PARTIAL ISOLATION AND CHARACTERIZATION OF THE MOUSE CALMODULIN GENES

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(ABSTRACT)

Three genes encode identical calmodulin proteins in mammals. One possible reason for this unique situation is that it allows differential regulation of calmodulin gene expression. In order to study the promoter regions of the different genes, the 5' ends of three mouse calmodulin cDNAs were isolated using a modified 5' Rapid Amplification of cDNA Ends (RACE) method. Using these cDNAs as probes, the promoter regions of the *Cam I* and *Cam III* genes as well as a *Cam II* pseudogene were isolated from a 129SVJ mouse genomic library. When the *Cam II* cDNA was used to determine the chromosomal location of the *Cam II* gene, the results indicated that at least two likely pseudogenes exist in addition to the functional gene. Using a probe that contained only sequence from the promoter region and first intron of the *Cam II* gene, the chromosomal location of the bona fide *Cam II* gene was identified.

Evidence indicates that conflicting growth signals in cells can lead to apoptosis. To determine if calmodulin, a proliferation signal, would induce apoptosis in cells cultured in differentiation conditions, C2C12 myoblasts were transiently transfected with

a calmodulin expression vector. Half of the transfected cells were treated with calcium ionophore to determine if the calcium levels would have an effect. Overexpression of calmodulin did not lead to apoptosis, and the calcium ionophore caused death of the control- and calmodulin-transfected cells.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AP-2 - activator protein 2

β-gal - β-galactosidase

bp - base pair(s)

BSA - bovine serum albumin

Cam - DNA or RNA encoding calmodulin

CMV - cytomegalovirus

CRE - cyclic AMP-responsive element

dNTPs - deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)

FBS - fetal bovine serum

kb - kilobase(s)

LB - Luria-Bertani broth

NP-40 - nonidet P-40

nt - nucleotide(s)

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

RACE - rapid amplification of cDNA ends

SDS - sodium dodecyl sulfate

TAE - tris-acetate/EDTA electrophoresis buffer

TBE - tris-borate/EDTA electrophoresis buffer

TE - tris/EDTA buffer

X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactoside

INTRODUCTION

Calmodulin is a small, calcium-binding protein that plays a role in a diversity of cellular processes including cyclic nucleotide metabolism, glycogen metabolism, cell cycle regulation and apoptosis in eukaryotes. In response to calcium levels, calmodulin regulates the activities of many enzymes (Fig. 1). Calmodulin is a dumbbell-shaped protein with two heads connected by an α-helix. In the presence of calcium and a substrate, this α-helix becomes flexible and the two heads bind to the substrate (Fig.2) which induces a conformational change in the target protein that leads to its activation. Through its effects on kinases such as phosphorylase kinase and calcium/calmodulin-dependent protein kinase II (Payne et al., 1988), and phosphatases such as calcineurin (Hurwitz et al., 1988), calmodulin can indirectly activate or inactivate an even broader range of enzymes. Calmodulin is also important in cell signalling. Calcium-pumps such as the plasma membrane Ca^{2+}-ATPase and ion channels including sodium and potassium ion channels are affected by calmodulin as is nitric oxide synthase (Schmidt et al., 1992), a second messenger generator. Clearly, calmodulin plays an integral role in the transduction of the calcium signal in the cell (For review see Klee et al., 1986; Manalan and Klee, 1984).

Calmodulin is encoded by three active genes in mammals. This situation alone would not be novel, except that the genes do not encode isoforms of calmodulin, but encode identical proteins (SenGupta et al., 1987; Nojima et al., 1987; Nojima, 1989). Apparently, the specific amino acid sequence of calmodulin is very important for its proper function. One plausible explanation for the gene multiplicity is that it allows

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Calmodulin function

protein kinase

protein phosphatase

adenylate cyclase

phosphodiesterase

Ca^{+2} channels/pumps

$+ Ca^{+2}$

Calmodulin

$- Ca^{+2}$

$+ Ca^{+2}$

Active

phosphorylase kinase
nitric oxide synthase
(inactive)
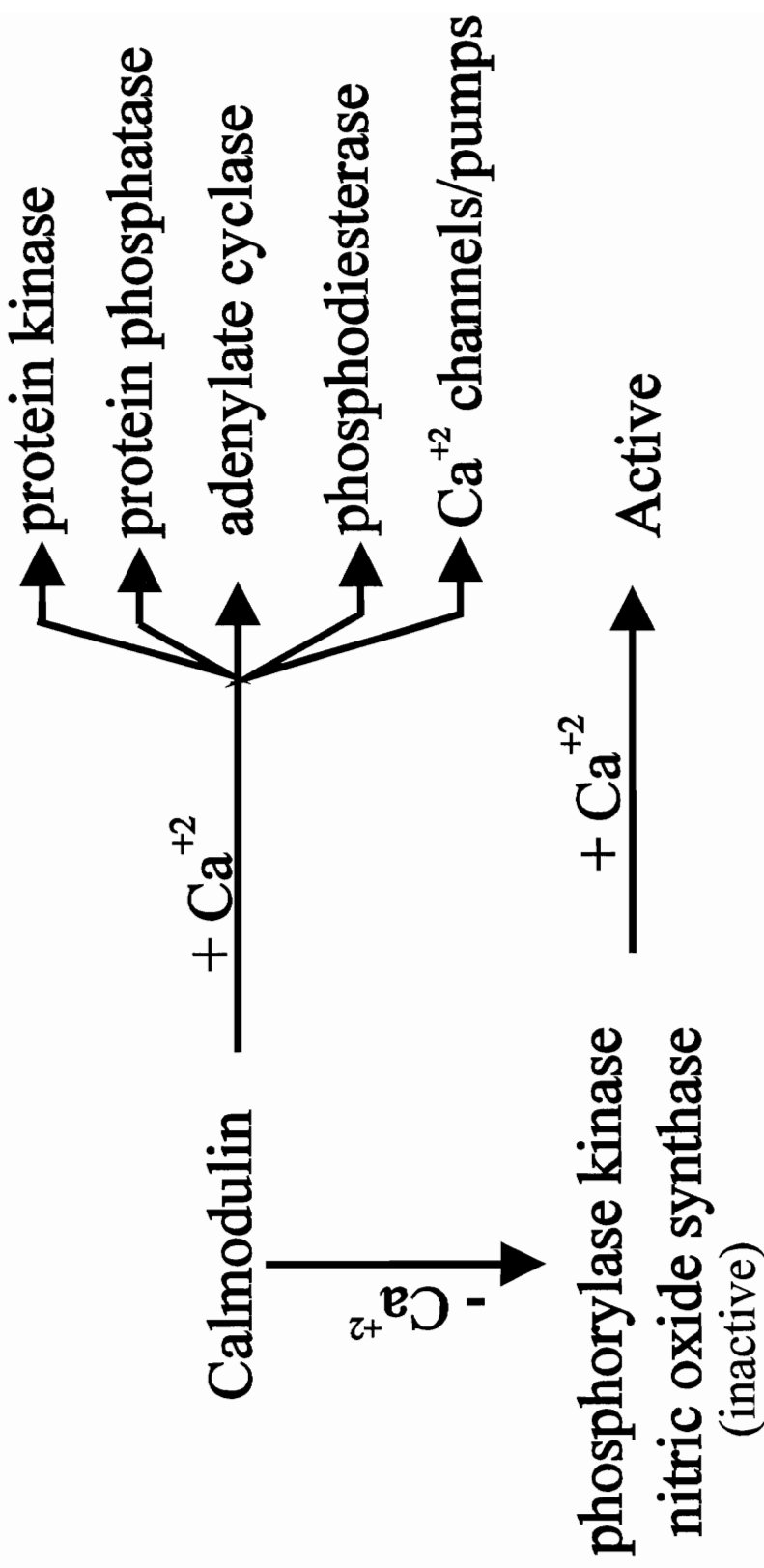
Figure 1. **Target enzymes for calmodulin regulation.** In the presence of calcium, calmodulin binds to many proteins and affects their enzymatic activities. In the absence of calcium, calmodulin is associated with the inactive states of phosphorylase kinase and nitric oxide synthase. When calcium is added, these enzymes convert to their active forms.

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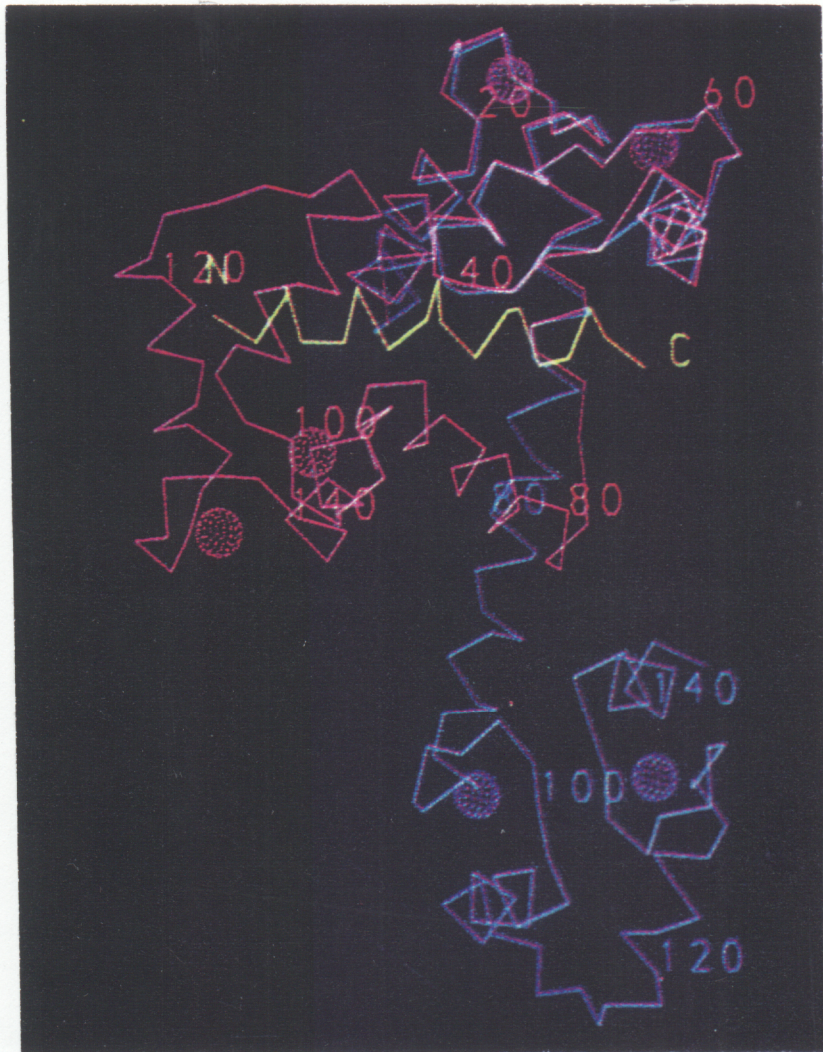
Figure 2. **Structures of calmodulin and calmodulin bound to substrate.** Calmodulin is shown in blue; myosin light chain kinase substrate sequence is in yellow; and calmodulin bound to substrate is in magenta (from Meador et al., 1992).

differential transcriptional regulation of the calmodulin genes in order to provide the correct amount of calmodulin protein in response to physiological factors, in different tissues and at different times during development.

The three calmodulin genes have been isolated from the rat and are designated *Cam I*, *Cam II* and *Cam III* (Nojima et al., 1987; Nojima, 1989). *Cam I* and *Cam III* consist of six exons and five introns, whereas *Cam II* contains five exons and four introns. Interestingly, in all three genes the ATG start codon is immediately followed by a large (3-4 kilobase) intron. A functional role for this intron is likely, but has not yet been identified. Only relatively small portions (600-1400 bp) of the promoter regions of the three rat *Cam* genes have been isolated, and little is known about the cis-acting sequences regulating their transcription.

While the rat calmodulin genes have been partially characterized, even less information is known about the mouse genes. Although rats and mice are related, they are not similar enough for use in some experiments. For example, to date, null or "knockout" mutations in higher organisms have only been successfully performed in mice. For such experiments, it is necessary to have precise genetic information, as reflected by the necessity to have DNA from syngeneic mice to inject into the embryonic stem cells when generating a null mutant. Thus, isolation of the mouse genes is desirable to allow further characterization of the calmodulin genes and their regulation.

There is considerable evidence that the mammalian calmodulin genes are differentially expressed in different tissues and at different times during development. Expression of each of the calmodulin genes can be identified since transcription of each

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gene results in at least one transcript of a unique size. More than one message from individual genes results from different polyadenylation sites in the case of the *Cam I* and *Cam III* primary transcripts. The *Cam I* gene produces two transcripts. The major mRNA is 1.7 kb and is abundantly expressed in rat brain, liver, skeletal muscle, testis, small intestine, kidney and less abundantly in the spleen (Nojima et al., 1987; Nojima, 1989). In mouse, the 1.7 kb mRNA is expressed in the skeletal muscle, heart, liver and brain (Bender et al., 1988) (Fig. 3). A minor 4 kb *Cam I* transcript is found only in skeletal muscle and cardiac muscle in rats and mice (Bender et al., 1988; Nojima et al., 1987; Nojima, 1989). Only one message of 1.4 kb is transcribed from the *Cam II* gene. This mRNA is found primarily in the brain of mouse and rat, but also has been reported in several other tissues of the rat (Bender et al., 1988; Nojima et al., 1987; Nojima, 1989). Two mRNAs are transcribed from the *Cam III* gene. Both the 2.3 kb and 1.0 kb mRNAs are found in mouse skeletal muscle, heart, liver and brain, although the 1.0 kb mRNA is in a lesser amount in skeletal muscle and liver (Bender et. al., 1988) (Fig. 3). In rat, however, it has been reported that the 2.3 kb transcript is only in the brain, small intestine and testis (Nojima et al., 1987; Nojima, 1989). The reason for the discrepancies between rat and mouse calmodulin gene expression is unknown; however, results from Northern blot analysis of mouse tissues indicate that the *Cam I* and *Cam III* genes are expressed permissively in most tissues, while the *Cam II* gene is expressed significantly only in the brain (Bender et al., 1988) (Fig. 3).

