 9. (D) shows similar staining with anti-D4.1 antibodies during stage 10. Scale bars = 10 μm in (A) and (B), and 30 μm in (C) and (D).
calmodulin localization has been observed in follicle cells during this process. While maintaining a strong peripheral localization, calmodulin becomes highly concentrated at every point of intersection between three follicle cells covering the oocyte (Figure 5-2C). This unusual staining pattern is seen only in the oocyte follicle cells since the follicle cells that remain over the nurse cells do not display this localization, but do retain high levels of calmodulin protein.

Striking similarity is seen between localization of calmodulin and the protein 4.1 homolog of Drosophila, D4.1, in follicle cells during stage 9. Protein 4.1, like spectrin and adducin, is associated with the membrane cytoskeleton in vertebrates and is also a calmodulin-binding protein (Bennett and Gilligan, 1993). D4.1 is also found at the periphery of the follicle cells with greater localization seen at the intersections of every three cells (Figure 5-2D). For both proteins, this focus of intense staining is located apically on the lateral follicle cell membrane. Fehon et al. (1994) reported that D4.1 is associated with septate junctions, which are thought to be the equivalent of vertebrate tight junctions. A similar localization pattern in stage 10 follicle cells is seen for the Drosophila tumor suppressor Discs-Large, another protein associated with septate junctions (Dlg; Woods and Bryant, 1991). The concentration of Dlg and D4.1 at three cell intersections appears to persist longer than calmodulin concentration, since the latter has only been observed in stage 9 egg chambers. D4.1 staining at three cell intersections has been observed in stage 10 egg chambers.

Calmodulin protein was also detected in very intense spots within the follicle cells that are believed to be the centrosomes of these cells. The
spots always appear in pairs either within a single cell or in two adjacent cells (Figure 5-3). The single cells with two intense spots appear by morphology to be in mitosis, while the adjacent cells with single spots are possibly the daughter cells of a recent cell division. Since this localization is not seen in all follicle cells, calmodulin association with the centrosome must be transient during cell division (from metaphase through telophase) and may indicate a role for calmodulin in regulating mitosis. Centrosomal staining is only seen in egg chambers up to stage 5, when cell division ceases in follicle cells (Spradling, 1993).

3. Calmodulin localization in the nurse cells and oocyte

Calmodulin is found in moderate levels in the cytoplasm of both the nurse cells and oocyte from the earliest stages. During stages 1 through 6 of oogenesis a very intriguing localization of calmodulin to the germinal vesicle (oocyte nucleus) was detected. During these stages, the germinal vesicle is positioned at the posterior of the oocyte. Calmodulin was detected at high levels around the periphery of the germinal vesicle (Figure 5-4A). This localization appeared to be in a gradient that is strongest at the extreme posterior face of the germinal vesicle and was not observed in egg chambers beyond stage 6, after the germinal vesicle migrates to the anterior of the oocyte.

The presence of calmodulin at the posterior of oocytes during early embryogenesis may reflect association with microtubule organizing centers. During stages 1 through 6, the microtubule network extending through the oocyte/nurse cell syncytium originates from a loosely
Figure 5-3. Calmodulin localization to the centrosomes during follicle cell divisions. A confocal image of a stage 4 egg chamber stained with anti-calmodulin antiserum. Arrowhead shows a cell in mitosis with both centrosomes visible. Arrows point to adjacent cells that each have one intensely staining spot. These are believed to be the centrosome remnants in daughter cells of a recent cell division. The asterisk marks the germinal vesicle (oocyte nucleus). Anterior is up. Scale bar = 20 μm.
Figure 5-4. Localization of calmodulin in the developing oocyte. (A) An image of calmodulin localization at the periphery of the oocyte nucleus (arrow) in a stage 5 egg chamber. Scale bar = 20 μm. (B) A stage 7 egg chamber showing elevated levels of calmodulin in the cytoplasm of the oocyte (arrow). Scale bar = 30 μm. (C) Calmodulin localization to the cortex (arrows) of a stage 9 egg chamber. Scale bar = 40 μm.
organized MTOC in the posterior of the oocyte (Theurkauf et al., 1992). After stage 6, when the germinal vesicle has moved toward the anterior of the oocyte and calmodulin is no longer detected at the germinal vesicle surface, the microtubule network undertakes a major rearrangement such that microtubules are organized from centers located at the anterior of the oocyte.

The intensity of anti-calmodulin fluorescence in nurse cells and oocytes appears equal in the earliest stages of oogenesis. By stage 4, however, some egg chambers exhibit a small relative increase in calmodulin levels in the oocyte (Figure 5-4B). Calmodulin is concentrated at the plasma membranes of the nurse cells and oocytes throughout oogenesis. While this may be analogous to the peripheral staining seen in follicle cells, the staining at the germ cell membranes is much weaker and less defined.

As the oocyte begins to expand rapidly after stage 8, calmodulin is concentrated more strongly at the cortex of the oocyte where it contacts the follicle cells (Figure 5-4C). Although it appears that this concentration of calmodulin is within the oocyte, the boundary between the follicle cells and the oocyte consists of deeply interdigitated microvilli (King, 1970), making a precise determination beyond the capabilities of fluorescent microscopy.

4. Some elements of the calmodulin localization pattern in the ovary are not consistently repeatable

All of the patterns of calmodulin localization discussed above were observed repeatedly in the first few attempts at calmodulin localization. In
addition, both RC20 and RC23 antisera were used and compared with each other and negative controls to ensure that the patterns identified were not spurious. However, after several months some of the pattern elements became difficult to reproduce. In particular, the germinal vesicle localization in the oocyte and the centrosomal staining in the follicle cells was no longer observable. Attempts were made to restore the staining, which included fresh purification of antiserum, adjustments to the fixation protocol, and changes in the amount of calcium in the dissection buffer. None of these was able to restore these elements of the calmodulin localization pattern. The follicle cell localization near the plasma membrane has been consistently observed, while the spectrosome/fusome localization has been repeated often. It is unclear why some aspects of this pattern have been difficult to repeat. One possibility is that the epitopes recognized by the antibodies may be difficult to detect when localized around the germinal vesicle or the centrosome of follicle cells. Although it is also possible that these pattern elements derive from cross reaction to some other protein, calmodulin localization to centrosomes during embryogenesis (see below) suggests that a more likely explanation involves variations in experimental conditions, such as crowding of the flies or conditions during tissue collection.

B. Calmodulin Localization in Embryos

1. Overview of calmodulin protein expression during embryogenesis
Figure 5-5 shows the immunolocalization of calmodulin at various stages throughout embryogenesis. Calmodulin was detected in all cells observed and was present predominantly in the cytoplasm, although some fluorescence was detected in most nuclei. In many cells there appeared to be some enrichment at the periphery, which may be analogous to the cortical membrane staining observed in follicle cells. The specific localizations of calmodulin during embryogenesis will be detailed below.

Qualitative comparison of the amount of anti-calmodulin fluorescence in embryos at different stages of embryogenesis reveals higher levels during stages 1 through 5, which encompass the cleavage and blastoderm divisions (Figure 5-5A). After cellularization, global levels of calmodulin appear lower than during earlier stages (Figure 5-5B) and remain at lower levels throughout embryogenesis in most tissues (Figure 5-5C-F).

Two tissues showed higher levels of calmodulin protein relative to the rest of the embryo, the midgut and the CNS. The midgut forms from two invaginations during gastrulation, one at the anterior and one at the posterior end of the embryo (Costa et al., 1993). These midgut primordia show moderately higher levels of calmodulin that persist until late in embryogenesis, after the two sets of cells meet to form the larval midgut (Figure 5-5C-E). No specific subcellular localization has been detected within these cells. Transcript levels persist slightly longer in the midgut primordia during gastrulation than in most tissues (Kovalick and Beckingham, 1992), which may result in the increased calmodulin protein detected in this tissue.
Figure 5-5. Immunolocalization of calmodulin during embryogenesis. (A) Stage 4, syncitial blastoderm embryo. (B) Stage 5, cellular blastoderm embryo. Most calmodulin is located in the cytoplasm that lies beneath the peripheral nuclei (C) Stage 8, germ band elongation. In (C) and (D) arrows denote anterior and posterior midgut primordia. (D) Stage 12. germ band retraction. (E) Stage 14 embryo. Anterior and posterior midgut primordia have united to form one continuous tissue. (F) Stage 16 embryo showing high levels of calmodulin protein in cells of the CNS. For all panels anterior is to the left and dorsal is up. Scale bar = 60 μm.
Prior to stage 14, neural calmodulin levels are not elevated relative to other tissues (Figure 5-5C-E), even though cells of the neural lineages show high levels of calmodulin transcripts from the point at which they are first identifiable at least 8 hours earlier (Kovalick and Beckingham, 1992). During stage 14, when the central nervous system is fairly well organized, calmodulin begins to be detected in higher levels in the CNS than in other tissues (Figure 5-5F). This increase is not seen in all cells, but appears in a large number of cells in a segmentally repeating pattern of cell bodies located at the ventral surface of the ventral nerve cord (Figure 5-6A). At least some of cells may be neurons since anti-calmodulin fluorescence is also observed in the axon tracts (Figure 5-6C). Within the CNS there are two major sets of axon tracts: The connectives are the long tracts on either side of the midline that run in an anterior-posterior direction. Commissures are short tracts, two per segment, that cross the midline between the connectives. Surprisingly, the connectives are labeled non-uniformly with anti-calmodulin antibodies, while the commissures show very little specific localization of calmodulin (Figure 5-7).

As discussed in the introduction to this chapter, one of the reasons for performing immunolocalizations was to compare levels of calmodulin protein with the pattern of calmodulin transcript production during embryogenesis. Kovalick and Beckingham (1992) demonstrated that maternally-supplied calmodulin transcripts are distributed uniformly throughout embryos until the beginning of gastrulation. These transcripts are gradually degraded until the only calmodulin transcripts detected after five hours of development are those produced by zygotic transcription in the developing CNS and PNS. These two tissues display high levels of
Figure 5-6. Calmodulin localization in the embryonic ventral nerve cord. Ventral views of the ventral nerve cord taken at three different focal depths. (A) Image taken near the ventral surface of the ventral nerve cord. (B) A focal plane 7 μm deeper in the ventral nerve cord. (C) A focal plane 10 μm deeper than in A that shows the nerve tracts near the dorsal surface of the nerve cord. Connectives run horizontally, commissures run vertically. Anterior is to the left. Scale bar = 20 μm.
Figure 5-7. Calmodulin localizes to connectives but not commissures during late embryogenesis. Embryos were double-labeled with anti-CaM and BP102 to stain axons. (A) Anti-calmodulin labeling of the CNS. Calmodulin is found at higher levels in the connectives. (B) BP102 staining of the axon tracts. Arrowheads - connectives, arrows - commissures. (C) Overlay of calmodulin staining in green and BP102 in red. Scale bar = 15 μm.
calmodulin mRNA through the end of embryogenesis. As reported in Chapter 2, global transcription of the calmodulin gene is activated just prior to hatching.

Surprisingly, the pattern of calmodulin proteins differs significantly from the calmodulin mRNA pattern. First, in both experiments the levels of calmodulin protein and transcripts are high in very early embryos, however, the amount of protein as determined by immunolocalization appears to decrease in advance of the degradation of calmodulin transcripts. Calmodulin protein levels reach a lower level at cellularization whereas transcript levels are not detectably lower until gastrulation is well underway (Kovalick and Beckingham, 1992). Second, calmodulin transcripts are present at high levels in the CNS and PNS throughout embryogenesis, but increased calmodulin protein levels are not detected in the CNS until stage 14. Increases in PNS staining were not observed before stage 17, when embryos become refractive to whole mount staining due to the production of cuticle. Furthermore, only a subset of CNS cells displayed an increase in calmodulin even though all cells of the CNS possess calmodulin transcripts. The CNS cells showing increases in calmodulin protein levels may correspond to those that display greater amounts of calmodulin transcripts (Kovalick and Beckingham; 1992).

2. Calmodulin localizes to the spindles and pericentrosomal cytoplasm in syncytial embryos

The first two hours of embryogenesis in Drosophila consist of rapid synchronous mitoses of nuclei in the common cytoplasm of the syncytial
egg (Foe et al., 1993). In order to examine the subcellular localization of calmodulin during early embryogenesis, embryos were double stained to detect calmodulin and either β-tubulin, actin or DNA. Double staining of calmodulin and DNA revealed predominantly free cytoplasmic calmodulin during the early stages of embryogenesis. Embryos in metaphase or anaphase of mitosis had large areas that were devoid of calmodulin staining that appeared to correspond to the position of the mitotic spindles (data not shown). Embryos were then double stained for calmodulin and β-tubulin to further examine this pattern. Detection of intact microtubules in Drosophila embryos requires stabilization of microtubules which can be accomplished with the drug taxol. The staining of taxol-treated embryos with anti-calmodulin and anti-β-tubulin antibodies reveals a dynamic pattern of calmodulin localization during these mitoses. During interphase, calmodulin is found weakly concentrated in loosely organized structures that appear to be the microtubule organizing centers (MTOCs). Calmodulin protein is detected around the base of the spindles during metaphase and anaphase of mitosis (Figure 5-8A). The highest intensity of fluorescence lies just distal to the spindle ends. Calmodulin localization also appears to extend along the spindles toward the metaphase plate and in a cloud around the base of the spindles, where the centrosome is located. A small region in the center of this cloud appears to have a much lower level of calmodulin (Figure 5-7B), suggesting that calmodulin is concentrated in the pericentrosomal material but may be excluded from the core of the centrosome.