Not only is calmodulin gene expression tissue-specific, but the distribution of the mRNAs within the rat brain also varies (Gannon and McEwen, 1994). Even on the
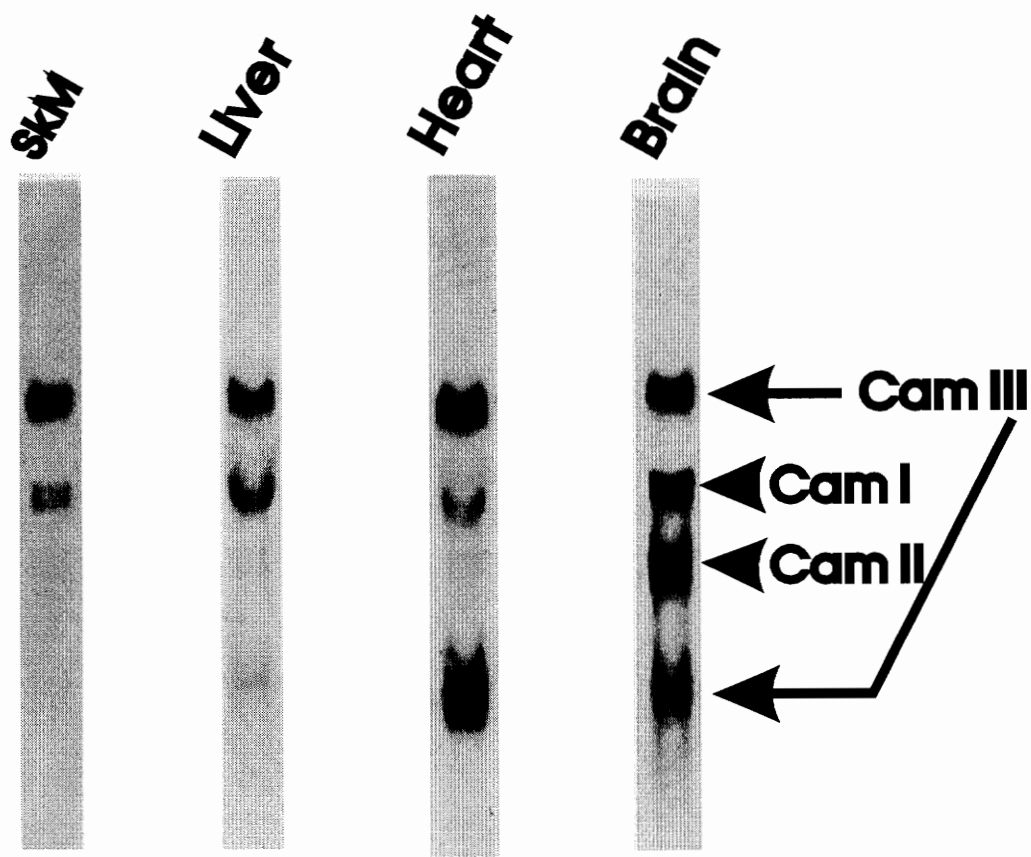
Figure 3. **Northern blot analysis of calmodulin expression in mouse tissues.** Lanes of total RNA from mouse skeletal muscle (SkM), heart, liver and brain tissues from a Northern blot hybridized with a probe that hybridizes to all calmodulin messages are shown. The gene origin of each message is also shown.

cellular level, calmodulin mRNAs vary in their distribution. In neurons, *Cam I* and *Cam II* mRNAs are localized in the neurites as well as the cell bodies, while the *Cam III* message is only found in the cell body (Zhang et al., 1993). This specialized localization of the messages might be a way to vary the levels of calmodulin within different regions of the cell.

Calmodulin levels also vary through the course of the cell cycle. An increase in calmodulin is important in the transition from G1 to the S phase (Chafouleas et al., 1982). Calmodulin is also involved in the re-entry of cells from quiescence (Go) into the cell cycle (Chafouleas et al., 1984). Calmodulin levels drop shortly after release from Go and then increase five to eight hours later, as the cells enter S phase. In addition, treatment of cells with a calmodulin antagonist causes a block in re-entry into the cell cycle. The calmodulin levels are thought to be controlled through regulation of gene expression, since changes in mRNA levels precede the same changes in protein levels.

Calmodulin's role in the cell cycle has been further studied by expressing the protein in high levels in mouse C127 cells. High calmodulin levels lead to an increase in cell proliferation by shortening of G1 in the cell cycle (Rasmussen and Means, 1987; Rasmusen and Means, 1990). This evidence suggests that calmodulin could be a signal for proliferation. In addition, increased calmodulin levels are associated with virally-transformed cells (Chafouleas et al., 1981; Zendegui et al., 1984) and virally-induced sarcomas in the rat (MacManus et al., 1989). This increase may be related to the increase in proliferation of these transformed cells.

Interestingly, in the virally-induced sarcomas, transcription of *Cam I* and *Cam II*

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increased, while *Cam III* remained unchanged (MacManus et al., 1989). This finding lends further support to differential regulation of the calmodulin genes. It also demonstrates that the normal tissue-specific expression of the *Cam II* gene can be disrupted in transformed cells. In the same study, the investigators monitored the transcription of the *Cam* genes during prenatal development in the rat. As the embryo developed, *Cam I* and *Cam III* transcription remained constant except in the liver where *Cam III* expression increased somewhat. However, overall transcription of *Cam II* decreased during development of the rat embryo. Changes in the transcription of the *Cam* genes have also been noted, specifically in the brain and skeletal muscle of rats during development (Weinman et al., 1991). In the developing rat brain, the amounts of all calmodulin mRNAs increase steadily until they reach a peak at 8 days after birth. There is a slight decrease of the *Cam II* and *Cam III* mRNAs at 17 days and then an increase in the *Cam I* and *Cam II* messages at the adult stage. In the skeletal muscle of the developing rat, calmodulin message levels remain constant during fetal development, then *Cam II* and *Cam III* mRNAs decrease abruptly shortly before birth. After birth, *Cam I* and *Cam III* mRNAs gradually increase until the adult stage.

At the cellular level, expression of the calmodulin genes has been shown to vary according to cell type and developmental stage during spermatogenesis (Slaughter et al., 1989) and during differentiation. In myoblast cell lines BC_3H1 and C2C12, calmodulin gene expression and protein levels decrease during differentiation (Christenson and Means, 1993). Conversely, calmodulin mRNA levels increase in PC12 cells when they are induced to differentiate into neurons (Bai and Weiss, 1991).

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Although there are patterns of expression of the calmodulin genes during development and differentiation, these patterns are dependent on the tissue or cell type. Logical candidates to mediate this differential regulation would be tissue-specific factors. This idea is supported by evidence that the promoter and leader sequence of the rat *Cam II* gene is able to drive neuro-specific expression of a β-galactosidase reporter gene in transgenic mice (Matsuo et al., 1993).

Identification of specific effectors of *Cam* gene expression has been sought by sequence analysis of the promoter regions of the genes. Two potential CREs (cAMP Responsive Elements) and one possible AP-2 (Activator Protein-2) site were found in the rat *Cam II* gene (Nojima, 1989) and a potential AP-2 site was also found in *Cam I* (Bai et al., 1992). The possibility that at least one of these sites in each of the genes is real is supported by the finding that treatment of PC12 cells with dibutyryl cAMP results in an increase in transcription of the *Cam II* and *Cam I* genes (Bai et al., 1992); however, functional mapping of the CRE sequences or any other cis-acting sequences has not been done.

Other possible hormonal effectors of *Cam* gene expression include the corticosteroids. When rats undergo adrenalectomy, *Cam III* expression is attenuated in certain areas of the brain (Gannon and McEwen, 1994). This effect is reversible by treatment with corticosterone; however, this phenomenon may be associated with the variation in the levels of CaM-dependent enzymes as reported previously (Bender et al., 1988; Thacore et al., 1988). Thus, changes in calmodulin gene expression might not have been directly affected by corticosterone, but may have resulted from feedback

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regulation dependent on the expression of calmodulin target enzymes.

Additional evidence for hormonal control of *Cam* gene expression comes from glucocorticoid-treated WEHI7.2 cells, a mouse T-lymphosarcoma cell line. When these cells are treated with dexamethasone, *Cam* mRNA levels increase ten-fold after four to six hours of exposure to the drug (Dowd et al., 1991). Interestingly, dexamethasone treatment also leads to apoptosis in these cells.

Apoptosis, or programmed cell death, is a natural, active process that is important in normal processes such as cell turnover in healthy tissues, clonal deletion of self-reactive lymphocytes in the immune system, embryonic development and metamorphosis. Because of the nature of apoptosis as an active process requiring protein synthesis, the idea that apoptosis could be triggered in specific cells makes it attractive for use in cancer treatment. In fact, many cytotoxic cancer chemotherapeutic agents and radiation treatment already in use cause cell death by apoptosis. The idea has been proposed that resistance to apoptosis is an important cause of the resistance of cancer cells to clinical treatment (Fisher, 1994). As the process of apoptosis is better understood, we will be better able to take advantage of it in cancer therapy.

There are biochemical and morphological changes characteristic of apoptosis that include chromatin condensation, nuclear fragmentation and cell blebbing which ultimately leads to the formation of apoptotic bodies (Wyllie et al., 1980). The apoptotic bodies, containing cytoplasm and sometimes nuclear fragments, break away from the dying cell and are phagocytosed by surrounding normal cells. A unique biochemical process in apoptosis is DNA fragmentation by an endonuclease. This endonuclease cleaves the

DNA inter-nucleosomally and thus when the DNA is subjected to gel electrophoresis, a characteristic "ladder" of DNA bands can be seen that differ in size by approximately 200 bp. A Ca^{2+}-dependent endonuclease has been isolated from apoptotic rat thymocytes that is distinct from other DNases in the cells (Gaido and Cidlowski, 1991). The calcium dependence of this enzyme is consistent with the findings that calcium is necessary for DNA fragmentation during apoptosis (McConkey et al., 1989). The fact that calcium is important in apoptosis (McConkey et al.,1989; Orrenius et al., 1992; Dowd et al., 1992; Nicotera et al., 1994) leads to the question of calmodulin's importance as a mediator of the calcium signal. The findings that calmodulin has been linked to Ca^{2+}-activated DNA fragmentation in rat liver nuclei (Jones et al., 1989) and induction of calmodulin gene expression occurs upon glucocorticoid-induced apoptosis of WEHI7.2 cells (Dowd et al., 1991) suggest a definite role for calmodulin in apoptosis.

Since calmodulin gene expression increases when WEHI7.2 cells are treated with glucocorticoids and glucocorticoid treatment leads to apoptosis, one could hypothesize that calmodulin is necessary to induce apoptosis. This hypothesis is supported by the finding that calmodulin antagonists inhibit the glucocorticoid-induced apoptosis of WEHI7.2 cells (Dowd et al., 1991). One experiment to study the role of calmodulin in apoptosis is to overexpress a calmodulin-encoding cDNA in WEHI7 cells without glucocorticoid treatment and assay the cells for DNA fragmentation as an indication of apoptosis. However, WEHI7 cells are resistant to transfection by the calcium phosphate method, by use of cationic lipids, and by electroporation (personal experience and personal communication with Dr. S. Bourgeois). Thus, experiments on the

overexpression of calmodulin in WEHI7 cells await the development of an effective transfection procedure.

An additional interesting aspect of apoptosis is the relationship between differentiation and apoptosis in different cell types. In many cases, differentiation protects cells from death by apoptosis. During normal development, as proliferating neuronal precursors stop growing and differentiate into neurons, there is extensive cell death. To study this phenomenon, researchers have used a proliferating neuronal cell line, ND7, which differentiates into neurons upon shifting to serum-free media. A portion of these cells die by apoptosis rather than differentiate (Howard et al., 1993). This suggests that within the population of cells, a subpopulation is susceptible to apoptosis while the remaining cells are able to differentiate. A distinguishing factor between these subpopulations might be the position of the cells in the cell cycle. Cells that have withdrawn from the cell cycle and are in interphase would be able to differentiate, while those cells still active in the cell cycle would be susceptible to apoptosis when they are shifted to serum-free media.

In another neural-related example, when PC12 cells are treated with nerve growth factor, they can differentiate into neurons, while in serum-free media, PC12 cells undergo apoptosis. When the proto-oncogene protein bcl-2, a noted inhibitor of apoptosis in some systems (Bissonette et al., 1992, reviewed in Williams, 1991), is overexpressed in PC12 cells, the cells are able to survive in serum-free media. Furthermore, the cells expressing bcl-2 show delayed and limited neurite outgrowth (Sato et al., 1994). Although differentiation is limited, this example still links differentiation

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with protection from apoptosis.

In contrast to the effects of the bcl-2 protein, overexpression of a different proto-oncogene, c-*myc*, in C2C12 mouse myoblasts actually leads to apoptosis in a fraction of the cells when the cells are cultured in differentiation medium (Crescenzi et al., 1994). This effect of c-*myc* overexpression also occurs in fibroblasts (Evan et al., 1992). Since Myc is a proliferation signal (Luscher and Eisenman, 1990), it probably increases the number of cells in the cell cycle. If being active in the cell cycle makes the cells susceptible to apoptosis when they are shifted to differentiation medium as suggested above, it might explain how Myc could cause an increase in the number of cells undergoing apoptosis. However, there also must be a signal for apoptosis present in the case of the C2C12 cells, because unlike neuronal cells, C2C12 cells do not usually undergo apoptosis when cultured in differentiation medium. Instead, these cells usually differentiate into myotubes. From the results of overexpression of c-*myc* in C2C12 cells or fibroblasts, it has been suggested that the conflict between signals for proliferation versus cell cycle withdrawal leads to apoptosis (Hoffman and Liebermann, 1994). c-*myc* provides a signal for cell proliferation, while the differentiation medium signals withdrawal from the cell cycle.

The fact that high levels of Myc under growth-limiting conditions causes apoptosis forms the basis of the hypothesis that expression of calmodulin, a signal for proliferation, would serve a similar role as that of c-Myc in C2C12 cells cultured under differentiation conditions and induce apoptosis.

There are many unresolved questions concerning the role of calmodulin in the

regulation of different cellular processes. I chose to investigate the effects of overexpression of calmodulin in C2C12 cells to make a contribution towards answering some of the questions concerning a role for calmodulin in apoptosis. It is well established that the expression and intracellular concentration of calmodulin vary in response to physiological conditions, and in many instances, changes in calmodulin expression are necessary for correct cellular responses. Yet it is not understood what cellular mechanisms regulate calmodulin expression and why three genes have evolved in mammals. Although these genes are redundant with respect to their coding sequences, they clearly exhibit differential transcriptional regulation.

To further our understanding of the regulation of the three calmodulin genes, I undertook the isolation of the necessary elements to map the cis-acting sequences regulating the transcription of each calmodulin gene. Mapping the cis-acting sequences would help to determine the functional differences between the three calmodulin genes. I concentrated my efforts on the analysis of the mouse calmodulin genes because: (1) differential expression of the calmodulin genes has clearly been shown in the mouse; (2) there are a large assortment of mouse cell lines that serve as models for tissue specific responses; and (3) most importantly, only in mice can the transgenic approach employing knock-out mutations be done to specifically investigate the importance of each calmodulin gene in the context of the organism.

The specific objectives in isolating the elements necessary for mapping of the cis-acting sequences were: (1) to isolate the 5' ends of the three calmodulin cDNAs from the mouse; and (2) to use these cDNAs as probes in screening a 129SVJ mouse genomic

library to isolate the promoter regions of the genes. Isolation of the gene promoter regions will also contribute to creating a knockout mutation, since the 129SVJ mouse is syngeneic with the embryonic stem cells used in generating a null mutant. In addition, mapping the chromosome locations of the calmodulin genes to identify the relative positions of the calmodulin genes and any related genes or pseudogenes will further our knowledge for future creation of a null mutant. A final objective was to study the function of calmodulin in apoptosis; specifically, to determine if the action of calmodulin as a proliferation signal would lead to apoptosis of C2C12 myoblasts under differentiation conditions.

MATERIALS AND METHODS

Agarose and polyacrylamide gel electrophoresis

For analysis of DNA greater than 500 bp, 1% or 1.5% agarose gels were run using a Hoefer HE33 submarine horizontal mini-gel apparatus (San Francisco, CA). For assay of DNA fragmentation during apoptosis, 2% agarose gels were used. Samples were electrophoresed at 150 V for approximately 1 hour, and the DNA was visualized after ethidium bromide staining by a UV transilluminator. Alternatively, agarose gels were run using a vertical Hoefer Mighty Small Model SE 200 apparatus at 22 mA. The agarose gel electrophoresis buffer consisted of 40 mM Tris acetate, pH 7.8, 0.2 mM EDTA (TAE). For analysis of DNA less than 1000 bp, 4% polyacrylamide gels were run in the same vertical apparatus at 22 mA for 0.5 hour in 8.9 mM Tris, 8.9 mM boric acid, 0.2 mM EDTA (TBE). Polyacrylamide gels were stained and viewed as described above.

Modified 5' RACE method

Three 5 μL annealing reactions containing 0.2μg poly(A)$^+$RNA from mouse brain and 50 fmol of the degenerate primer (GSP-1) (Fig. 4) in 0.4 M KCl, 10 mM PIPES (pH 6.4) were sealed in capillary tubes and each tube was incubated at a different temperature, 52°C, 56°C or 60°C for 24 h. The contents of each annealing reaction were expelled into a separate microfuge tube containing the components for a 50-μL reverse transcription reaction (20 mM Tris (pH 8.3), 2.5 mM MgCl$_2$, 1mM each dNTP,

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0.1 mg per mL BSA, 2 mM DTT, 1.25 μg actinomycin D, 80 units RNasin, 40 units reverse transcriptase). Reverse transcription to synthesize the cDNAs was carried out at 42°C for 1 hour. The template RNA was removed by incubating the mixture at 55°C with 7.5 ng RNase A for 15 min. The enzymes were inactivated in a 10-min incubation at 95°C. The buffer in the mixtures was exchanged with TE buffer (1mM Tris, pH 8, 0.1 mM EDTA) by 3 repetitions of dilution and concentration on microconcentrators (Microcon-100, Amicon, Beverly, MA). The 50 μL retentates were then concentrated to approximately 10 μL in a vacuum concentrator. Three tailing reactions (20 μL each) containing 5 μL of the concentrates and buffer components to give 10 mM Tris, pH 8.4, 25 mM KCl, 1 mM $MgCl_2$, 0.2 mM dATP, 0.05 mg per mL BSA were prepared. Terminal deoxytransferase (8 units) was added and the tailing reaction proceeded at 37°C for 15 min. To terminate the reaction, the mixture was heated to 65°C for 10 min. 2.5 μL of each poly(dA)-tailed cDNA mixture was included in three corresponding 50 μL PCR reactions with Vent DNA polymerase buffer (New England Biolabs, Beverly, MA) supplemented with 1.0 mM $MgSO_4$, 0.12 mg per mL BSA, 0.4 mM DTT, 0.2 mM each dNTP, 100 pmol of GSP-1, 10 pmol Anchor dT_{17} primer (5' CGACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3'), 25 pmol Anchor primer (5' CGACTCGAGTCGACATCG 3'). After denaturation at 94°C for 3 min the temperature was held at 80°C and 1 unit of Vent DNA polymerase (NEB, Beverly, MA) was added. Two cycles of 52°C for 5 min, 72°C for 5 min, 94°C for 30 s were followed by 35 cycles of a three temperature PCR: 56°C for 30 s, 72°C for 90 s and 94°C for 30 s. PCR products (2.5 μL) originating from annealing at 52°C were

subjected to a second PCR using one of the nested gene specific primers (GSP-2A, GSP-2B or GSP-2C) (Fig. 4) and the Anchor primer.

The individual calmodulin cDNAs were purified by Wizard PCR Preps (Promega, Madison, WI). The *Cam II* (Cam C) and *Cam III* (Cam A) cDNAs were subcloned into a *Not*I/*Sal*I site in the pBluescript KS+ plasmid (Stratagene, La Jolla, CA) and the *Cam I* (Cam B) plasmid was blunt-end cloned (Maniatis et al., 1982) into the same vector.

Screening the 129SVJ mouse genomic library

The lambda library was titered and plated according to the manufacturer's protocol (Stratagene, La Jolla, CA). Plaque lifts were made on BA-85 nitrocellulose circles (Schleicher and Schuell, Keene, NH) (Sambrook et al., 1989). Typical prehybridization solutions consisted of 6X SSC, 5X Denhardt's solution, 0.1% SDS, 20 mM sodium phosphate, pH 6.8, 1 mM EDTA, 100 μg/mL denatured, fragmented salmon sperm DNA, 5 μg/mL tRNA and 30% formamide. 10% dextran sulfate was also included in some hybridizations. Hybridization solutions were the same except they contained 2.5X Denhardt's solution (instead of 5X). ^{32}P-labeled probe generated using the Prime-a-Gene kit (Promega, Madison, WI) was added to at least 4 X 10^6 cpm/mL. Prehybridization was for 4 hours and hybridization was for 17-22 hours at 38°C to 42°C. Filters were washed in 2X SSC, 0.1% SDS warming to the hybridization temperature over a 20 min period, then in 0.1X SSC, 0.1% SDS at increasing temperatures until the background radiation was low. The filters were autoradiographed at -70°C with a Cronex Lightning Plus intensifying screen for 1-2 days. Plaques exhibiting positive

hybridization were cored and the phage was eluted from the agarose by overnight incubation in SM buffer (0.1 M NaCl, 8 mM MgSO$_4$, 50 mM Tris, pH 7.5, 0.01% gelatin). After secondary screening or tertiary screening, the positive plaques were chosen for DNA isolation and sequence analysis.

Lambda DNA isolation

Lambda DNA was isolated from plate lysates in SM buffer. After an overnight incubation at 4°C of the SM buffer on two 150 mm plates with near confluent lysis, the buffer was decanted and a few drops of chloroform were added. After centrifugation at 8000 g for 10 min, the supernatant was loaded on a glycerol step gradient (Maniatis et al., 1982). The pellet was resuspended in STE (10 mM Tris, pH 7.7, 1 mM EDTA, 100 mM NaCl) and was treated with 100 μg/mL proteinase K in 0.1% SDS for 3 hours at 37°C, then 1 hour at 52°C. The DNA was extracted with phenol/chloroform and ethanol precipitated. The DNA pellet obtained by centrifugation was resuspended in TE.

DNA sequencing

Sequencing of plasmid DNA was performed using either the Sequenase kit (USB, Cleveland, OH) using [α-^{35}S]-dATP (DuPont-NEN, Boston, MA) or CircumVent PCR Sequencing kit (New England Biolabs, Beverly, MA) using [γ-^{32}P]ATP (6000 Ci/mmol) (DuPont-NEN, Boston, MA) according to the manufacturers' protocols. Lambda DNA was sequenced using the CircumVent PCR Sequencing kit under the following conditions: 95°C for 3 min, followed by 25 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for

30 s. Sequencing products were electrophoresed on 6% polyacrylamide/7.5M urea sequencing gels, the gels were dried and exposed to X-ray film for autoradiography.

Southern blot analysis

Purified lambda DNA (2.5 μg) from the positive plaque was digested with the appropriate restriction enzymes and was electrophoresed on a 0.5% horizontal agarose gel. The DNA was transferred (Sambrook et al., 1989) to Nytran nylon membrane (Schleicher and Schuell, Keene, NH) under alkaline conditions using a Hoefer Trans-Vac TE 80 vacuum blotter (Hoefer Scientific Instruments, San Francisco, CA). Using Ambion's MAXIScript kit (Ambion, Austin, TX) and [α-^{32}P]-UTP (DuPont-NEN, Boston, MA) a riboprobe was synthesized from the *Cam III* (Cam A) plasmid that included essentially only the 5' untranslated region of the *Cam* cDNA. Prehybridization and hybridization were carried out as described for the library screening except that 40% formamide was used instead of 30% and hybridization was at 42°C.

PCR amplification and modification of a portion of the *Cam II* gene

In order to construct a probe for the bona fide mouse *Cam II* gene, PCR was used to amplify a portion of the gene including a partial promoter region, exon 1 and part of intron 1 from mouse genomic DNA. The primer sequences for PCR were derived from the partial mouse *Cam II* gene sequence given in GenBank (Accession # D12623). The primer designated "Promoter I" is a 24-mer with the sequence $^{5'}$TGA GTG TGT GCG TGT GTC TGT GGC$^{3'}$. The "Intron I" primer is a 23-mer with the sequence $^{5'}$CCT CCT

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GCT CTG CTC TCT CGA CC³'.

A 50 μL PCR reaction consisted of 100 ng of ICR mouse genomic DNA, 25 pmol of Promoter I primer, 25 pmol of Intron I primer, and was 0.2 mM in each dXTP in Vent DNA polymerase buffer (NEB). The mixture was heated to 95°C for 3 min and then was held at 85°C while 2 units of Vent (exo-) DNA polymerase (NEB) was added. The mixture was subjected to 30 cycles of a two-temperature cycle consisting of 95°C for 1 min, then 75°C for 4 min. After a final extension at 75°C for 10 min, the reaction mixture was stored at 4°C. Agarose gel electrophoresis showed a single product of approximately 900 bp which agreed with the predicted size of the product. The identity of the DNA was confirmed by restriction enzyme analysis.

The 900 bp PCR product was purified using Wizard PCR Preps (Promega) and was blunt-end subcloned (Maniatis et al., 1982) into a SmaI restriction enzyme site in the pBluescript KS+ plasmid (Stratagene). Wizard Mini-Preps (Promega, Madison, WI) were used to isolate the plasmid DNA from five white colonies and restriction enzyme analysis allowed identification of the correct clone.

To modify the plasmid so that a probe could be synthesized that would be less likely to hybridize with a pseudogene, a 400 bp portion of the insert including the first exon was excised by digestion of the plasmid with SacII. The plasmid was re-ligated and cloned in DH5α cells as described above. Colonies were screened by PCR (Gussow et.al., 1989) with the Promoter I and Intron I primers mentioned above to identify the plasmid with the correct sized insert. The identity of the correctly modified plasmid was confirmed by restriction enzyme analysis.

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Cells and tissue culture media

C2C12 cells (Blau et al., 1983) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (Intergen, Purchase, NY). Differentiation medium was DMEM supplemented with 0.5% horse serum (Sigma, St. Louis, MO). All media were supplemented with 0.58 g/L glutamine. Cultures were grown at 37°C with 10% CO_2 in a humidified Napco Model 5410 incubator (Tualatin, OR).

Preparation of the *Cam I* coding insert

PCR was used to amplify a 5' portion of the *Cam I* coding sequence from the *Cam I* plasmid (Cam B isolated by 5' RACE) using primers designated as Cam I 5' ($^{5'}$CGGGATCCTCTAGAAGCGGAGGCACCTCGGC $^{3'}$) and Cam I 3' ($^{5'}$TCCCATCCTTGTCAAACACTCG $^{3'}$). A separate PCR reaction was used to amplify the 3' portion of the *Cam I* coding sequence from a previously isolated cDNA containing the 3' non-translated and carboxy-terminal coding region of the *Cam I* mRNA (Bender et al., 1988) using primers designated as B3A 5' ($^{5'}$CGGGATCCTCTAGAGGGGGAAAAGTAGTTG $^{3'}$) and B3A 3' ($^{5'}$GGCCTTCCGAGTGTTTGACAAGG $^{3'}$). The primers were designed so that a portion of the coding region of each target cDNA overlapped. The primers were removed from the first two PCR reactions by spin column chromatography and aliquots of each were combined in another PCR containing the Cam I 5' and B3A 3' primers. Thus, the strands were filled in from the overlapping section to create a full-length coding sequence

and then amplification of the full-length coding sequence occurred with the Cam I 5' and B3A 3' primers.

The PCR products (20 μL) were run on a 1.5% low melting point agarose gel and the band at 588 bp was excised. The DNA was extracted from the gel using the Wizard PCR Preps kit (Promega, Madison, WI) and then was digested with *Xba*I. After phenol/chloroform extraction and ethanol precipitation (Maniatis et al., 1982), the insert was ready for ligation with the Rc/CMV vector.

Preparation of Rc/CMV expression vector for ligation

Three mL of LB medium supplemented with 5 mM glucose and 80 μg/mL ampicillin was inoculated with one colony of DH5α transformed with Rc/CMV (Boshart et al., 1985) from a LB-agar plate containing ampicillin. After overnight growth at 37°C with aeration, the Rc/CMV plasmid was isolated from the cells using the Wizard Mini-Preps Kit (Promega, Madison, WI). The Rc/CMV vector was digested with *Xba*I, ethanol precipitated and then treated two times with calf intestinal alkaline phosphatase (Maniatis et al., 1982). The vector then was extracted with phenol/chloroform (Maniatis et al., 1982) and precipitated with ethanol. The pellet obtained by centrifugation was resuspended in TAE and electrophoresed on a 1% agarose gel. The linear DNA band was excised from the gel and the Rc/CMV vector was extracted using the GlassMax kit (BRL, Gaithersburg, MD).

Ligation of the *Cam I* coding insert and Rc/CMV vector

Approximately 200 ng of the prepared Rc/CMV and a 3-fold molar excess of the prepared insert were combined and heated to 65°C for 2 min and then cooled to room temperature. Ligation buffer (Promega) and 4.5 units of T4 DNA ligase (Promega) were added. The ligation mixture was left at room temperature for 1 hour and then was transferred to a water bath and left to cool at 4°C overnight.

Transformation of *E. coli*

One-third of the ligation mixture was combined with 200 μL of subcloning-efficiency transformation competent DH5α cells (BRL, Gaithersburg, MD) and transformation was done according to the method described by Hanahan (1985). Transformed cells were spread on LB-agar plates containing 80 μg/mL ampicillin and incubated at 37°C overnight.

Selection of the *Cam* expression vector

Thirteen colonies were screened by PCR using the Cam I 5' and B3A 3' primers essentially as reported by Gussow and Clackson (1989). The PCR products were analyzed on a 4% polyacrylamide gel. Six colonies gave a band of the correct insert size (588 bp). Overnight cultures were grown with each colony and Wizard Mini-Preps (Promega, Madison, WI) were used to purify the plasmids from each culture. In order to determine the orientation of the inserts in these plasmids, the plasmids were digested with *Hin*dIII. *Hin*dIII was chosen because it cuts once in the vector (100 bp 5' of the

*Xba*I site) and once in the *Cam I* insert (132 bp 3' of the 5' *Xba*I site). Thus, if the insert was in the correct orientation, a band at 232 bp would be expected, while a 534 bp band would be expected if the insert was in the opposite orientation. The digest products were analyzed on a 4% polyacrylamide gel. This analysis showed that three of the plasmids contained the insert in the correct orientation and the other three contained the insert in the anti-sense orientation. One colony containing the *Cam* expression vector (Rc/CMV-Cam I) and one colony containing the anti-sense *Cam* expression vector were picked and grown overnight. An aliquot of each culture was used to make a frozen stock in 15% glycerol at -70°C.