This contrasts with the localization of the calmodulin to centrosomes in follicle cells of the ovary in which calmodulin appears to be present
Figure 5-8. Immunolocalization of calmodulin during syncytial blastoderm nuclear divisions. Embryos were double stained with anti-CaM and anti-β-tubulin. (A) Nuclei that are between mitoses and are therefore in M phase. Staining is seen in the nuclei and around the periphery of the nucleus. (B) Staining of cells in mitosis in syncytium. Left - anti-calmodulin, middle - anti-β-tubulin, Right - overlay of both channels. Scale bar = 10 μm.
throughout the centrosome. This may reflect differences in the organization of centrosomes in the nuclear divisions of early embryogenesis and other cell divisions in Drosophila. Unlike other cell divisions the nuclear divisions of cleavage stage embryos occur very rapidly and the centrioles duplicate and begin to separate before mitosis is completed (Foe et al., 1993). Thus, the lack of calmodulin staining in the centrosome core observed in the embryonic nuclear divisions may be due to this early duplication and separation of the centrioles that results in a relatively loose organization of the centrosomes.

Since calmodulin antibodies failed to localize to the centrosome and spindles in embryos not treated with taxol, localization of calmodulin to the pericentrosomal cloud and the mitotic spindles appears to be dependent upon intact microtubules.

Calmodulin localization was also compared to the localization of actin, α-spectrin and D4.1. Although calmodulin is somewhat enriched at the cortex of cells after gastrulation begins, calmodulin localization is less defined than these proteins. No evidence was found for specific colocalization of calmodulin with any of these proteins prior to and during cellularization. Therefore, in comparison to the cells of the ovary, localization of calmodulin at the cortical cytoskeleton is not as robust.

In most tissues observed, the cytoplasm contains higher levels of calmodulin protein than the nucleus. One exception has been observed, however, during early embryogenesis. In some syncytial embryos, nuclei have been observed to have higher levels of calmodulin in the nucleus than in the cytoplasm (Figure 5-8B). These nuclei appear by morphology to be in interphase, indicating that they are in the process of DNA replication.
No specific localization within the nucleus is observed.

C. Discussion

1. The role of calmodulin in cell division

Calmodulin protein has been detected at the centrosomes and spindles during mitosis in early embryos and in ovarian follicle cells, suggesting a role for calmodulin in the regulation of cell division. In the early embryo this localization is strongest during metaphase and anaphase, although calmodulin appears to associate with the MTOC during interphase and prophase as well. In follicle cells, prominent localization to the spindle poles is observed during what appears to be metaphase and anaphase, however, in contrast to embryonic nuclear divisions, the localization to the spindle is weaker and no discontinuity is detected in staining of the centrosome. Also, the localization of calmodulin to centrosomes of follicle cells appear to remain strong long after completion of mitosis. These differences are probably a result of differences between typical cell divisions in Drosophila, as represented by follicle cell divisions, and the unique nuclear divisions of early embryogenesis.

Calmodulin localization to mitotic spindles has been observed previously in many organisms including mammals (Welsh et al., 1984; Stemple et al., 1988), invertebrates (Hamaguchi and Iwasa, 1980), plants (Vantard et al., 1985), and yeast (Stirling et al., 1994). As was observed in Drosophila, calmodulin localization to the spindle depends upon the
presence of intact microtubules, at least in mammalian cells (Stemple et al., 1988).

The interaction of calmodulin with microtubules and centrosomes in cycling cells differs slightly between Drosophila and other species. In Drosophila calmodulin does not appear to interact with interphase microtubules and mitotic spindles along the entire length of the microtubules. Rather, calmodulin localization in Drosophila appears strongest at the poles of mitotic spindles and decreases approaching the metaphase plate. In interphase cells, the localization of calmodulin to microtubules is detected primarily at the periphery of the nucleus where the MTOCs are located. MTOC and microtubule localization has been observed only in cells that are actively cycling, with the possible exception of staining at the periphery of the oocyte nucleus.

Most evidence suggests that calmodulin does not interact with tubulin directly, but affects microtubules through certain microtubule associated proteins (MAPs), that were originally identified based on their ability to enhance and stabilize microtubule polymerization (Lee and Wolfft, 1984; Stemple et al., 1988). High calcium concentrations inhibit microtubule formation and enhance rapid depolymerization of preformed microtubules in vivo (Karr et al., 1980). These effects have been separated into two mechanisms (Berkowitz and Wolfft, 1981): an intrinsic calcium-dependent destabilizing effect that is inhibited by MAPs and a second microtubule destabilizing mechanism is calmodulin- and MAP-dependent (Lee and Wolfft, 1982). Thus, MAPs have opposite effects on microtubules depending on the presence or absence of Ca\(^{2+}\)-calmodulin.

Some MAPs, including MAP2, MAP4 and \(\tau\), are calmodulin binding
proteins (Lee and Wolf, 1984). Several MAPs have been identified in Drosophila (Kellogg et al., 1989), some of which share very similar localization to calmodulin during early embryogenesis. Two of these MAPS, CP60 and CP190, form a complex with γ-tubulin that is localized to the pericentriolar material in a pattern very reminiscent of the localization of calmodulin (Raff et al., 1993; Kellogg et al., 1995; Oegema et al., 1995). γ-tubulin has been shown to be essential for proper microtubule nucleation from centrosomes in Drosophila (Debec et al., 1995) and Aspergillus (Oakley et al., 1990). None of these Drosophila proteins have been assayed for the ability to bind calmodulin, but their similar localization to calmodulin and homology to mammalian MAPs suggest that these or other homologous complexes may be important targets of calmodulin during the cell cycle.

Calmodulin has been shown to be important for proper assembly and function of spindles during cell division in the yeast Saccharomyces cerevisiae. Calmodulin null mutations cause lethality due to defects in mitotic spindles and aberrant chromosome segregation (Davis, 1992; Ohya and Botstein, 1994). This essential function of calmodulin involves Spc110p, a protein localized to the spindle pole body (SPB), which acts as the MTOC in S. cerevisiae (Geiser et al., 1993). Furthermore, calmodulin localization to the SPB is the result of binding to Spc110 (Spang et al., 1996) and this interaction is necessary for proper assembly of the SPB and the mitotic spindle (Sundberg et al., 1996).