Transient transfection of C2C12 cells

For use in the transfection, the CMV/β-galactosidase plasmid (a β-galactosidase expression vector in which expression is driven by a cytomegaloviral promoter), the Rc/CMV vector alone and the *Cam* expression vector (Rc/CMV-Cam I) were each isolated from 400 mL cultures in LB plus 80 μg/mL ampicillin using an alkaline lysis method and purified by equilibrium centrifugation in a CsCl gradient (Sambrook et al., 1989). The CMV/β-gal plasmid was used as a control for transfection efficiency, because of the ability to stain the cells for β-galactosidase activity (MacGregor, 1992).

Twenty-four hours prior to transfection, C2C12 cells were seeded at 3 X 10^5 cells per 100 mm dish or 1 X 10^5 cells per 60 mm dish. The cells were transfected using a modification of the calcium phosphate method (Kriegler, 1990) with 30 μg/mL DNA in the calcium phosphate precipitation solution. In the case of transfection with CMV/β-gal

or Rc/CMV-Cam I, the Rc/CMV vector alone comprised half of the total DNA in the solution to act as carrier DNA. After an overnight incubation (22 hours) at 37°C, 5% CO_2 in the presence of the $CaPO_4$/DNA coprecipitate, the cells were washed one time in 37°C PBS (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). Then 4.4 mL or 1.7 mL of a room temperature 15% glycerol solution in HBS (250 μM KCl, 277 μM glucose, 35 μM Na_2HPO_4, 8.5 mM NaCl, 2 mM HEPES, pH 7.05-7.10) was added to 100 mm plates or 60 mm plates, respectively. After 5 min, the glycerol solution was removed by aspiration and the cells were washed one time in warm PBS. Differentiation medium was added to the plates and the cells were incubated at 37°C, 10% CO_2. After 30 hours, half of the cells transfected with Rc/CMV and half of the cells transfected with CMV/Cam I were removed from the incubator. A 2 mM solution of the calcium ionophore A23187 in DMSO was added to the media of these cells to a final concentration of 1 μM. The cells were returned to the incubator for 20 hours before harvesting all cells.

Staining CMV/β-gal-transfected cells for β-gal activity

Cells were stained for β-galactosidase activity using the chromogenic substrate X-gal according to the protocol described by MacGregor (1992), with the exception that the cells were fixed in formaldehyde instead of glutaraldehyde. Cells were viewed and photographed using a Zeiss Axiovert 35 inverted microscope (Oberkochen, Germany).

Isolation and Analysis of DNA from transfected C2C12 cells

DNA was isolated from one 100 mm dish of transfected cells under each condition (i.e. Rc/CMV-transfected, Rc/CMV-transfected + A23187 treatment, CMV/*Cam I*-transfected, and CMV/*Cam I*-transfected + A23187). The remaining cells were washed two times with warm PBS and stored at -70°C. The media from the cells used in the DNA preparation was transferred to 15 mL centrifuge tubes and centrifuged at 500 g for 5 min. The plates were washed with warm PBS which was then used to resuspend the pellets in the centrifuge tubes. The tubes were again centrifuged at 500 g for 5 min. Lysis buffer (0.5 mL of 0.3 M Tris, pH 8, 0.1 M NaCl, 10 mM EDTA, 0.2 M sucrose, 0.5% SDS) was added to each plate and then transferred to the corresponding pellet in the centrifuge tube. Proteinase K was added to each lysate to a concentration of 50 μg/mL and the mixtures were incubated at 37°C for greater than or equal to 2 hours. After transferring to 65°C for 30 min, 8 M potassium acetate was added to each mixture to a final concentration of 1 M and the mixtures were incubated on ice 30-60 min. The solutions were transferred to ultracentrifuge tubes and centrifuged at 4°C at 40,000 g for 20 min. The supernatants were decanted into microcentrifuge tubes and the DNA was phenol/chloroform extracted twice followed by chloroform extraction and ethanol precipitation. Following centrifugation, each sample was resuspended in 300 μL of TE buffer (10 mM Tris, pH 7.2, 0.1 mM EDTA) and RNase A was added to 5 μg/mL. After incubating the samples at room temperature for 20 min, another phenol/chloroform extraction followed by chloroform extraction and ethanol precipitation were done. The DNA from each sample was resuspended in 60 μL of TE.

10 μL of each resuspended sample was analyzed by electrophoresis on a 2% agarose mini-gel.

Northern blot analysis of RNA from transfected cells

Total RNA was isolated from two 100 mm dishes of each condition of transfected cells using the acid guanidine-phenol extraction method outlined by Chomczynski (1994). Then, the RNA was resuspended in 0.3 mL of RNase-free H$_2$O, 10X Buffer D (Promega) was added to a 0.5X concentration and the RNA was treated with 5 units of RQ1 DNase (Promega, Madison, WI) for 40 min at 37°C. This incubation was followed by a phenol/chloroform extraction (Maniatis et al., 1982) and ethanol precipitation by adding 3 M sodium acetate to 0.3 M and adding 3 volumes of 100% ethanol. After storing at -20°C for more than 1 hour, the RNA was precipitated by centrifugation, resuspended and the absorbance at 260 nm was determined using a Perkin-Elmer Lambda 2 UV/Vis Spectrophotometer (Norwalk, CT). From this value, the concentration was estimated and 15 μg of each sample was ethanol precipitated. The remaining RNA was ethanol precipitated and stored at -70°C.

The RNA pellets were resuspended by heating at 68°C for 3-5 min in 15 μL of a solution consisting of 1X MOPS buffer (40 mM morpholinopropanesulfonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 6% formaldehyde and 55% formamide. After adding 3 μL of RNA loading buffer (20% ficoll, type 400, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol), the samples were loaded on a formaldehyde/agarose gel (Maniatis et al., 1982) and electrophoresed in the 1X MOPS

buffer in a Hoefer MAX Submarine Agarose Gel Unit Model HE 99 (Hoefer Scientific Instruments, San Francisco, CA) at 80 V with buffer recirculation until the dye front had migrated 12-14 cm. The gel was washed for 20-30 min in Transfer buffer (25 mM Tris acetate, pH 8.0, 1 mM EDTA) with one buffer change and the RNA was transferred to Nytran nylon membrane (Schleicher and Schuell, Keene, NH) using a Bio-Rad Trans-Blot Apparatus. Transfer was carried out at 250 mA for 0.5 hour, then 450 mA for 3 hours, followed by 1 hour at 650 mA. Then, the membrane was rinsed in 5X SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7) for 5 min and the RNA was fixed to the membrane by baking at 70-80°C for 1 hour.

Prior to probing, the Northern blot was wet in 5X SSC, 0.5% SDS for 30 min at 37°C. The membrane was sealed in a hybridization bag with prehybridization solution (40% formamide, 20 mM sodium phosphate, pH 7, 6X SSC, 5X Denhardt's solution, 0.5% SDS, 1 mM EDTA, 5 μg/mL tRNA and 100 μg/mL fragmented denatured salmon sperm DNA) and was incubated in a Hot Shaker water bath (Bellco, Vineland, NJ) at 42°C for 4 hours. The prehybridization solution was removed and the hybridization solution (15 mL prehybridization solution except 2.5X Denhardt's instead of 5X) containing approximately 7 X 10^6 cpm/mL of ^{32}P-labeled probe was added. Hybridization was carried out at 42°C for 17 hours. The membrane then was rinsed in 2X SSC, 0.5% SDS, 1 mM EDTA and washed in the same buffer for 5 min at room temperature. A wash in 0.1X SSC, 0.2% SDS, 1 mM EDTA warming to 42°C for 20 min was followed by a wash in the same buffer warming to 46°C for 20 min when necessary. If higher stringency was desired, additional washes were done at 52°C in 0.1X SSC, 0.1% SDS,

1mM EDTA. At this point, the membrane was exposed to Fuji RX X-Ray Film with an intensifying screen at -70°C.

³²P-labeled probe synthesis

³²P-labeled probes were generated using the Prime-a-Gene kit (Promega, Madison, WI) using [α-³²P]-dCTP (3000 Ci/mmol) (DuPont-NEN, Boston, MA) according to the manufacturer's protocol. For use of this kit, template DNA for probe synthesis had to be prepared. Plasmids containing an insert to be used as template DNA (i.e. Cam B for *Cam I* cDNA, pActin for actin cDNA, or Cam A for *Cam III* cDNA) were digested with appropriate restriction enzymes to excise the insert. The products were run on a 1% Ultrapure low melting point agarose gel (BRL, Gaithersburg, MD). The band corresponding to the size of the insert DNA was excised from the gel and the DNA was extracted using Wizard PCR Preps (Promega, Madison, WI). An aliquot of the recovered insert DNA was run on a 4% polyacrylamide gel to estimate the concentration of sample. The purified insert DNA was used as template DNA for probe synthesis.

RESULTS

Isolation of *Cam* cDNAs and *Cam* genes

Previously, two partial calmodulin cDNAs from *Cam I* and *Cam III* had been isolated from the mouse (Bender et al., 1988). Using a modified 5'-RACE method (Rapid Amplification of cDNA Ends), the 5' ends of the two partial cDNAs and a third cDNA corresponding to *Cam II* were isolated (Skinner et al., 1994).

Initially, the conventional method of 5'-RACE as reported by Frohman et al. (1990) and modified by Jain et al. (1992) was attempted in order to isolate the three calmodulin genes from total RNA from brain, where all three genes are expressed. This method consists of an initial reverse transcription reaction in which a gene-specific oligonucleotide primer and target RNA are incubated in the presence of reverse transcriptase. RNase is then added to remove the template RNA, leaving cDNAs. A poly(dA) tail is added to the cDNAs using terminal deoxytransferase and dATP. Then, the tailed cDNAs are subjected to a polymerase chain reaction using the same gene specific primer and an "Anchor" primer containing a poly(dT) tail. A portion of the first PCR products are used in a second PCR in which a second nested gene-specific primer and an "Anchor" primer without the poly(dT) tail are used to amplify the cDNA of interest.

For use in amplification of the calmodulin cDNAs, the first gene specific primer was a degenerate primer that was complementary to all three *Cam* mRNAs. The sequence selected for this primer is at a position where the three mRNA sequences would be most similar. The sequences of the two partial cDNAs previously isolated were used

along with the sequence of the rat *Cam II* cDNA which was believed to be homologous to the mouse *Cam II* cDNA to design the primer. A 22-nt sequence containing only three nucleotide differences was chosen for the degenerate gene-specific primer (GSP-1). Thus, eight different sequences are represented in the GSP-1 primer. Just upstream from the sequence chosen for the degenerate primer is a region in which every codon differs between at least two of the three cDNAs. This region was used to create three separate gene-specific nested primers for use as the second primer in the procedure and were designated GSP-2A (corresponding to *Cam III*), GSP-2B (corresponding to *Cam I*) and GSP-2C (corresponding to *Cam II*) (Fig. 4).

This conventional 5'-RACE method resulted in the amplification of nonspecific products. When GSP-2B was used as the nested primer, only low molecular weight bands were apparent, and multiple DNA species were amplified by GSP-2A and GSP-2C. Two of the products of a size possible for the calmodulin cDNAs were isolated and sequenced, but neither encoded calmodulin. In order to increase the stringency of the initial primer annealing and reduce the number of nonspecific products, the 5' RACE method was modified by separating the primer annealing step from the reverse transcription reaction. This change allowed the annealing temperature to be increased from 42°C, which is optimal for reverse transcriptase activity, to the more stringent temperatures for annealing of 52°C, 56°C or 60°C. A lower concentration of the degenerate primer was used to decrease nonspecific annealing. The decreased primer concentration slows the kinetics of annealing and was compensated for by allowing the annealing step to proceed overnight. After the overnight incubation, the annealing

Figure 4. **Primer design for 5' RACE.** The 3' ends of the calmodulin coding DNAs from mouse and rat were used to design the oligonucleotides that were used in the 5' RACE method. Differences in the three sequences are indicated (|). GSP-1 is the degenerate gene specific primer that consists of 8 different sequences. The GSP-2 primers are the nested gene specific primers that have an additional sequence (shown in lower case letters) that contain restriction enzyme sites for use in cloning. Mouse CamA and GSP-2A correspond to *Cam III*, mouse CamB and GSP-2B correspond to *Cam I* and rat Cam II and GSP-2C correspond to mouse *Cam II*.
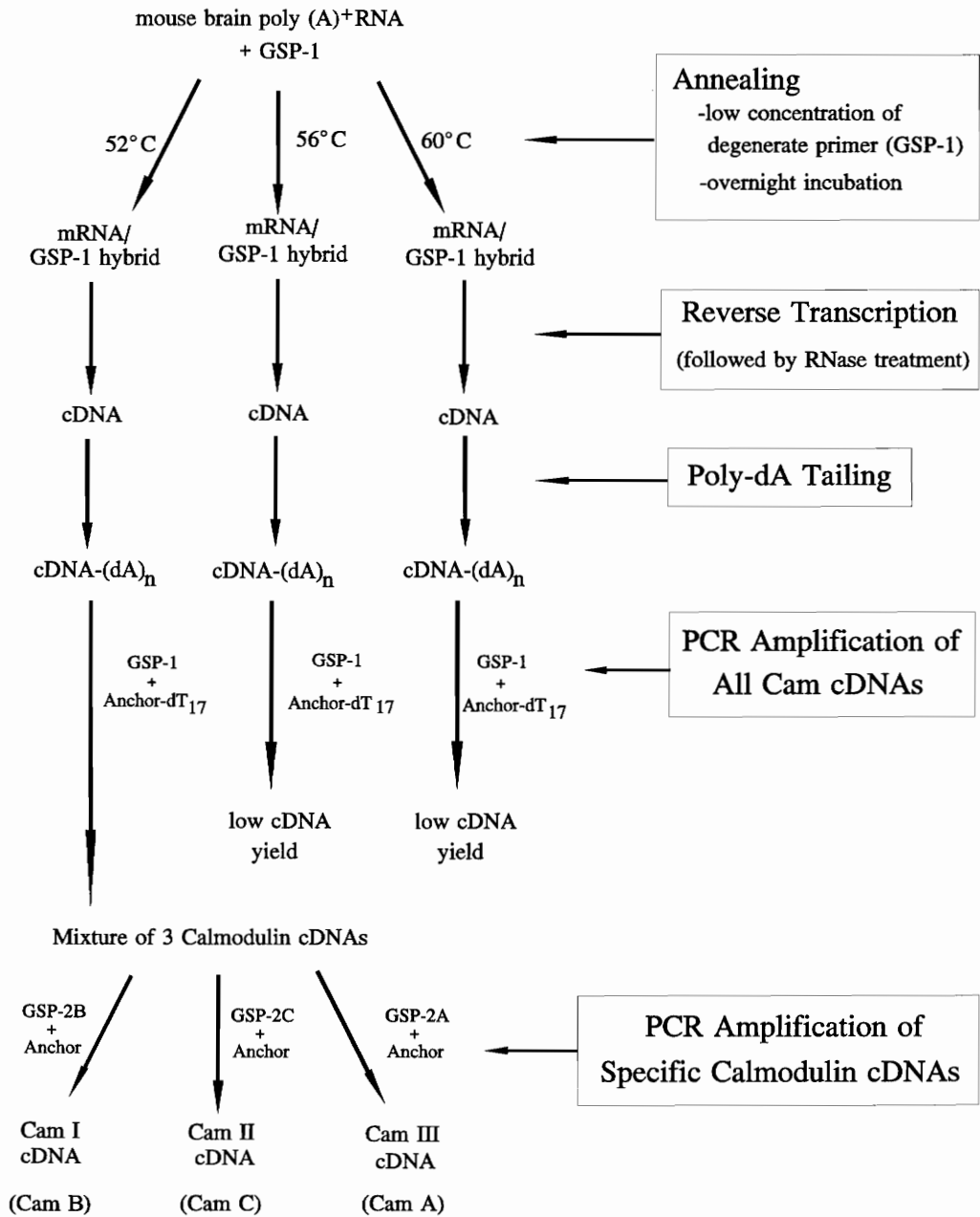
reaction was diluted into a reverse transcription reaction buffer and first strand cDNA synthesis was allowed to proceed at 42°C for 1 hour. (Skinner et al., 1994) Subsequent steps were performed essentially as in the conventional 5' RACE method (Fig. 5). The results of this modified 5' RACE method are shown in Figure 6. Only the annealing reaction at 52°C led to significant products after the first PCR. The second PCR using the gene-specific nested primers yielded three different products. These products were subcloned and sequenced, and each was found to encode a different calmodulin cDNA. The sequences have been submitted to GenBank with the following accession numbers: *MuscalmdB* alias *Cam I*, M1931; *MusCam* alias *Cam II*, L31642; and *MuscalmdA* alias *Cam III*, M19380 (Appendix).

Comparison of the 5'-untranslated sequences of each mouse cDNA with the rat cDNAs allowed the assignment of homologs (Fig. 7). Interestingly, each mouse cDNA is more similar to its rat homolog than it is to the other mouse calmodulin cDNAs.

The isolation of the 5' ends and the majority of the coding portion of the three mouse calmodulin cDNAs allowed three further developments: (1) using the newly isolated sequence and the previously isolated sequence, a full-length coding sequence was generated using PCR (which will be discussed later); (2) the cDNAs were used to synthesize probes that were used to screen a genomic library for isolation of the mouse calmodulin genes; and (3) the 5' leader sequences from each cDNA allowed Southern mapping of the adjacent promoter region from each genomic isolate.

The genomic library chosen for screening was from the 129SVJ mouse. This library was chosen because the 129SVJ mouse is syngeneic with the embryonic stem cells

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mouse brain poly $(A)^+$ RNA
+ GSP-1

52°C   56°C   60°C

```
Annealing
  -low concentration of
    degenerate primer (GSP-1)
  -overnight incubation
```

mRNA/   mRNA/   mRNA/
GSP-1 hybrid   GSP-1 hybrid   GSP-1 hybrid

```
Reverse Transcription
(followed by RNase treatment)
```

cDNA   cDNA   cDNA

```
Poly-dA Tailing
```

cDNA-$(dA)_n$ cDNA-$(dA)_n$ cDNA-$(dA)_n$

GSP-1   GSP-1   GSP-1
+   +   +
Anchor-dT_{17} Anchor-dT_{17} Anchor-dT_{17}

```
PCR Amplification of
All Cam cDNAs
```

low cDNA   low cDNA
yield   yield

Mixture of 3 Calmodulin cDNAs

GSP-2B   GSP-2C   GSP-2A
+   +   +
Anchor   Anchor   Anchor

```
PCR Amplification of
Specific Calmodulin cDNAs
```

Cam I   Cam II   Cam III
cDNA   cDNA   cDNA

(Cam B)   (Cam C)   (Cam A)

Figure 5. **Diagram of the modified 5' RACE method.** Separation of annealing and reverse transcription allowed specific first strand synthesis and amplification of all three of the mouse calmodulin cDNAs by PCR. A second PCR with nested primers allowed the specific amplification of each calmodulin cDNA.

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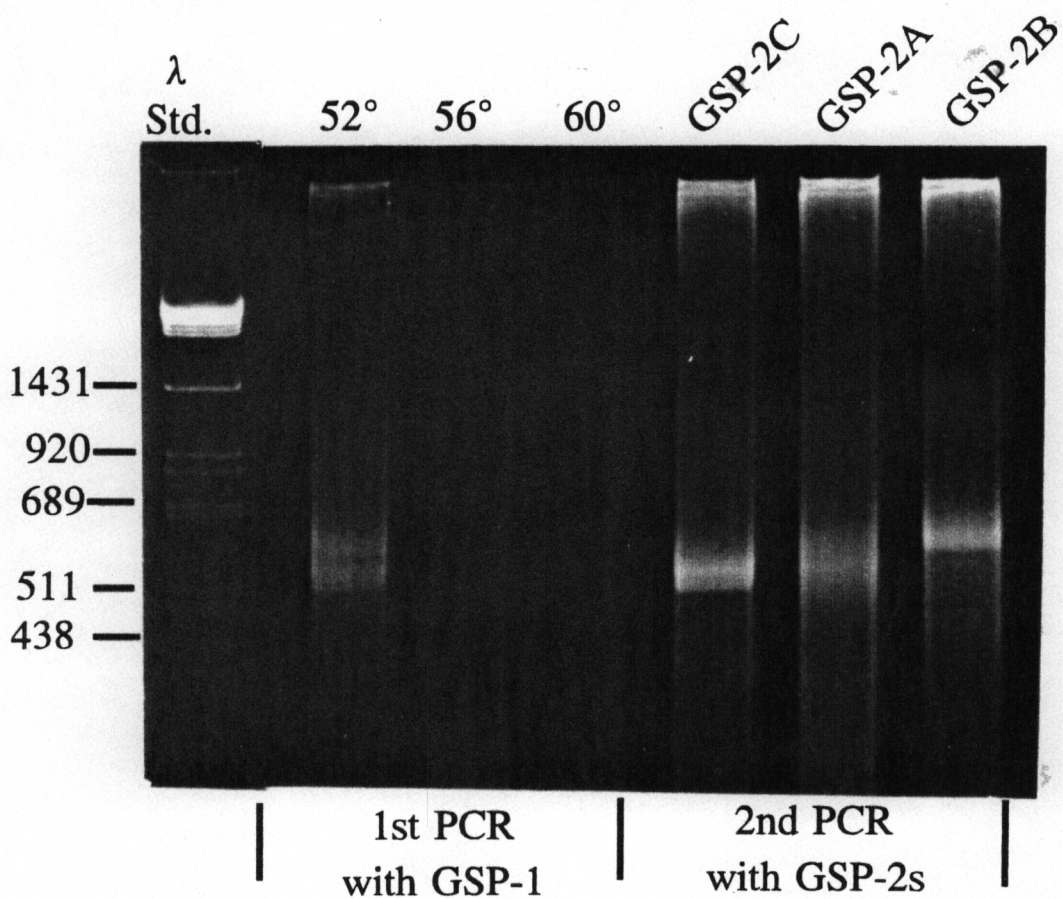
Figure 6. **5' RACE products.** Samples were electrophoresed on a 4% polyacrylamide gel. Molecular weight markers were run in the far left lane. Products of the first PCR of cDNAs using the degenerate GSP-1 primer were loaded in the next three lanes, and products from the second PCR reactions using the nested GSP-2 primers and the first PCR products from the 52°C annealing/reverse transcription mix were loaded in the last three lanes. The temperatures shown above the lanes indicate the temperature used in annealing of the GSP-1 primer to RNA for first strand cDNA synthesis. The gene specific nested primers used in the second PCR are indicated above their respective lanes. GSP-2C is specific to *Cam II*, GSP-2A is specific to *Cam III* and GSP-2B is specific to *Cam I*.

```
                                        M   A   D   Q   L   T

Rat Cam I       73..CACCCTCGCCTGCTCCGCTCTTCCTTCCTTCGCTCGCACC ATG GCT GAT CAG CTG ACT...3'
                                --
Mouse Cam I    146...CACCCTCGCCTGCTCCGTTCTTCCTTCCTTCGCTCGCACC ATG GCT GAT CAG CTG ACT...3'
(Cam B)


Rat Cam II      30..GTTGTCTGTTCTGGTCTCGGAAACCGGTAGCCCTTGCAGC ATG GCT GAC CAA CTG ACT...3'
                             --  ---
Mouse Cam II    35..GTTGTCTGGTCGGCGTCTCGGAAACCCGTAGCCCTTNCAGC ATG GCT GAC CAA CTG ACT...3'
(Cam C)


Rat Cam III     62..GTAACCTCGATCCCGAGCTCCGGATACGGTGCCTCGCC ATG GCT GAC CAG CTG ACC...3'
                                                                              --
Mouse Cam III   69..GTAACCTCGATCCCGAGCTCCGGATACGGTGCCTCGCC ATG GCT GAC CAG CTG ACT...3'
(Cam A)
```

Figure 7. **Alignment of the 5' ends of corresponding mouse and rat calmodulin cDNAs.** A portion of each mouse calmodulin cDNA is aligned with its corresponding rat homolog using the Clustal Program (IntelliGenetics, Mountain View, CA). Sequence differences are marked (|). The number of nucleotides in the 5' untranslated sequences that are not included in the figure are given at the beginning of each sequence. Comparison shows that the sequences from the mouse are closer to their rat counterparts than to each other.

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used in creating knockout mutations. Thus, any genetic information obtained from the library might later be used in such an experiment. Another reason for screening the library was to isolate the promoter regions of the genes for use in studies to elucidate the genetic elements necessary for the transcriptional regulation of the genes. Use of the 5' ends of the cDNAs as templates for probes increased the chances of identifying a bacteriophage that would have the adjacent promoter region of the *Cam* gene. The library screening resulted in the isolation of *Cam I* and *Cam III* genomic fragments that contain 8 kb and 3.5 kb of 5' flanking sequence, respectively.

Southern mapping of the 5' flanking sequence

DNA from the lambda phage encoding the *Cam III* sequence was digested with *Not*I, *Sac*I and *Sal*I and the digests were run on an agarose gel. The DNA was transferred to nylon membrane and probed to determine which DNA bands represented 5' leader sequence and the promoter region of the gene (Fig. 8). *Not*I excised the entire insert showing that it was 20-25 kb, since the lambda arms are 9 kb and 20 kb. *Sal*I excised the insert and also appeared to cut once in the insert which resulted in two bands in addition to the lambda arms. There is a *Sac*I site 25 bp upstream of the ATG start codon determined from the cDNA sequence and the probe was specific for the 5' untranslated region of the Cam III cDNA up to the ATG start codon. The lambda vector also contains two *Sac*I sites bordering the insert. The band at 3.5-4 kb is 5' flanking sequence and the small band of about 0.5 kb is part of the end of exon I and the beginning of intron I. These band assignments are based on the fact that there is only
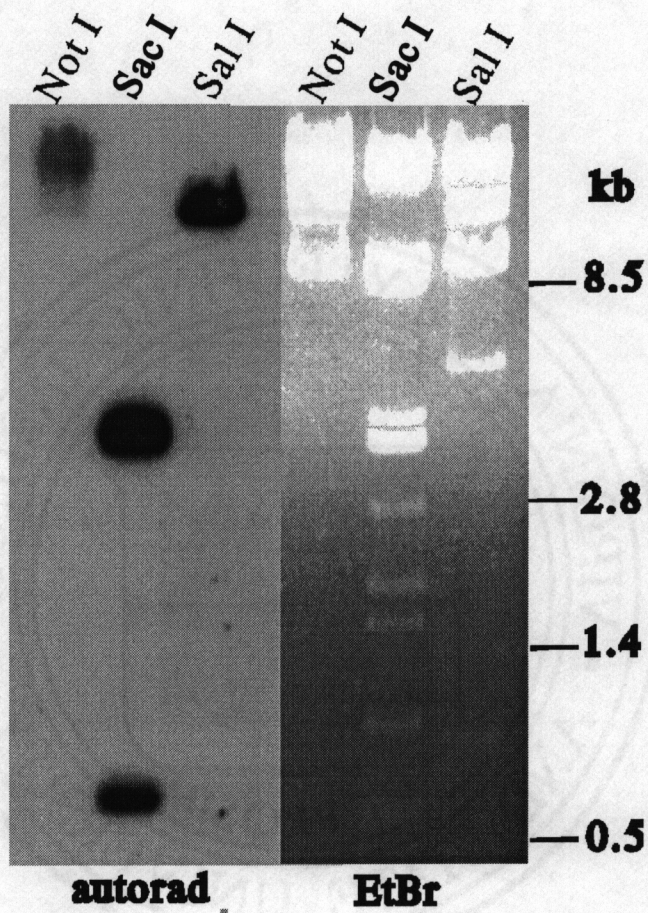
Figure 8. **Southern blot of the *Cam III*-encoding recombinant phage DNA.** Lambda DNA was digested with the restriction enzymes indicated and then subjected to agarose gel electrophoresis. After visualization of the DNA with ethidium bromide (EtBr), the DNA was transferred to nylon membrane and probed with a 5' untranslated portion of the *Cam III* cDNA (Autorad). *Not*I and *Sac*I excise the insert from the lambda DNA.

a small portion of the probe that would hybridize to sequence 3' of the *Sac*I restriction enzyme site in the 5' untranslated region. The weaker signal from the 0.5 kb band indicates that the probe did not hybridize as strongly with it as with the 4 kb band. Thus, the 4 kb band represents the 5' flanking sequence of the *Cam III* gene. Similar Southern analysis of the DNA from the lambda phage containing the *Cam I* gene indicated that the insert contained approximately 8 kb of 5' flanking sequence.

Cam II Pseudogenes and Chromosome Mapping

At least one partial pseudogene corresponding to *Cam II* was isolated as well, but no sequences from a bona fide *Cam II* gene were identified. The pseudogenes were identified by the fact that the sequence matched the *Cam II* cDNA sequence in the coding region, but were missing at least the third intron of the gene by comparison to the structure of the rat *Cam II* gene. The existence of multiple calmodulin pseudogenes has been noted previously in the rat and human (Nojima et al., 1987; Nojima, 1989; Koller and Strehler, 1988). Also, the presence of *Cam II*-related pseudogenes in mouse had been suggested during chromosome mapping of the *Cam II* gene.