The observations outlined above suggest that calmodulin functions to regulate the nucleation and polymerization/depolymerization of microtubules during mitosis. The observation that Drosophila calmodulin
is localized to the centrosomes and spindles during mitosis predicts the existence of calmodulin targets involved in assembly and functional behavior of mitotic spindles in Drosophila.

Additional roles for calmodulin in cell division have been elucidated by studies which disrupt Ca\(^{2+}\)-calmodulin signaling. Several major checkpoints in the cell cycle are calmodulin dependent in various species including the Go/G1, G1/S and G2/M transitions as well as the triggering of anaphase (Means, 1994; Berridge, 1995; Homa, 1995). Calcium/calmodulin-dependent protein kinase II (CaMKII) appears to be an essential target of calmodulin signaling at these cell cycle transitions (Morin et al., 1994; Means, 1994; Takuwa et al., 1995).

The subcellular localization of CaMKII in actively cycling mammalian cells is very similar to that observed for calmodulin as discussed above. Ohta et al. (1990) report that CaMKII localizes to the centrosomes and spindles during metaphase and anaphase of mitosis. Furthermore, as observed for calmodulin, localization is dependent upon intact microtubules (Ohta et al., 1990).

Based on the above data, it seems likely that the localization pattern of calmodulin to the mitotic apparatus in Drosophila is a reflection of its activity in regulating cell and nuclear cycles. Since centrosomal localization is seen in embryos and in follicle cells, the function of calmodulin in cell cycle control is probably not limited to the rapid mitoses of cleavage and blastoderm embryos, but is a feature of all cell divisions in Drosophila.

Although many of the components of the cell cycle are conserved between many eukaryotes, the early mitoses of Drosophila embryogenesis
are unique in several ways that make predictions of the specific roles of calmodulin more complicated. The first 10 cycles of cell division are extremely rapid and lack G1 and G2 phases (Foe et al., 1993). Measurement of endogenous calmodulin and experimental manipulation of calmodulin expression and activity have established a correlation between calmodulin concentration and the rate of cell division (Means, 1994). Calmodulin levels in precellular Drosophila embryos observed by immunolocalization (Figure 5-5) are qualitatively higher than in later stages suggesting that the high levels of maternal calmodulin may contribute to the rapid cell cycle during early embryogenesis. Since cyclic degradation and synthesis of cyclins, which normally sets the pace of the cell cycle, has not been observed during the first ten nuclear divisions (Foe et al., 1993), calmodulin regulation of cell cycle transitions could be a primary determinant of cell cycle progression.

2. Calmodulin interaction with the cytoskeleton

Immunolocalizations revealed calmodulin interaction with elements of the cytoskeleton other than mitotic spindles. Ovarian follicle cells show calmodulin localization to the peripheral cytoskeleton (Figure 5-2). In germ cells calmodulin is associated with the spectrosome/fusome (Figure 5-1). Localization of calmodulin at the cortex of oocytes after stage 8 of oogenesis may also be due to cytoskeletal interactions (Figure 5-4C).

The localization pattern of calmodulin in Drosophila ovaries overlaps with various aspects of the localization patterns of actin (Riparbelli and Callaini, 1995), α-spectrin (Figure 5-1C,2B; Deng et al.,
1995), adducin homologs from the hts locus (Lin et al., 1994; Zaccaï and Lipshitz; 1996), D4.1 (Figure 5-2D, Fehon et al., 1994), and Dlg (Woods and Bryant, 1991). Some differences are seen between the localization patterns of these proteins. In follicle cells, actin, spectrin and Hts proteins are concentrated in the region just beneath the plasma membrane and have a filamentous appearance, which is very similar to calmodulin localization at most stages. The localization of D4.1 and Dlg are more discrete, being tightly associated with the septate junctions in the cell membrane. Calmodulin localization seems more diffuse than D4.1 and Dlg except during stage 9, when all three proteins are concentrated at the intersections between follicle cells. This suggests that calmodulin associates with the septate junction during this stage, and may play a role in the cell shape changes that occur at this stage to cause migration of the majority of follicle cells to cover the oocyte.

Actin and one Hts isoform are components of the ring canals that join the cytoplasm of germ cells within each cyst (Robinson et al., 1994), however, calmodulin does not appear to associate with these structures at any point in oogenesis. Spectrin is virtually absent from oocytes after stage 9 (Lin et al., 1994) while calmodulin (Figure 5-4C), actin (Riparbelli and Callaini, 1995), and at least one hts gene product (Zaccaï and Lipshitz, 1996) are localized at the cortex of the oocyte.

Adducin localization in the embryo is similar to calmodulin localization in that two isoforms show enrichment in the developing midgut and CNS (Zaccaï and Lipshitz, 1996). However, the neural cell bodies containing high levels of hts protein are found primarily near the dorsal surface of the ventral nerve cord whereas calmodulin levels are higher in
more ventral cells (Figure 5-6A; Zaccai and Lipshitz, 1996).
Furthermore, staining of hts protein in the axon tracts is more prominent
in the commissures than in the connectives, which is opposite of the
predominantly connective localization seen for calmodulin (Figure 5-6C).

No convincing colocalization of calmodulin with either spectrin,
actin was observed during embryogenesis. In addition, D4.1 and Dlg
associate tightly with the plasma membranes of most epithelial cells (Fehon
et al., 1994; Woods and Bryant, 1991), a pattern not observed with anti-
calmodulin staining.

Adducin and α-spectrin were identified as components of the
erthrocyte plasma membrane cytoskeleton and found to be calmodulin
binding proteins (Bennett and Gilligan, 1993). α-spectrin and β-spectrin
combine with actin to form cytoskeletal filaments that associate with the
plasma membrane and a number of different proteins, including adducin
and protein 4.1 (Bennett and Gilligan, 1993). The spectrin-based
cytoskeleton is believed to function in controlling cell shape and cell-cell
contacts by correctly positioning proteins at the plasma membrane (Bennett
and Gilligan, 1993; Lee et al., 1993; Deng et al., 1995). Adducin is
thought to promote the assembly of spectrin/actin fibers in mammals
(Hughes and Bennett, 1995) and to cap the ends of actin filaments
preventing elongation and depolymerization (Kuhlman et al., 1996). Both
functions of adducin are inhibited by Ca^{2+}-calmodulin (Kuhlman et al.,
1996), as is the affinity of spectrin for actin (Bennett and Gilligan, 1993).

The staining of the fusome with anti-α-spectrin and anti-Hts
antibodies is very discrete and persists throughout regions 2a and 2b of the
germarium (Lin et al., 1994) whereas anti-calmodulin staining of the
fusome is more diffuse and is seen only in region 2a. Actin is not a major component of the fusome (Lin et al., 1994).