The two previously isolated calmodulin cDNAs had been used to map the chromosome locations of the *Cam I* and *Cam III* genes to chromosome 8, 19p13 and chromosome 7, 19q12-13.2, respectively, in Dr. Nancy Jenkins' lab (ABL-Basic Research Program, Frederick Cancer Research and Development Center, Frederick, MD) (Fig. 9). Upon isolation of the third cDNA, chromosome mapping of the *Cam II* gene was attempted; however, three loci were detected on chromosomes 6, 17 and X. This

finding suggested that there are *Cam II*-related pseudogenes in the mouse, and the library screening confirmed it. In order to create a probe that would be as specific as possible for the bona fide *Cam II* gene and not complementary to the pseudogenes, PCR was used to amplify a portion of the promoter region, exon I and intron I of *Cam II* from mouse genomic DNA. The primers used in this PCR were generated using the partial *Cam II* gene sequence information from Matsuo et al. (1993) in GenBank (Accession Number D12623).

This PCR gave a product that was consistent with the predicted size of 900 bp. The identity of the product was confirmed by restriction enzyme analysis and the DNA was subcloned. In order to make the sequence specific to the bona fide gene, a portion of the sequence containing exon I was excised and the plasmid was religated and cloned. Thus, a probe made from this clone should contain only a partial 5' flanking region and a portion of the first intron and should not hybridize to a retropseudogene. This construct was successfully used in Dr. Nancy Jenkins' lab to generate a probe that identified the chromosome locus of *Cam II* as 2p16 on chromosome 17, one of the three loci previously identified as *Cam II*-related (Fig. 9).

Overexpression of Calmodulin in C2C12 Myoblasts

Since overexpression of the proliferation signal c-Myc in C2C12 cells under differentiation conditions leads to apoptosis (Crescenzi et al., 1994), overexpression of calmodulin, another signal for proliferation, in C2C12 cells under differentiation conditions was undertaken to determine if the conflicting growth signals would lead to
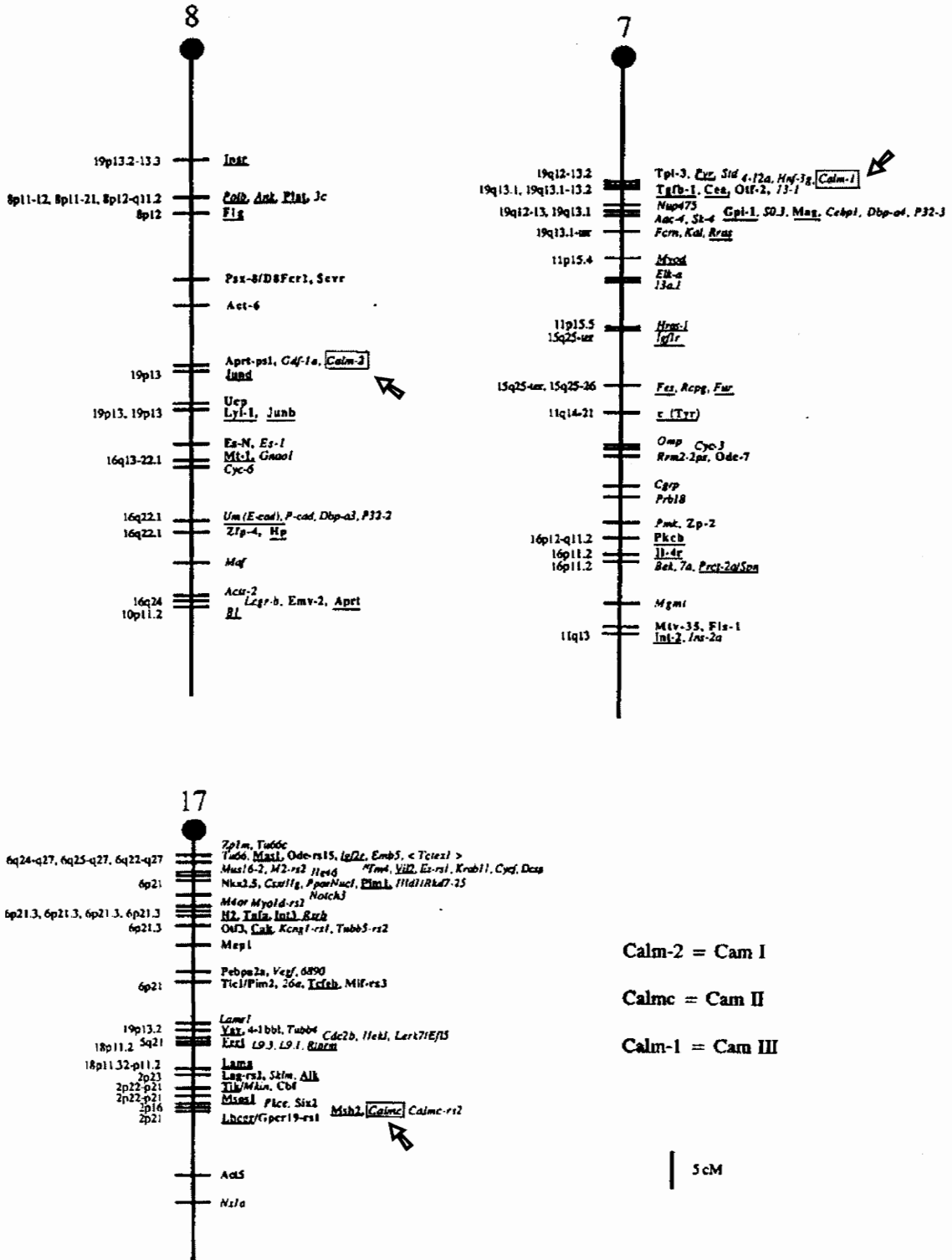
Figure 9. **Chromosome maps showing the positions of the *Cam I*, *Cam II* and *Cam III* loci.** Chromosome maps were provided by Dr. Nancy Jenkins (Frederick Cancer Research and Development Center, Frederick, MD).

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apoptosis. In order to construct the calmodulin expression vector to use in the transfection of C2C12 cells, a full length calmodulin coding sequence was necessary. This DNA was generated from the previously isolated 3' portion of the *Cam I* cDNA (Bender et al., 1988) and the 5' end of the *Cam I* cDNA isolated as described above (Skinner et al., 1994) using a recombinant PCR procedure often used for site-directed mutagenesis by PCR (Higuchi, 1990). The 5' portion of the coding sequence was amplified by PCR using two specific primers. Likewise, the 3' portion of the coding sequence was amplified in a separate PCR. The primers were designed so that the 3' end of the 5' portion of the sequence and the 5' end of the 3' portion of the sequence would overlap. The unincorporated primers were removed from the PCR products and a final PCR was done using the products from the first two PCRs and the primers to amplify the entire coding sequence. Because the first PCR products overlap, the DNA polymerase was able to fill in the complementary strands to create the complete coding sequence. The primers included in the reaction then allowed amplification of the full coding sequence (Fig. 10).

The *Cam I* coding sequence was subcloned into an *Xba*I restriction enzyme site in the mammalian expression vector Rc/CMV using an *Xba*I restriction enzyme site derived from the 5' primer used in constructing the *Cam I* PCR product and a natural *Xba*I restriction enzyme site following the TGA stop codon in the 3' end of the *Cam I* PCR product (Fig. 11).

Before transfecting the C2C12 cells with the calmodulin expression vector, transfection conditions were optimized using a CMV/β-galactosidase expression vector.
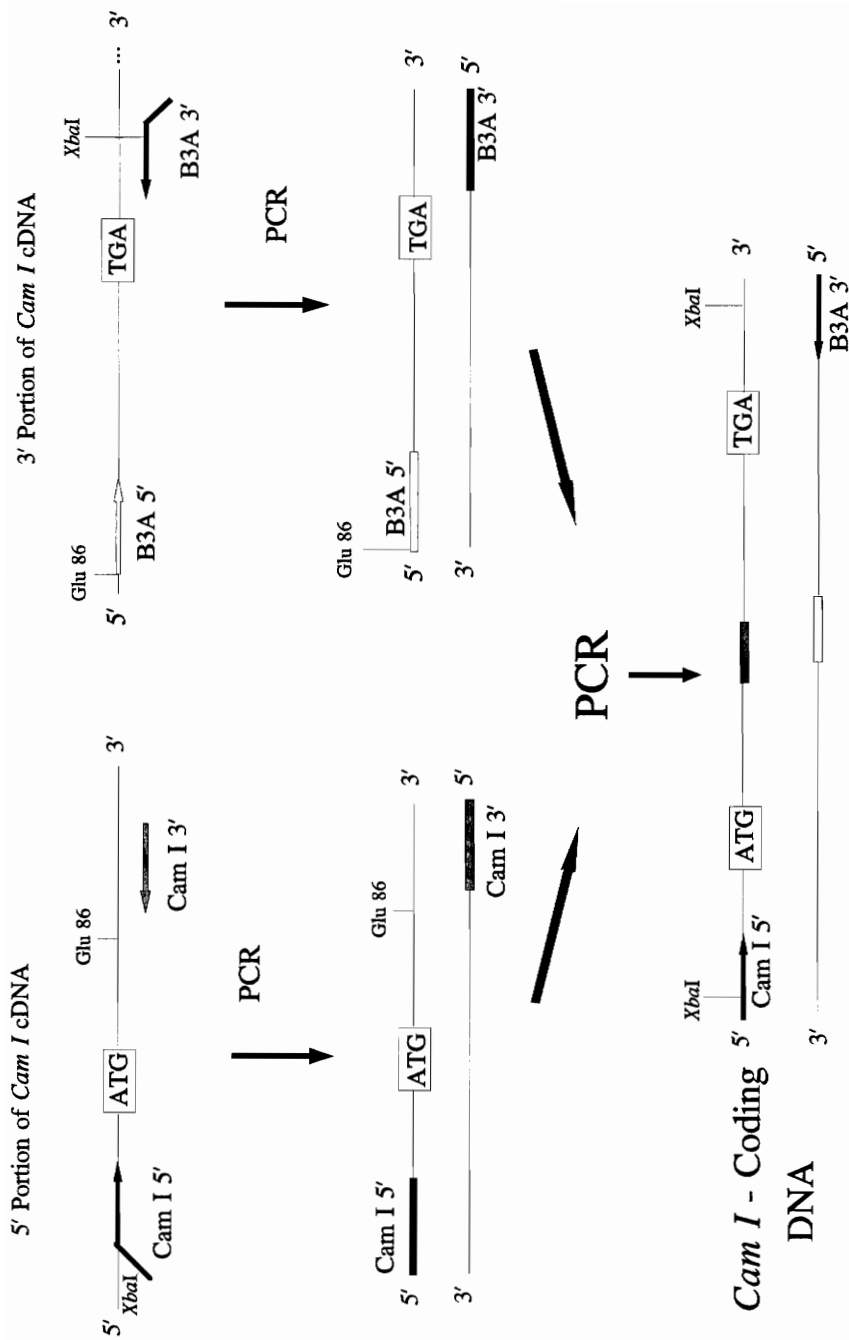
Figure 10. **Diagram of the synthesis of complete coding calmodulin cDNA.** Cam I 5′, Cam I 3′, B3A 5′ and B3A 3′ are primers used in PCR. The position of Glu 86 is included as a point of reference. Arrows represent primers and filled boxes indicate incorporated primer sequences.
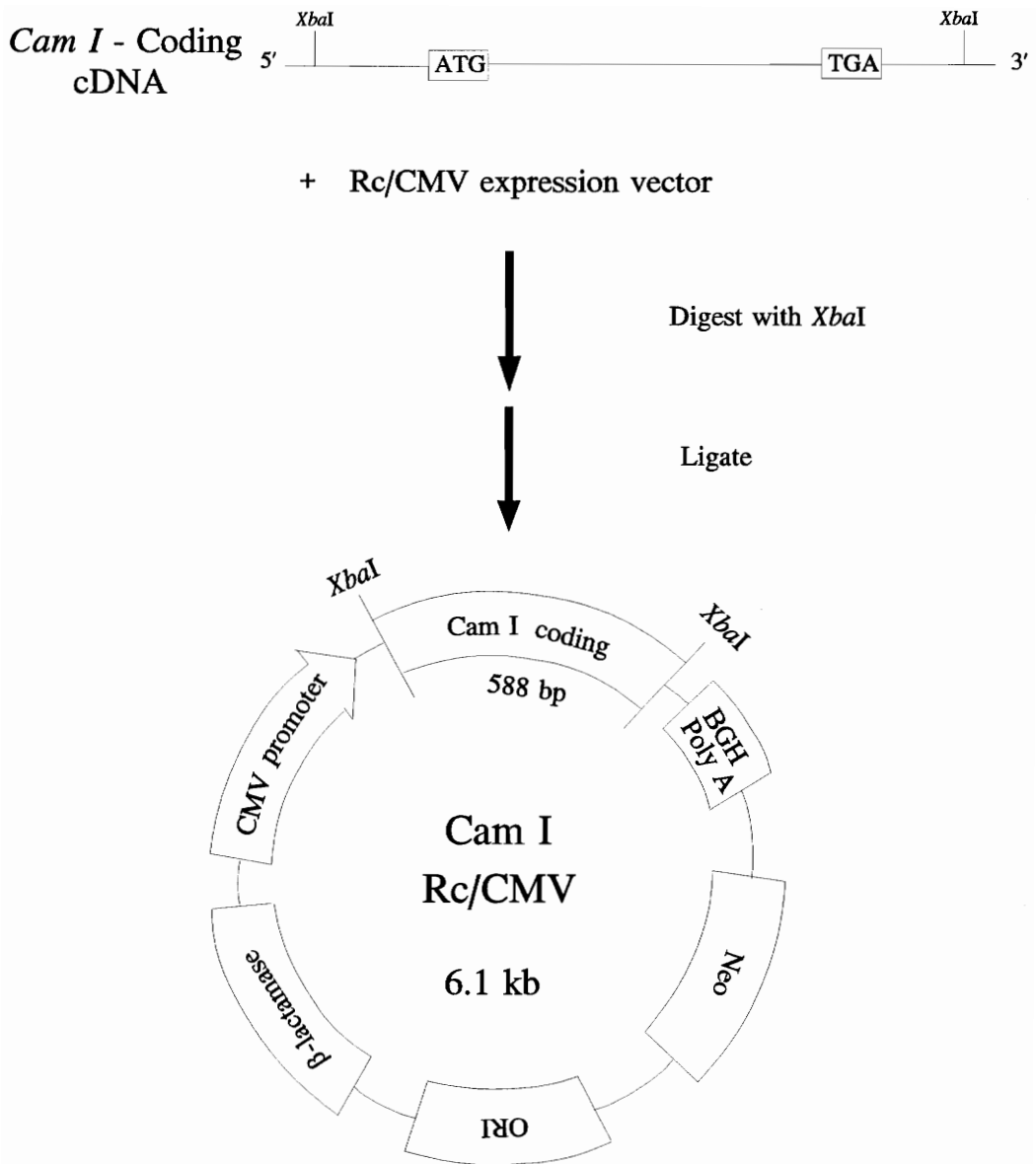
Figure 11. **Diagram of the synthesis of the calmodulin expression vector.** The diagram shows that the calmodulin-encoding DNA was subcloned into the Rc/CMV vector using an *Xba*I restriction enzyme site. The vector contains a cytomegaloviral promoter to drive calmodulin expression and a region from the bovine growth hormone gene to provide a polyadenylation signal for the message.

Use of this vector to transfect C2C12 cells allowed staining of the cells for β-galactosidase activity as an indicator of the transfection efficiency (Fig. 12). The optimal conditions which gave a transfection efficiency of 30 to 40% involved the use of the calcium phosphate method with cesium chloride purified plasmid DNA and a glycerol shock step.

Using this method, the C2C12 cells were transfected with the calmodulin expression vector or with the Rc/CMV vector alone as a control. The *Cam*-transfected cells did not appear to differ from the control-transfected cells morphologically or with respect to cell viability. The transfected cells treated with 1 μM A23187 showed cell death after 24 hours of exposure. Death seemed to be delayed somewhat in the *Cam*-transfected cells, which might have been due to a calcium-buffering activity of the calmodulin; however, the significance of the small difference is questionable. Expression of the exogenous calmodulin message in the *Cam*-transfected cells was confirmed by Northern blot analysis (Fig. 13).

The transfected cells not treated with the calcium ionophore appeared to differentiate as some fusion of the cells was apparent. The ionophore-treated cells were not of sufficient density to fuse, because of cell death (Fig. 14). Northern blot analysis confirmed that the control- and *Cam*-transfected cells were biochemically differentiated by showing a greater amount of α-actin message than β-actin message (Fig. 15). Consistent with the cell death observed, this pattern was not seen in the ionophore-treated cells and actin mRNA levels were also low in the treated cells. RNA from 23A2 myoblasts was included on the blot as an example of proliferative myoblasts and shows
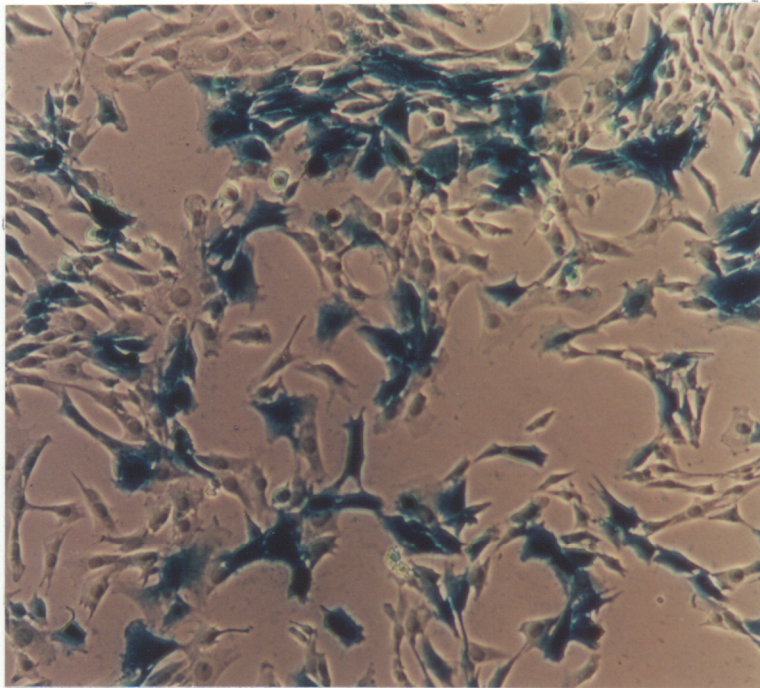
Figure 12. **CMV-β-galactosidase transfected C2C12 cells stained for β-galactosidase activity.** C2C12 cells were transfected with a β-galactosidase expression vector driven by a cytomegaloviral promoter and after 24 hours were stained using X-gal (MacGregor, 1992). Cells positive for β-galactosidase activity are blue. Magnification is 100X.
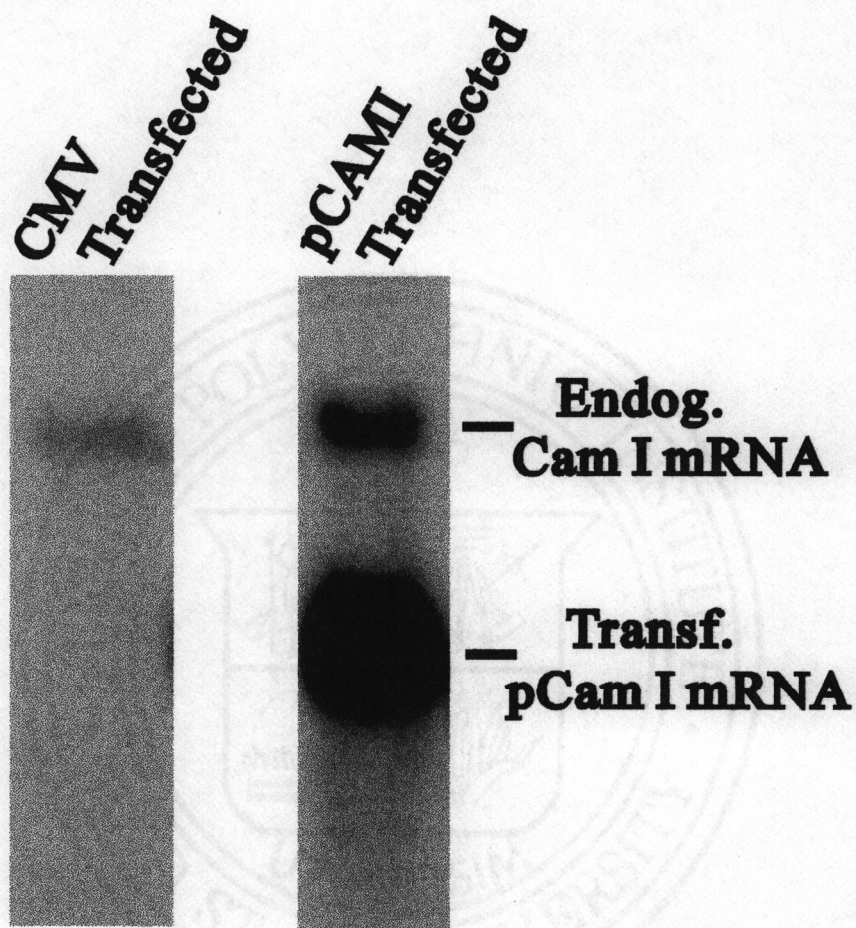
Figure 13. **Northern blot of RNA from transfected cells probed with *Cam I* cDNA.**
Lanes from a Northern blot containing RNA from C2C12 cells transfected with the
Rc/CMV vector alone (CMV Transfected) and cells transfected with the calmodulin
expression vector and the Rc/CMV vector (pCAMI Transfected) that was hybridized with
a *Cam I* cDNA probe are shown. A band representing the endogenous 1.7 kb *Cam I*
message is visible in both lanes (Endog. Cam I mRNA). The *Cam I* mRNA from the
calmodulin expression vector is visible as a strong band only in the RNA sample from
cells transfected with the calmodulin expression vector (Transf. pCamI mRNA).

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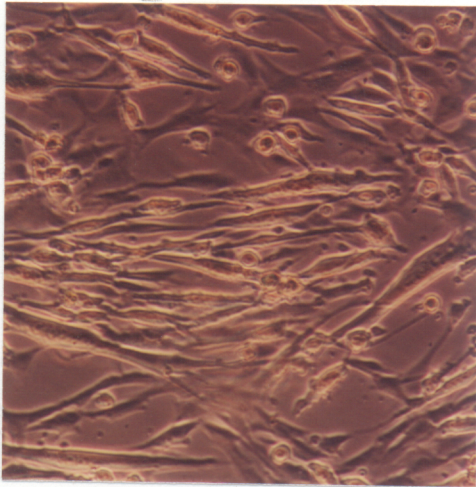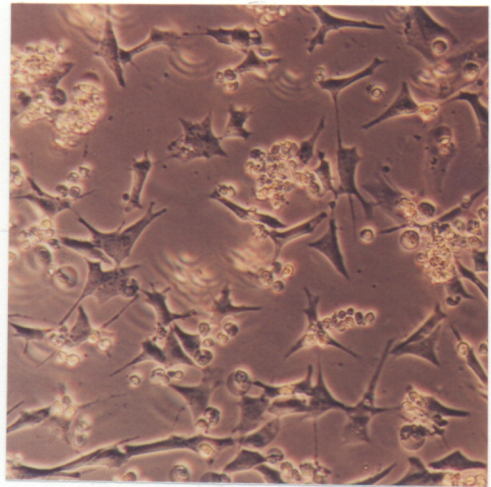
Rc/CMV

Rc/CMV + A23187
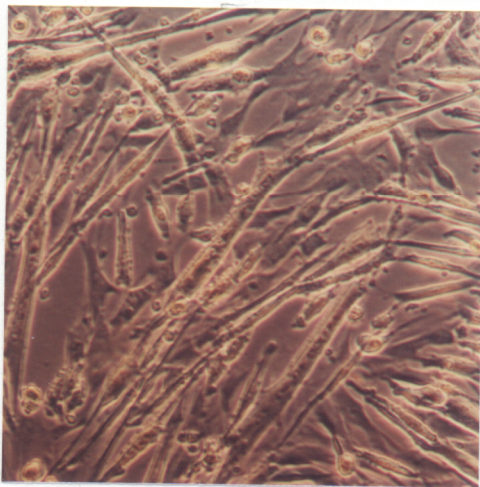
Rc/CMV-Cam I
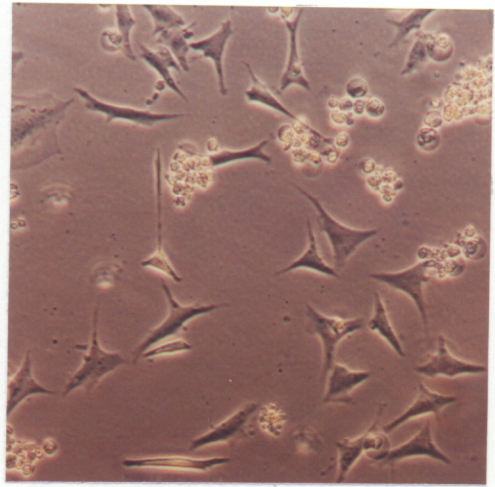
Rc/CMV-Cam I + A23187

Figure 14. **Transfected C2C12 cells.** Photographs were taken 3 days after glycerol shock. **Rc/CMV** are cells transfected with the expression vector only as a control. **Rc/CMV + A23187** are cells transfected with the expression vector and treated with calcium ionophore. **Rc/CMV-Cam I** are cells transfected with the calmodulin expression vector containing a cytomegaloviral promoter and with the vector alone. **Rc/CMV-Cam I + A23187** are cells transfected with the calmodulin expression vector, with the vector alone and treated with calcium ionophore. Magnification of the cells is 100X.
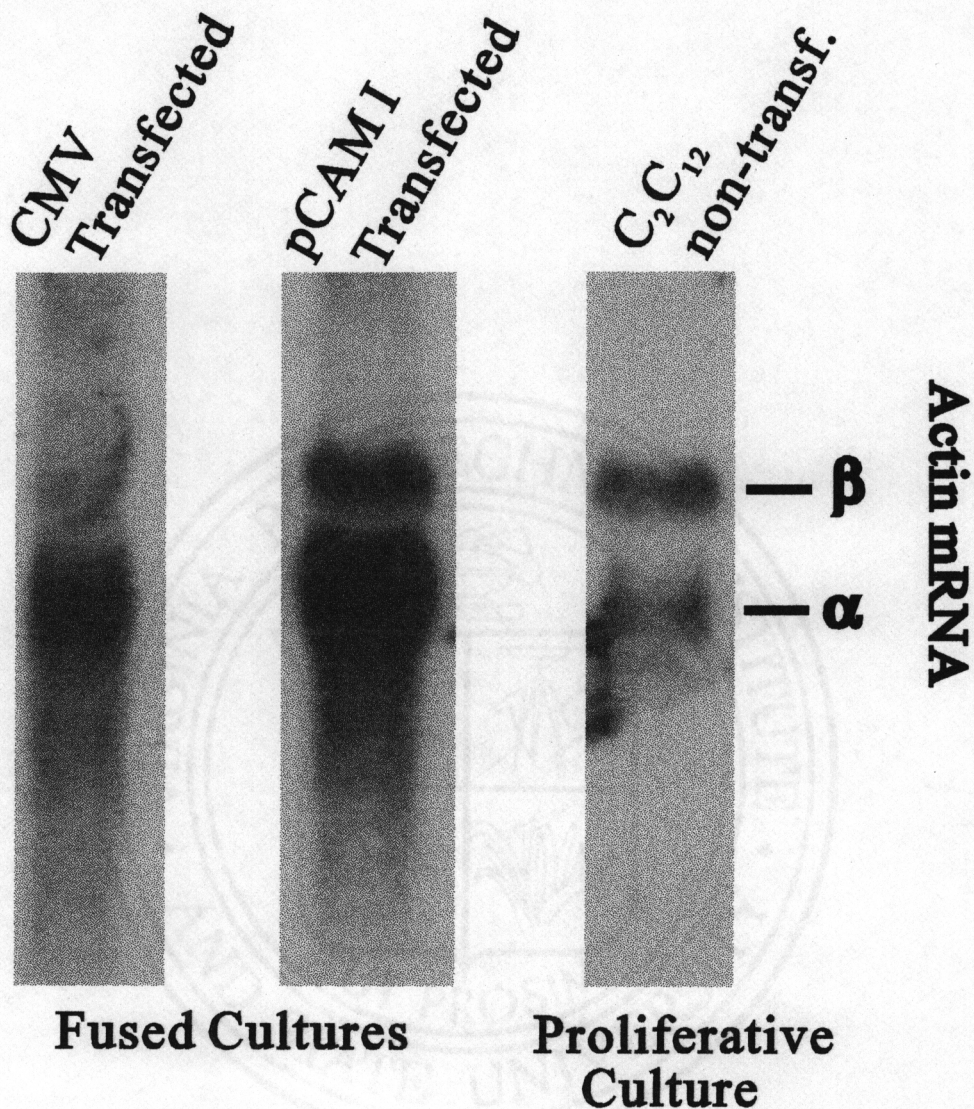
Figure 15. **Northern blot of RNA from transfected cells probed with actin cDNA.**
Lanes from a Northern blot probed with actin cDNA including RNA from C2C12 cells
transfected with the Rc/CMV vector alone (CMV Transfected), cells transfected with the
calmodulin expression vector and Rc/CMV (pCAM I Transfected), and proliferative non-
transfected myoblasts (C2C12 non-transf.) are shown in the figure. In proliferative
myoblasts, the amount of the β-actin message is greater than that of α-actin (Proliferative
Culture). In differentiated muscle cells, the amount of α-actin mRNA is greater than that
of β-actin (Fused Cultures).

a greater amount of β-actin message than α-actin message. Interestingly, this result shows that overexpression of calmodulin did not antagonize the differentiation of the transfected C2C12 cells as assayed by α-actin expression.