In Drosophila, the fusome and spectrosome are membrane containing organelles believed to regulate the divisions of the germ line stem cells and cystocytes. Mutations in genes encoding fusome products result in either too many or too few divisions of stem cells and cystocytes (Lin and Spradling, 1995; McKearin and Ohlstein, 1995). Some fusome protein mutations also result in the inability of cystocytes to properly differentiate into nurse cells and oocyte, possibly through the failure to properly target certain proteins or RNAs to the presumptive oocyte.

3. Calmodulin in the central nervous system

Immunolocalization of calmodulin in the nervous system revealed a segmentally repeated pattern of cells that show high level of calmodulin protein expression after stage 14 of embryogenesis (Figure 5-6). Most of these cells are located at the ventral surface of the CNS. These cells have not been identified but at least some of them are likely to be neurons as specific localization of calmodulin can be detected in the neuropil at the same stage as calmodulin levels increase in the cell bodies. Many of the better characterized neurons and glia of the embryonic CNS are located on the dorsal surface making identification of most of the calmodulin expressing cells unlikely at this time (Seeger et al., 1993; Menne and Klämbt, 1994).

Calmodulin has been shown to have diverse effects upon neuronal function in numerous organisms. Many of these effects involve the role of
calmodulin in regulating phosphorylation and dephosphorylation of numerous downstream targets via calcium-calmodulin dependent kinases, particularly CaMKII and calcineurin (Braun and Schulman, 1995). In Drosophila, calmodulin targets CaMKII (Griffith et al., 1993) and adenylate cyclase (rutabaga; Levin et al., 1992) are known to affect learning and memory and neuronal plasticity.

Observation of the localization of calmodulin in the embryonic CNS reveals the surprising finding that greater amounts of calmodulin are localized in connectives, the long anterior to posterior axon tracts in the CNS, than in the commissures connecting the connectives across the midline. Since calmodulin localization is also absent from axon bundles leaving the CNS, higher calmodulin levels may be limited to CNS interneurons. Malfunction of these neurons due to low levels of calmodulin may cause the behavioral defects observed in calmodulin null larvae (Heiman et al., 1996). Expression of high levels of calmodulin protein in the nervous system occur too late in neurogenesis to suggest a role for zygotic calmodulin in the formation of the nervous system, but instead suggest the importance of calmodulin in proper neuronal function.

4. Regulation of calmodulin levels

Based upon in situ hybridization data (Kovalick and Beckingham, 1992), it was initially expected that expression of calmodulin protein would be high in the developing CNS and PNS of the embryo. As will be discussed in chapter 6, immunoblot analysis suggested that the nervous system may not contain higher levels of calmodulin as originally believed
because null mutant embryos and their heterozygous siblings have similar calmodulin protein levels.

Consistent with the generally accepted opinion that calmodulin is necessary in all eukaryotic cells, this immunolocalization work revealed no cells that completely lack detectable amounts of calmodulin protein during embryogenesis. Since many tissues of Drosophila embryos lack detectable levels of calmodulin mRNA (Kovalick and Beckingham, 1992), calmodulin protein must be very stable in these cells, perduring up to 20 hours from maternal contributions. This evidence supports immunoblotting results (presented in Chapter 6) indicating that calmodulin levels during embryogenesis are determined solely by maternal contribution.

Immunoblotting experiments detailed in Chapter 6 also indicate that zygotic calmodulin transcripts produced in the embryonic CNS are not translated during most of embryogenesis, since even relatively late in embryogenesis embryos homozygous for the calmodulin null mutation contained essentially equal amounts of calmodulin compared to heterozygous embryos. Immunolocalization of calmodulin in the embryonic CNS largely confirms this interpretation since increases in calmodulin protein levels within the nervous system are not detected early in neurogenensis. Higher calmodulin levels are detected in the CNS during later embryogenesis, after stage 14, indicating translation of the zygotic transcripts. This discrepancy may be due to the relative sensitivities of the two different types of experiments, as the increase in the amount of calmodulin in the CNS may be small in comparison with the total amount of calmodulin in the embryo.
D. Summary

The immunolocalization of calmodulin has indicated roles for calmodulin in the regulation of the cell cycle, the cytoskeleton and neural function. The role of calmodulin in cell cycle regulation is likely to occur at several points, particularly in the proper assembly and function of mitotic spindles, DNA synthesis and in determining the timing of mitotic divisions during early embryogenesis. The extensive co-localization of calmodulin with both adducin and spectrin supports the prediction that calmodulin regulates cytoskeletal structure and influences cell shape and migration. The timing of increases in calmodulin levels in the nervous system suggests regulation of CNS function during larval life.
Chapter 6: Examination of Calmodulin Levels by Quantitative Immunoblotting Techniques

In addition to immunolocalization, the availability of anti-calmodulin antibodies permits the examination of calmodulin expression by immunoblotting methods. While immunolocalization is useful for determining the location of the protein within individual cells and for visual comparisons of protein levels in different tissues in the organism, immunoblots have the advantage of permitting quantitation of overall calmodulin levels. Immunoblot analysis was therefore used to study the expression and stability of maternal and zygotic calmodulin in the embryo. One issue addressed by immunoblotting was whether the level of calmodulin protein is determined solely by the amount of calmodulin mRNA present or does some other mechanism exist for regulating calmodulin expression. Examination of calmodulin levels in null embryos also allowed the issue of the perdurance of the protein during embryogenesis to be explored.

Protein extracts from staged collections of wild-type and mutant embryos were used to make developmental immunoblots which were probed using affinity purified anti-calmodulin antiserum (RC11, see chapter 4). To control for the amount of protein, a fixed number of embryos, usually 20, was collected and the whole extract was used to prepare the immunoblot. This method was used because the procedure used to isolate homozygous mutant embryos produced low numbers of embryos, thereby preventing preparations large enough to be standardized by other methods.
Quantitative data was collected by laser scanning densitometry of photographic films produced by chemiluminescent detection of the anti-calmodulin antibodies. The results of volume integration of each band was used to calculate the relative amount of calmodulin in each sample. Averages of multiple experiments were used to reduce errors between samples.

A. Calmodulin Levels are Essentially Constant Through Embryogenesis

Previous in situ hybridization studies (Kovalick and Beckingham, 1992; see Chapter 3) revealed high levels of maternally-supplied calmodulin transcripts in the early embryos that fade away over the first four to five hours of embryonic development. At this time, zygotic transcripts begin to accumulate in the cells that will form the CNS and PNS of the larva. By late embryogenesis, these cells have high levels of transcripts, while other tissues lack detectable calmodulin mRNA. Based on this data and assuming a correlation between RNA and protein level, a dynamic pattern of calmodulin protein expression would be expected with high levels during early and late embryogenesis and much lower levels in mid-embryogenesis beginning four to five hours after egg laying (AED).

Surprisingly, quantitative immunoblotting showed that, although there were some fluctuations in the calmodulin protein levels detected, these do not coincide with the marked changes in calmodulin transcript expression established previously. Figure 6-1A shows a representative immunoblot of extracts from two-hour staged embryos. Quantitation of the results from three separate experiments is shown in Figure 6-1B.
Figure 6-1. Developmental immunoblot of Calmodulin in Drosophila embryos. A. Representative immunoblot of calmodulin in extracts from 20 OreR embryos collected at each interval. B. For each time period the band was quantitated as a percentage of the total calmodulin detected for all time points. The graph shows the average of three experiments. Error bars show standard deviation.
Overall, calmodulin levels remain relatively constant throughout embryogenesis, with no large variations in the amount of protein detected at any stage. Statistical analysis determined that the variations observed were not statistically significant (P > 0.1).

Two mechanisms that could give rise to more or less constant calmodulin protein levels in a background of large changes in transcript levels can be imagined. The first possibility is that, as maternally supplied calmodulin is lost in the early stages, equivalent levels of calmodulin are produced from transcripts in the nervous system. This model would require that most tissues in the embryo be capable of surviving long periods of development, up to 20 hours, without significant amounts of calmodulin. Alternatively, calmodulin protein may be very stable and persist in tissues long after the disappearance of detectable transcripts. In this case calmodulin levels would be expected to increase later in development due to the accumulation of calmodulin transcripts in the nervous systems. Although no increases were seen, this model could be valid if the transcripts in the CNS and PNS are largely not translated or if calmodulin protein is turned over rapidly in the nervous system.

B. Calmodulin Levels are Similar in Embryos Homozygous and Heterozygous for a Calmodulin Null Mutation

One way to distinguish between these two models is to compare the amount of calmodulin protein found in calmodulin null mutant embryos with that found in non-mutant embryos. Homozygous Camn339 embryos are zygotic RNA nulls, with no transcripts detected in the embryos after
the clearance of maternal transcripts during the first four to five hours of embryogenesis (Heiman et al., 1996). In order to make protein extracts from null mutant embryos, a method was needed to determine the genotype of the embryos resulting from the cross of heterozygous Cam^{339} flies. A balancer chromosome that expresses β-galactosidase was used so that the embryos can be genotyped based on the detection of β-galactosidase activity and the morphology of the embryos (CyO wg LacZ; Kassis et al., 1992).

Methods to detect β-galactosidase activity in embryos normally involve fixation of the embryos followed by treatment with colorimetric substrates or antibodies. Since fixation would interfere with the ability to analyze the proteins by immunoblot, another alternative had to be found that would allow staining without sacrificing the ability to extract the proteins. Two protective coverings separate the embryo from its environment. The chorion, the outer cuticular structure, can be removed from the embryos with bleach using a standard Drosophila procedure that does not kill the animal (Ashburner, 1989b). The vitelline membrane lies beneath the chorion and provides a waterproof barrier around the embryo. Fixation procedures commonly used in Drosophila place the embryos in a biphasic solution containing an aqueous fixative in the lower phase and heptane in the upper phase (Ashburner, 1989b). The embryos will migrate to the interphase and become permeable to the fixative. This method was adapted to allow penetration of a colorimetric substrate into the live embryos by placing a small volume of heptane over the buffer containing the substrate. In addition, detergent (0.05% Triton X--100) was added to ensure permeability of embryos to the substrate solution. Embryos were separated based upon production of colored substrate and morphology, and
proteins were extracted from both homozygous Cam\textsuperscript{n339} and heterozygous embryos. Since the \(\beta\)-galactosidase gene is not expressed until after four hours of development from this construct, extracts were taken only from collections aged six hours or longer.

Figure 6-2 shows the results of quantitation of calmodulin immunoblots for calmodulin null and heterozygous embryos. Calmodulin protein levels do not decrease early during development in homozygous null embryos, and, surprisingly, the null embryos contain comparable levels of calmodulin to their heterozygous siblings at most time points. This condition persists until at least 18 hours AED, after which statistically significant divergence (P<0.01) is seen between the wild-type and mutant animals. This correlates well with the observations by immunolocalization that some cells in the CNS begin expressing higher amounts of calmodulin during late embryogenesis (see Chapter 5).

Two conclusions can be drawn about calmodulin regulation during embryogenesis from these findings. First, the observation that calmodulin levels do not decrease rapidly in the Cam\textsuperscript{n339} embryos demonstrates that maternally-supplied calmodulin protein is highly stable and perdures throughout embryogenesis. This may explain the initially surprising finding that Cam\textsuperscript{n339} embryos survive embryogenesis and hatch as first instar larvae even though no calmodulin transcripts are present for the last twenty hours of embryogenesis (Heiman et al., 1996).

Secondly, since protein levels are approximately equal in mutant and heterozygous embryos at most stages, the calmodulin transcripts being abundantly produced in the CNS appear to not be translated into significant amounts of protein. It is possible that these transcripts are not required
Figure 6-2. Comparison of calmodulin levels in heterozygous and homozygous Cam^{339} embryos during embryogenesis. For each time period immunoblots from twenty Cam^{339}/Cam^{339} (shaded) and Cam^{339}/CyO wgLacZ (open) embryos were quantitated and expressed as a percentage of the amount detected in 6-8 hr heterozygous embryos. At least two experiments were averaged for each time period. Error bars show standard deviation.
until after the CNS becomes active prior to hatching. The phenotype of the null mutation clearly indicates the need for neural expression of calmodulin immediately after hatching. Cam^{n339} first instar larvae are sluggish and display behavioral phenotypes that appear to be neural in origin (Heiman et al., 1996). Immunolocalization of calmodulin, as discussed in Chapter 5, reveals higher levels in the embryonic CNS late in embryogenesis compared to other tissues. This increase in calmodulin, presumably produced by translation of zygotic transcripts, is observed by stage 14 (around 16 hours of development) which is earlier than increases are detectable by immunoblot. However, these increases are significantly delayed relative to calmodulin transcript accumulation in the embryonic CNS.