To assay for apoptosis, DNA was isolated from the C2C12 transfected cells transfected with the control Rc/CMV vector alone or the calmodulin expression vector (Rc/CMV-Cam I) and either untreated or treated with the calcium ionophore A23187 and analyzed by agarose gel electrophoresis. As shown in Figure 16, none of the samples showed the characteristic ladder of DNA. This result indicated that apoptosis was not occurring in any of the cultures. The calmodulin protein levels were not quantitated in the cells, because of complications in the assay; however, assuming that the abundant calmodulin mRNA transcripts were translated into protein, it can be concluded that a high level of calmodulin is not sufficient to prevent differentiation or cause apoptosis in the C2C12 cells. Also, the mechanism of cell death caused by the calcium ionophore A23187 was not apoptosis.
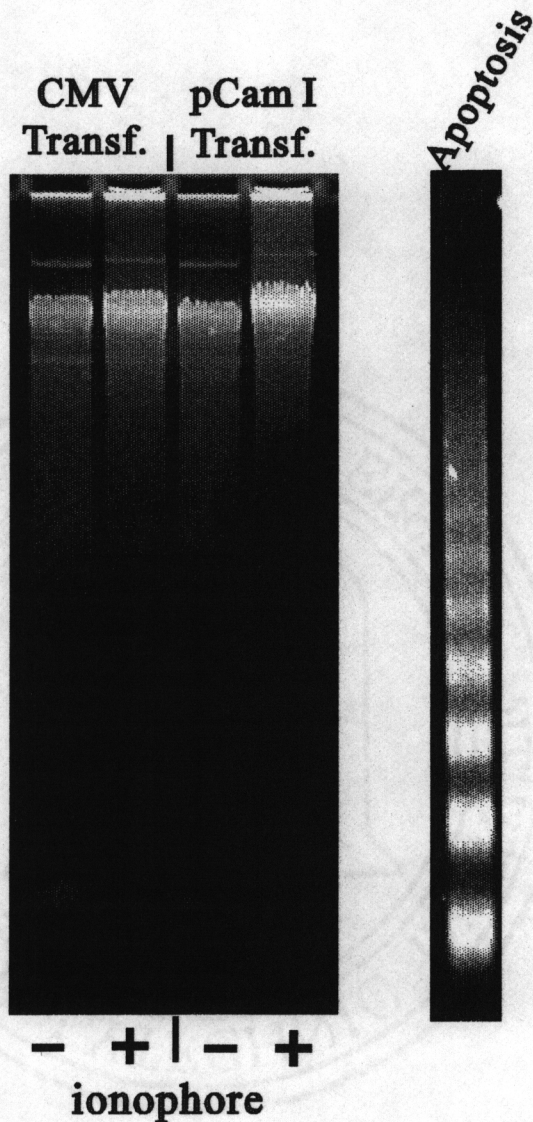
Figure 16. **Agarose gel of DNA from transfected cells.** DNA was isolated from cells transfected with the Rc/CMV expression vector alone (CMV Transf.), cells transfected with the Rc/CMV expression vector and treated with calcium ionophore, cells transfected with the calmodulin expression vector and Rc/CMV vector (pCam I Transf.) and cells transfected with the calmodulin expression vector and the Rc/CMV vector and treated with calcium ionophore. Approximately 4 μg of the DNA was electrophoresed on a 2% agarose gel. A control sample of DNA for apoptosis was isolated from dexamethasone-treated WEHI7 cells, and shows the "ladder" characteristic of DNA from apoptotic cells (Apoptosis).

DISCUSSION

Isolation of the 5' ends of the three calmodulin genes from mouse using a modified 5'-RACE method allowed the assignment of the mouse genes to their rat homologs. Furthermore, it allowed library screening which led to the isolation of the promoter regions of the *Cam I* and *Cam III* genes, as well as identification of at least one *Cam II* pseudogene in the mouse. Chromosome mapping of the mouse calmodulin genes in Dr. Nancy Jenkins' lab showed that the three calmodulin genes are not linked, but are on different chromosomes. Mapping also identified two *Cam II*-related sequences that are likely to be pseudogenes. Since at least one of these pseudogenes was shown to be missing an intron by sequence analysis, it is likely that it is intronless. This fact taken with the finding that a probe consisting only of promoter and first intron partial sequence did not identify the *Cam*-related loci is important when creating a knockout mutation. If the pseudogene had contained introns, it would likely have the flanking regions of the gene which would create another site of possible homologous recombination. Since the pseudogenes appear to be intronless, this problem should not arise.

Sequencing of the isolated promoter regions of the *Cam I* and *Cam III* gene remains to be performed, but the availability of these regions will allow study of the cis-acting elements that regulate calmodulin gene transcription. Now that the probe from the spliced partial promoter region and first intron of *Cam II* has been shown to be specific for the bona fide *Cam II* gene, the library could also be screened again without the complications of isolating pseudogenes in order to isolate the promoter region of the *Cam II* gene.

The results of the transient transfection of C2C12 cells with the calmodulin expression vector under differentiation conditions indicated that a high level of calmodulin, even in the presence of a high level of intracellular calcium due to a calcium ionophore, does not lead to apoptosis. Although the calcium ionophore-treated cells underwent cell death, the control- and *Cam*-transfected cells differentiated. No DNA fragmentation was seen in any of the cultures as assayed by gel electrophoresis. Calmodulin did not inhibit differentiation as would have been predicted from the fact that it can act as a proliferation signal. It is possible that a small percentage of cells that did not differentiate died by apoptosis. If this was the case, the assay for DNA fragmentation may have not been sensitive enough to see the ladder. The assay for DNA fragmentation can detect the DNA ladder in as little as 1 μg of DNA from apoptotic WEHI7 cells. Since 4 μg of DNA from the transfected C2C12 cells were analyzed, the limit of detection for the DNA ladder would have been 25% of the cells. However, the abundance of the calmodulin message in the *Cam*-transfected cells suggests that if calmodulin acts as a proliferation signal, there should be an abundance of signal present in the *Cam*-transfected cells. It is possible that although the *Cam* message was present, it was not translated to make the calmodulin protein, since the protein was not assayed. This is unlikely; still, it is surprising that there were no visible differences between the *Cam*-transfected cells and the control-transfected cells.

As discussed in the introduction, calmodulin affects many different enzymes and processes in the cell. Although there may be a role for calmodulin as a proliferation signal, it may have other roles that conflict with proliferation. One example of this

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would be calmodulin's activation of nitric oxide synthase which produces the second messenger recently shown to be important in myoblast fusion (Lee et al., 1994). Thus, calmodulin plays a role in differentiation as well as proliferation.

Another possibility is that the effect of a high level of c-Myc in C2C12 cells may not result from conflicting growth and differentiation signals, but may be due to a direct activation of the apoptotic pathway by Myc. It is possible that in addition to providing a proliferation signal, Myc, as a transcription factor, could induce expression of another protein that is responsible for apoptosis.

The work presented here provides a foundation for the study of transcriptional regulation of the mouse calmodulin genes, information important in the creation of knockout mutations and provides a contribution to the study of the role of calmodulin in apoptosis. In order to map the cis-acting elements responsible for transcriptional regulation, the promoter regions of the *Cam I* and *Cam III* genes could be linked to a reporter gene such as β-galactosidase or luciferase. Cells representing a certain tissue in the mouse could be transfected with such constructs. By creating sequential deletions of the promoter regions that are linked to the reporter gene, portions of the promoter region and cis-acting elements necessary for tissue-specific expression could be identified. The results of such an experiment could help to explain the mechanism of differential calmodulin gene expression in the mouse. By using the probe now known to be specific for the bona fide *Cam II* gene, the genomic library could be screened again to isolate the *Cam II* gene and allow mapping of its promoter region as well. The large portions of the 5' flanking sequence and the first introns of the *Cam I* and *Cam III* genes that have

been isolated provide necessary reagents to allow the creation of knockout mutants. This type of experiment would provide information about the function of calmodulin in the cell as well as to determine whether transcription of each of the calmodulin genes is related; for instance, whether the removal of one of the functional calmodulin genes will result in an increase in transcription of the other genes to compensate for the decrease in calmodulin. Finally, in overexpressing calmodulin in C2C12 cells, a protocol for the transient transfection of C2C12 cells with high transfection efficiency was developed and the experiment established that the cytomegaloviral promoter is able to drive strong transcription in C2C12 cells. The results indicate that calmodulin does not induce apoptosis in C2C12 cells; however, further study should be done for confirmation. Many questions about calmodulin and its expression remain to be answered. The results from this work will allow some of them to be answered in the future.

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APPENDIX

Sequences of Mouse Calmodulin cDNAs Obtained by 5' RACE

```
Cam I   CGGCAGTGGTGCTGGGAGTCTCGTGTCCGTGGTGCCGTTACTCGA           45



AGTCGGGCGGCGGCTGAGGCTCAGCGCACAACGCAGGTAGCGCGTTACGAGCAGC    100
                   Cam III   CGCT-CG-GTAAAG-GTGTGGAG


AGAAGCGGAGGCACCTCGGCGGTCACAGCCCCTGCGCTGGTGCAGCCACCCTCGC    155
   Cam II   CCACAATTAGTC-GAGTGGAGCGAG-GAGTCG--CGTTGTCTGG
GCGC-GACG-CGGASTCGAGCTG-TGCAGCTGCTGC-GCCGCC--AGTAACCTCG


                               M   A   D   Q   L   T   E   E
CTGCTCCGTTCTTCCTTCCTTCGCTCGCACCATGGCTGATCAGCTGACTGAAGAG    210
TCG-GTCTCGGAAACCCGTAGCCCTTN-AG-ATG-----C--A--------A--G
ATC-CCGAGCTCCGGATACCGGTGCCT-GC-ATG-----C--G--------G--A


    Q   I   A   E   F   K   E   A   F   S   L   F   D   K   D   G   D   G
CAGATTGCTGAATTCAAGGAAGCTTTCTCCCTATTCGATAAAGATGGTGACGGCA    265
--------A--G-----A-----T-----A--A--T--C--G--C--G--T--G-
--------A--G-----G-----C-----C--C--C--C--G--T--A--T--C-


    T   I   T   T   K   E   L   G   T   V   M   R   S   L   G   Q   N   P   T
CCATCACAACCAAGGAACTGGGTACCGTCATGCGTTCACTTGGTCAGAACCCAAC    320
-A--A--A--A-----G-----G--A--G---A-G--C--G--G--------C--
-C--T--C--C-----G-----G--T--G---A-A--G--G--A--------C--


        E   A   E   L   Q   D   M   I   N   E   V   D   A   D   G   N   G   T
AGAAGCCGAGCTGCAGGATATGATCAACGAAGTGGATGCTGATGGCAATGGCACC    375
A--A--A--A--------C-----C--T--A--A-----G-----T-----C--A
T--G--G--G--------C-----T--T--G--G-----T-----C-----G--C


    I   D   F   P   E   F   L   T   M   M   A   R   K   M   K   D   T   D
ATTGACTTCCCAGAGTTCTTGACTATGATGGCTAGAAAAATGAAAGACACAGATA    430
-----T-----T--A--TC----A--------A-----A-----A--C-----C-
-----C-----A--G--CT----C--------C-----G-----G--T-----C-
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        S  E  E  E  I  R  E  A  F  R  V  F  D  K  D  G  N  G  Y
       GCGAAGAAGAGATCCGCGAGGCCTTCCGAGTGTTTGACAAGGATGGGAATGGTTA   485
       -T--A--A-----TA-A--A--A-----T--G-----T--------C-----C--
       -C--G--G-----AC-A--G--C-----T--C-----C--------G-----C--


         I  S  A  A  E  L  R  H  V  M  T  N  L  G  E  K  L  T
       CATCAGTGCGGCAGAACTGCGCCACGTCATGACAAACTTAGGAGAAAAGCTAACA   545
       T--T--T--AG-A--SS-T--C--T--G-----A---C-T--C--G---T-A---
       T--T--C--TG-C--GC-G--T--C--C-----G---C-G--G--G---C-G---


         D  E  E  V  D  E  M  I  R  E  A  D  I  D
       GATGAAGAAGTAGATGAAATGATCAGAGAAGCAGATATTGAT*GSP-2B*
       -----A--G--C-----G------A-G--A--A--C---NNN*GSP-2C*
       -----G--A--G-----G------C-A--G--C--C---GAT*GSP-2A*
```

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Plasmids, Genomic Isolates and Their Descriptions

Cam B = *Cam I* cDNA in pBluescript KS+ plasmid

Cam C = *Cam II* cDNA in pBluescript KS+ plasmid

Cam A = *Cam III* cDNA in pBluescript KS+ plasmid

CamIIPI = portion of *Cam II* gene amplified by PCR of ICR mouse genomic DNA including some promoter region, entire first exon and partial first intron subcloned in pBluescript KS+ plasmid

ModCamIIPI = same as CamIIPI except that 400 bp sequence between 2 *Sac*II sites including the entire first exon was removed so only some of the promoter region and some of the first intron are present

Cam 6 = Lambda FIX II vector containing *Cam III* insert (from 129SVJ mouse genomic library from Stratagene)

2CD₂ = Lamda FIX II vector containing *Cam I* insert (from 129SVJ mouse genomic library from Stratagene)

Rc/CMV = mammalian expression vector with cytomegaloviral promoter (Invitrogen)

CMV/Cam I = CMV/pCam I = Rc/CMV-Cam I = calmodulin expression vector
using coding sequence from *Cam I*

Rc/CMV-Cam I AS = Rc/CMV expression vector with *Cam I* coding sequence in orientation to produce the anti-sense calmodulin mRNA

VITA

Tricia Lynn Skinner was born January 8, 1971 in Havre de Grace, Maryland to Gregory and Diana Skinner. After graduating from North Harford High School in May 1989, she attended Harford Community College in Bel Air, Maryland where she received her A.A. in June 1991. She transferred to Western Maryland College in Westminster, Maryland where she was inducted into Phi Beta Kappa and graduated Summa Cum Laude and received her B.A. in Chemistry in May 1993. Ms. Skinner then entered the Department of Biochemistry and Anaerobic Microbiology at Virginia Polytechnic Institute and State University with a Cunningham Fellowship in June 1993. During her time at Virginia Tech, Ms. Skinner received an honorable mention for her application for a scholarship from the National Science Foundation and was awarded the Bruce M. Anderson Award. Ms. Skinner completed the requirements for her M.S. degree in August of 1995.

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