C. Calmodulin Protein Levels in Embryos Appear to be Determined by Maternal Gene Dosage

The observation that calmodulin null embryos appear to develop normally raises the question of whether these embryos receive an amount of maternal-supplied calmodulin similar to that of wild-type embryos. In Drosophila, RNA null mutations typically result in a 50% reduction in protein production from the mutant locus in heterozygous animals. Since no morphological defects or lethality is observed in homozygous Cam^{n339} embryos (Heiman et al., 1996), either the amount of protein produced in heterozygous adults is near wild-type levels, or the amount of calmodulin contained by wild-type embryos is in excess of the minimum required for completion of embryogenesis.
To address this question proteins were isolated from batches of zero to two hour embryos from wild-type and heterozygous Cam$^{n339}$ crosses. Although strain-specific or age-dependent variation may be responsible for some of the difference, immunoblots of these extracts showed that embryos from wild-type mothers have more than twice the amount of calmodulin than those from heterozygous Cam$^{n339}$ mothers (Figure 6-3). These data indicate that the amount of calmodulin in wild-type embryos is in excess of that needed to complete embryogenesis. This eliminates the possibility of a mechanism to sense and maintain calmodulin protein levels at a predetermined amount, at least during oogenesis, and suggests that calmodulin protein levels are determined primarily by gene dosage.

D. Model for Calmodulin Regulation During Embryogenesis

The immunoblot data discussed above results in the following model for calmodulin regulation: Calmodulin protein and transcripts are supplied to the egg during oogenesis, providing the embryo with an excess of calmodulin protein that will sustain all tissues throughout embryogenesis. Zygotic calmodulin transcripts are produced in the CNS and PNS, but are not translated into protein during most of embryogenesis. Just prior to or after hatching, when the nervous systems are activated, the need for calmodulin protein is higher and translation of the embryonic transcripts occurs. In mutants lacking these transcripts, rapidly diminishing levels of calmodulin during early larval life result in specific behavioral defects and eventually culminate in the death of the animal. No evidence of positive feedback in the regulation of calmodulin was detected; however, negative
Figure 6-3. Embryonic calmodulin protein levels are determined by maternal gene dosage. Crude protein extracts were prepared from ten 0-2 hr embryos from yw female flies (left lane) or Camh^{339}/CyO female flies (right lane). Extracts were processed for immuno-localization using RC11 antiserum.
feedback has not been eliminated and may be responsible for the observation that calmodulin transcripts are not translated in the embryonic CNS and PNS.
Chapter 7: Conclusions and Discussion

The experiments detailed in the preceding chapters examine the expression of the single calmodulin gene, in terms of both mRNA and protein levels, at different stages of development of Drosophila. These studies were initiated after the work of Kovalick and Beckingham (1992) revealed that calmodulin transcript expression during embryogenesis was not as simple as might have been predicted for a protein thought to be ubiquitously required. Those experiments showed that, after the degradation of maternal calmodulin transcripts, the only zygotic transcripts produced in the embryo are found in the developing CNS and PNS. The experiments in this manuscript were designed to further explore the pattern of expression of calmodulin transcripts and protein with the goal of greater understanding of the regulation and function of calmodulin during development.

A. Developmental Regulation of Calmodulin Expression

In situ hybridization studies were undertaken to examine the complexity of calmodulin transcript production in the tissues of larvae, pupae and adult flies. As was the case with embryos (Kovalick and Beckingham, 1992), a highly complex pattern emerged. Not only were variations seen between different developmental stages, but extensive differences in transcript expression were observed between tissues and even between cells in the same tissue at each different stage of development.
Calmodulin transcript expression in the CNS is highly dynamic. Transcripts are abundant in first instar larvae but the amount of calmodulin mRNA detected gradually declined resulting in undetectable levels by the third instar stage. During pupal development, the CNS again contains higher levels of calmodulin mRNA. By the adult stage, calmodulin transcripts appear to be maintained at a moderate level.

An interesting feature of calmodulin mRNA localization in the CNS is the variation observed between different regions. In pupae and adults, the optic lobes contain lower levels of calmodulin mRNA than other regions of the CNS. Such differences in expression levels may reflect either variations in the overall activity of these different regions or could reflect cells that have a greater requirement for calmodulin due to the specific processes being undertaken by those cells. Some examples of the latter might be neurons that respond to a certain neurotransmitter or have the capability of a high degree of plasticity.

The calmodulin transcription pattern in the CNS is interesting in that calmodulin transcripts are most abundant during periods in which the CNS is least active. During embryogenesis and pupation, periods when the structure and morphology of the CNS is being determined, calmodulin mRNA is quite abundant, while in the larval and adult stages, when the CNS is active and regulating behavior, calmodulin transcripts are at lower, or even, as for the third instar, undetectable levels. Given this correlation of mRNA expression with periods of formation of the nervous system, it was surprising that calmodulin null mutant larvae show no detectable morphological defects, but rather, exhibit behavioral abnormalities during larval life (Heiman et al., 1996). This paradox has been resolved by the
observation that maternally-supplied calmodulin protein is highly stable, and the indication that the zygotic calmodulin transcripts are largely untranslated during embryogenesis. This leads to the hypothesis that this is a general strategy for regulation of calmodulin expression in the CNS; calmodulin transcription is activated while the animal is inactive and the transcripts produced are translationally silent until just prior to the onset of neuronal activity. This preloading of transcripts may be necessary to provide a large pool of calmodulin to facilitate the challenging processes of hatching from the egg and eclosion from the pupal case.

The calmodulin transcript pattern observed in the pupal and adult musculature is similar to that of the CNS with levels being highest during pupal development when the tissue is developing but inactive. A similar mechanism of preloading of transcripts for later translation may occur in this tissue, although abundant calmodulin may also be required during formation of the myofibrillar structure.

The alimentary canal displays regional differences in the amount of calmodulin transcripts, however, unlike the CNS, calmodulin transcripts in the gut are most abundant during periods of activity and lowest during periods of fasting (embryogenesis and pupation). The regions of the gut that show the highest levels of calmodulin mRNA are the pharynx, proventriculus and posterior midgut of larvae and adults. These regions are the site of extensive secretory and endocytotic activity. In particular, the region of the proventriculus that shows the highest level of calmodulin transcripts in both adults and larvae is the primary site of secretion of components of the peritrophic membrane that lines the alimentary canal in Drosophila (King, 1988).
The correlation with secretory function extends beyond the gut to include cells in the ovary. Calmodulin mRNA is very abundant in follicle cells in the ovary. The follicle cells secrete the products that form the multilayered covering that isolates the egg from the environment (Spradling, 1993).

A further explanation for high calmodulin transcript levels in follicle cells was suggested by germline clone experiments performed by Clare Bolduc in the Beckingham laboratory. These experiments were aimed at examining the development of germ-line cells homozygous for the calmodulin null mutation Cam\textsuperscript{n339} and thus incapable of synthesizing calmodulin protein. In egg chambers in which all of the germ cells are homozygous for the null mutation, undetectable levels of calmodulin protein are seen in the oocyte and nurse cells during early oogenesis. Surprisingly, however, by late oogenesis the germ line has accumulated calmodulin protein to levels equivalent to heterozygous egg chambers. This calmodulin protein probably enters the germline from the surrounding follicle cells as has been suggested for the German cockroach, \textit{Blattella germanica} (Zhang and Kunkel, 1994). The eggs produced from calmodulin null clones were normal in shape and morphology but had defects in the chorion which appeared very thin and variable. Since no evidence has been found for a direct role of the oocyte in chorion formation, this is presumably a result of the failure of calmodulin-depleted follicle cells to produce or secrete chorion components properly. If this hypothesis is correct, it has several implications for calmodulin dynamics in the egg chamber. First, it implies that nurse cells normally make significant contributions to the calmodulin supply of the oocyte.
Furthermore, this interpretation suggests that some mechanism exists within the oocyte for monitoring calmodulin protein levels which can stimulate a compensating transport of calmodulin from the follicle cells. Alternatively, an oocyte-based signal involving calmodulin may be necessary for proper production, secretion, or assembly of chorion products by the follicle cells. Based on these data, the abundant levels of calmodulin transcripts in the mature egg are probably derived from the nurse cells, but some fraction of the protein may be derived from the maternal follicle cells.

This maternally-supplied calmodulin, abundant in the early embryo, is likely to play a pivotal role in the rapid, synchronous mitoses of cleavage and blastoderm stages. After 13 of the 14 synchronous nuclear divisions, calmodulin protein levels decrease slightly then remain essentially constant in most tissues of the embryo without production of zygotic calmodulin transcripts or protein. The decrease in calmodulin levels may contribute to slowing of the cell cycle during the last few synchronous nuclear divisions. It is noteworthy that this decrease precedes the exit of most embryonic cells from this unique nuclear cycling. Perdurance of maternal calmodulin appears to supply the needs of all tissues through most of embryogenesis. This includes the embryonic CNS and PNS where zygotic transcripts are not required for proper embryonic neurogenesis nor do they appear to be translated until just prior to the onset of neuronal activity.

As the end of embryogenesis nears, transcription of the calmodulin gene is activated in non-neuronal tissues, presumably in preparation for hatching. By the first instar stage of larval life, calmodulin transcripts are present at high levels in all tissues observed. As in embryos, transcripts
are degraded as larval development proceeds in most tissues, with the exception of the alimentary canal and salivary glands, which maintain high levels of calmodulin transcripts throughout the larval stages. Perdurance of calmodulin produced from the transcripts made during late embryogenesis and early in the first instar may supply the needs of the organism during the subsequent larval stages. Continued production of transcripts in the gut and salivary glands implies a need for high levels (or continuous replenishment) of calmodulin in secretory tissues.

B. Subcellular Localization and Intracellular Roles of Calmodulin

The subcellular localization of calmodulin protein suggests a role for calmodulin in several different processes during development. The most compelling is in the regulation of cell and nuclear division. Localization of calmodulin to centrosomes during mitosis was observed in early and late embryos and in follicle cells. Although many roles have been proposed for calmodulin in regulation of cell cycles, the subcellular localization of calmodulin suggests that regulation of the proper assembly of the centrosome and spindles and the triggering of anaphase are processes that may require localized calmodulin signals. Transient concentration of calmodulin in the nucleus may further suggest calmodulin as a regulator of DNA replication through action on the replication machinery. These processes are likely to involve a number of calmodulin targets including CaMKII, MAPs, or potential homologs of the yeast centrosomal protein Spc110. Spectosome and fusome staining with anti-calmodulin antibody during periods of cystocyte cell division suggests a role for calmodulin in
regulating these unique cell divisions as well.

Calmodulin may also regulate the structure of the cortical spectrin-based cytoskeleton. Calmodulin is enriched in follicle cells and germ cells of the ovary in a pattern with similarity to the expression and localization patterns of Drosophila α-spectrin, Hts (adducin) and D4.1 (Drosophila homolog of protein 4.1), all of which are homologs of calmodulin-binding proteins that interact with each other in the cortical cytoskeleton. Calmodulin regulation of these proteins may affect the activity of these proteins in the assembly and disassembly of cytoskeletal networks and cell-to-cell contacts necessary for cell shape changes and migrations during development. Calmodulin may also interact with α-spectrin and Hts to regulate key elements of differentiation of germline cystocytes in the ovary.

C. Translational Repression of Calmodulin mRNA

Immunoblot analysis of embryonic calmodulin levels, discussed in Chapter 6, showed that calmodulin protein levels remain relatively constant throughout embryogenesis. Although embryos homozygous for the calmodulin null mutation Cam\textsuperscript{a339} lack zygotic transcripts, comparison of calmodulin protein levels between homozygous and heterozygous embryos revealed no detectable differences. Since calmodulin protein levels, as examined by immunoblotting and immunolocalization, do not correlate with the production of calmodulin transcripts, it is concluded that the zygotic transcripts produced in the CNS and PNS of the embryo are not used in the production of calmodulin protein during most of
embryogenesis.

The mechanism of translational repression of calmodulin protein production in the CNS is not known. However, two calmodulin transcripts are produced by alternative polyadenylation site usage and are produced in a developmentally regulated pattern (Doyle et al., 1990; Kovalick and Beckingham, 1992). The longer transcript predominates in both late embryos and pupae, while the smaller transcript predominates in early embryos (Doyle et al., 1990; Kovalick and Beckingham, 1992). Although this remains to be tested, the longer transcript could contain a sequence element in the unique portion of the 3' UTR that mediates translational repression.

D. Conclusions and Future Directions

The studies reported here demonstrate the complexity of calmodulin expression throughout the development of Drosophila melanogaster, and indicate that regulation of calmodulin expression may involve control of translation as well as transcription. The pattern of expression and the subcellular localization of calmodulin have suggested an important role for calmodulin in processes such as neural function, secretion, cell division, and cytoskeletal dynamics.

Further experimentation will be required to determine the specific role of calmodulin in these processes and to define the mechanisms controlling calmodulin expression. Ongoing germline and somatic clone experiments with the calmodulin null allele hold great potential for characterizing the requirement for calmodulin in different cell types. In
particular, it will be interesting to examine the effects of calmodulin depletion on mitosis, especially in the rapid nuclear cycles of early embryogenesis.

It will also be interesting to address the questions of translational repression of CNS transcripts and the source of calmodulin contribution to the oocyte. Both questions can be addressed by the introductions of engineered constructs into flies. The first issue could be addressed by experiments to assay the translatability of mRNAs generated from fusion constructs of a reporter gene with two calmodulin 3' UTRs. Spatially restricted expression of a traceable calmodulin fusion protein, such as calmodulin fused to green fluorescent protein, would allow the determination of the extent and possibly timing of follicle cell and nurse cell contributions of calmodulin to the developing oocyte.
References


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